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Damage/Recovery by Additive on Lipid Membrane as a Mimicry of Human Stratum Corneum

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The effects of sodium dodecyl sulfate (SDS) on the model lipid membrane of human stratum corneum, composed of three main lipids of ceramide III, palmitic acid, and cholesterol, have been examined as a function of exposure period. Cholesterol first got to elute, palmitic acid followed it late, and the remaining solid was mainly ceramide III. The removal of lipids influenced the configurational structure of remaining lipid and the intralayer structure of lamellae. Monitoring of structural reorganization in the damaged membrane was carried out on the recovering procedure of palmitic acid and cholesterol. Both lipids were penetrated in the damaged membrane and recovered mostly the configurational lipid structure and the lamellar structure. Especially, it can be noted that cholesterol is more effective than palmitic acid on recovery.

Introduction

Surfactants are commonly used in house-, personal-, and skin-care products to increase cleaning performance and to create pleasant foam. Meanwhile, surfactants can penetrate the stratum corneum (SC), being adsorbed on the corneocyte keratin and mixed with the intercellular lipids.[1,2] Moreover, surfactants also damage the skin by removing skin lipids such as fatty acids, fatty acid glycerides, and cholesteryl esters, even if the removed lipids are in small quantity.[3−7] Therefore, it is almost certain that the penetration of surfactants and the selective removal of lipids lead to significant changes in the intercellular lipid profiles.

Much attention has been focused on the effects of surfactants on SC lipids in connection with the skin barrier function[8,9] and the change in the lipid composition associated with various skin symptoms.[10−12] The process or the mechanism of these effects remains relatively obscure, but the process, in general terms, involves the penetration of surfactants and the elution of lipid components through the lipid membrane. For elucidating the effects of surfactants on SC, the typical surfactant, sodium dodecyl sulfate (SDS), is frequently used.[13−15] Taking into account the fact that lipids of human SC consist predominantly of ceramides, free fatty acids, and cholesterol,[16,17] lipid mixtures, being composed mainly of three lipids described above, can be adopted as a simplified membrane model of SC to mimic the composition and structure of SC lipid lamellae and to study the influence of surfactants on it. It would therefore be very advantageous and useful to investigate the affect of additives by means of mimic lipid membrane as an alternative in vitro membrane model for skin response studies.

The present study focuses on the composition and the structure of lipid membrane, which was subjected the treatment with a SDS solution for different time course. Its objective is to shed light on the damage by surfactants on the lipid lamellae of SC. Furthermore, an effective approach has been exploited to recover the damaged membrane by eluted lipids, which was pursued as the time variation. To our knowledge, this would be the first report of time course investigation of a damage/recovery process by surfactant on mimic lipid membrane of human SC. The present results would be expected to bring critical information on the SC lipid profile in healthy and diseased in vivo human skin with relation to the SC lipid organization and to the skin barrier function.

Experimental Section

Materials. Ceramide III (95.9%) was purchased from Evonik Degussa (Essen, Germany). Palmitic acid, cholesterol, and SDS (99.0%) were supplied from Nacalai Tesque (Kyoto, Japan). Pyrene (a fluorescent agent) was obtained from Wako Pure Chemical Industries Ltd. (Richmond, VA).

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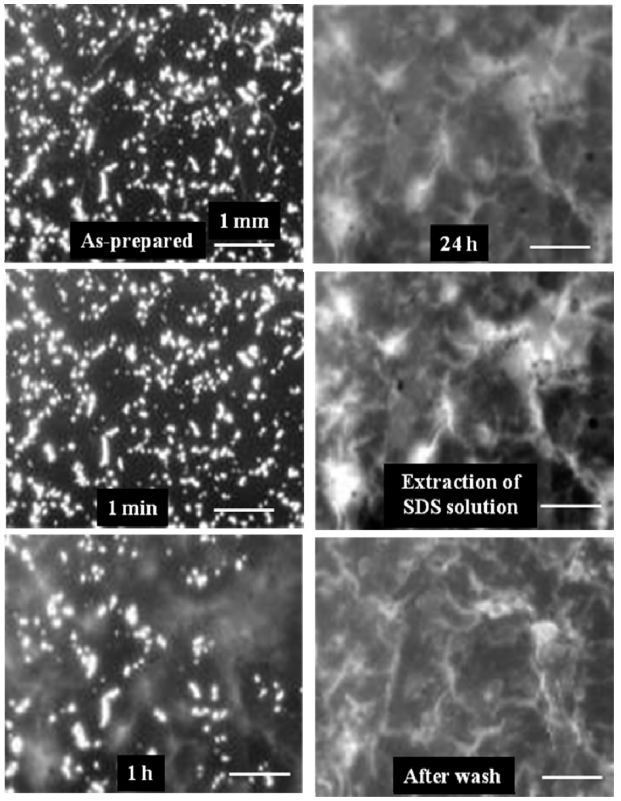

Figure 1. Fluorescence microscopic images of pyrene including lipid membranes exposed to an aqueous SDS solution at different periods.

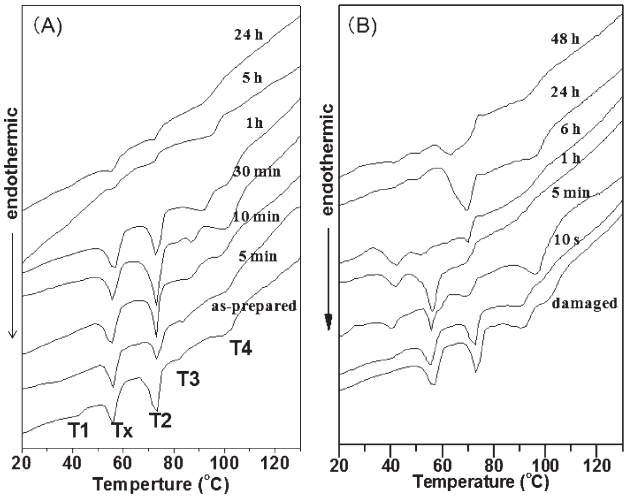

Figure 2. Thermal (DSC) behavior of lipid membranes. (A) Damage process of as-prepared membranes exposed to an aqueous SDS solution at different periods. (B) Recovery process of damaged membranes exposed to an ethanol solution of palmitic acid and cholesterol at different periods.

Preparation of Lipid Membrane. Ceramide III (0.3 g), palmitic acid (0.15 g), and cholesterol (0.15 g) were dissolved in a mixed solvent of chloroform (50 cm^3) and methanol (10 cm^3). Then 0.2 cm^3 of a lipid mixture solution was spread on 18 × 18 mm cover glass and dried. Sequentially, the lipid mixture membrane was melted at 120 °C in a glass tube oven, allowed to stand at that temperature for 1 h under nitrogen atmosphere, and cooled to room temperature. Then the cooling process, that is, whether the membrane was left to cool in a switched-off oven or out of the oven, does not produce the meaningful variation on the subsequent properties of membranes. Next the sample was immersed in water for 24 h. Finally, it was dried and stored in a desiccator containing saturated sodium chloride (at relative humidity of 75%) until use.

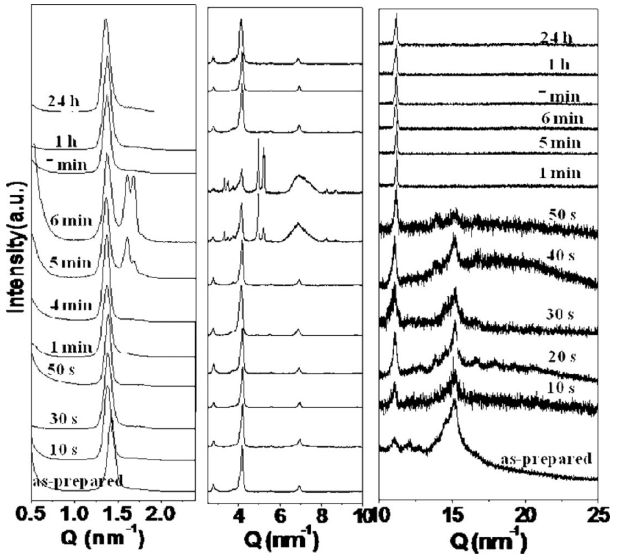

Figure 3. X-ray diffraction patterns of as-prepared lipid membranes exposed to an aqueous SDS solution at different periods.

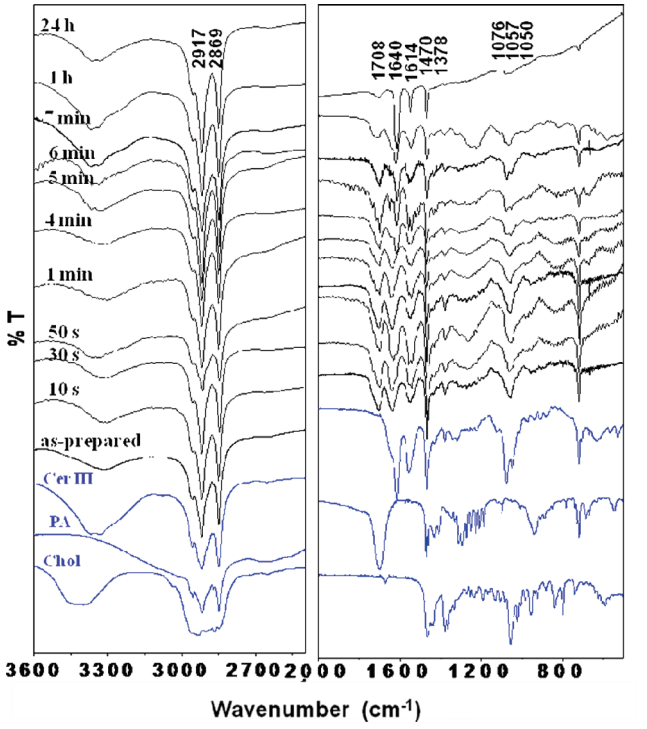

Figure 4. FT-IR absorption spectra of as-prepared lipid membranes exposed to an aqueous SDS solution at different periods.

Damage and Recovery Processes on Lipid Membrane. For the damage process, as-prepared lipid membranes were exposed to an aqueous SDS solution (5.0 mg/cm^3) and kept for different periods of time. The exposed membrane was washed with a small amount of water, dried, and used for measurement.

In the recovery process, damaged membranes (by SDS for 24 h as above) were exposed to an ethanol solution of cholesterol and palmitic acid (5.0 mg/cm^3) and kept for different periods of time. After washed and dried, the membranes were used for measurement.

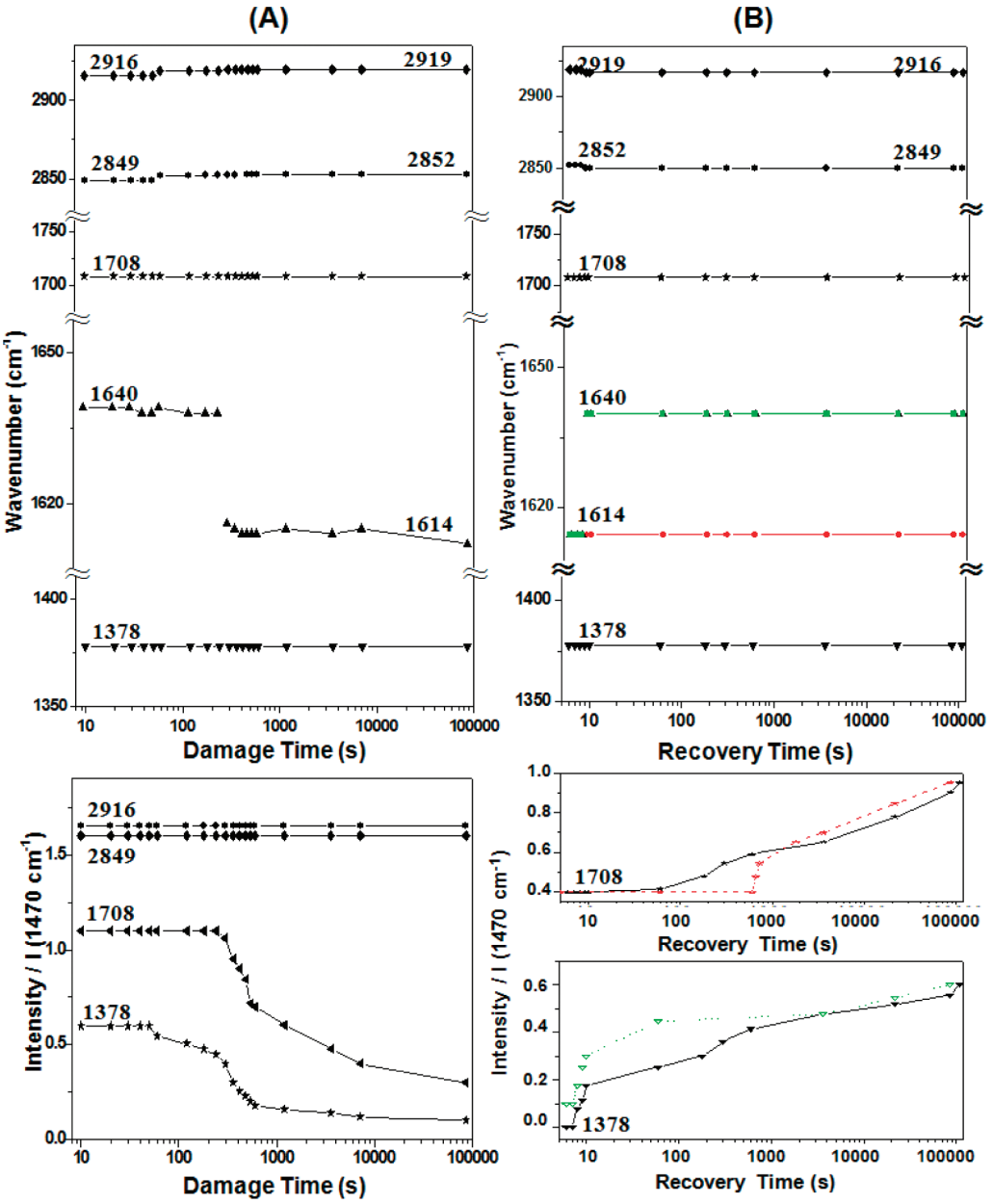

Figure 5. Wavenumber and intensity plots of IR absorption bands of lipid membranes as a function of exposed period. (A) Damage process of as-prepared lipid membranes exposed to an aqueous SDS solution at different periods. (B) Recovery process of damaged lipid membranes exposed to an ethanol solution of lipids at different periods: (black) palmitic acid and cholesterol, (red) palmitic acid, (green) cholesterol. Numerical values in the figure denote IR absorption band positions.

Measurements. The membranes on a cover glass were directly observed with a Nikon ECLIPSE microscope equipped with TE 2000-U stage and Canon pickup camera, from which the images were continuously captured at every 1 min. The microscope was operated under a halogen lamp and with a color-temperature correction filter. The differential scanning calorimetric (DSC) studies were performed using a DSC-100 (Seiko Instrument & Electronics Ltd., Japan) equipped with an SII SSC/5200 H analyzer. The membranes were heated over the temperature range of 5−150 °C at a heating rate of 2 °C/min. X-ray diffraction (XRD) measurements were performed with X-ray diffractometers (RAD-RC, RIGAKU, and D8 Discover, Bruker) with graphite-monochromated high-intensity Cu Kα radiation (λ= 0.154 nm) at 50 and 40 mA and 60 and 40 kV, respectively. Infrared (IR) absorption spectra were acquired with a Bio-Rad Digilab FTS-60A spectrometer in transmittance mode using a KBr pellet. All spectra were an average of 64 scans between 4000 and 400 cm^{-1}. Raman spectra were obtained using a Bomem FT-Raman accessory (Bomem Inc., Canada) mounted on a MB FT-IR optical bench fitted with a liquid nitrogen cooled germanium diode detector and a Nd:Yag laser providing a near-infrared exciting line at 533 nm.

Results and Discussion

Damage of As-Prepared Lipid Membrane. The effect of additives on lipid membrane could be visually detected on the microscope. The optical and fluorescence microscopic observation of lipid membrane during exposure to an aqueous SDS solution was carried out according to the procedure described in the Experimental Section. From optical microscopic images, it was clear that myelin texture was generated from the lipid membrane after exposure. In addition, there were some damages on the membrane (Figure S1 in the Supporting Information). Such effects of SDS on lipid membrane were observed more

obviously by using a fluorescent probe. When pyrene (1/3 weight of lipid mixture) was mixed with lipids before membrane preparation, the lipid membrane emitted fluorescence in domains (Figure 1). Part of the fluorescent domains defocused after 1 min exposure; the defocused parts enlarged after 1 h, and finally, whole membranes defocused after 24 h. Even after a SDS solution was extracted and the remaining lipid membrane was rinsed, the defocused surface was maintained. From these results, it was strongly supported that the embedded pyrene was gradually released from the membrane with damage of the membrane, indicating the simultaneous release of component lipids.

The removal of lipid components from the membrane, which was estimated from microscopy, should influence thermal and structural aspects of the membrane. In terms of DSC measurement of the lipid membrane (Figure 2), five characteristic endothermic peaks were observed for as-prepared lipid membranes. Weak T_1 peak at 41.2 °C is due to the thermal structural transition along the alkyl chain; T_2 and T_3 at 73.7 and 83.2 °C, respectively, are the phase transition temperature of lipids from gel to liquid crystalline state, and T_4 at 100.2 °C corresponds to the melting temperature of lipids.[18] Moreover, T_x at 55.5 °C is assigned to the loss of crystalline structure. After exposed to the SDS solution, the weak T_1 phase transition peak vanished within 5 min, indicating the disappearance of alkyl chain ordering. Meanwhile, the T_3 peak gradually shifted to higher temperature (to ~95 °C) and rather strengthened in intensity. The possibility is the shift of phase transition temperature arising from the variation of lipid components.

The variation in crystal structure of the lipid membrane during exposure to the SDS solution was examined by XRD. Figure 3 shows XRD results of lipid membranes after exposed to the SDS solution at various periods. There were Bragg peaks at Q= 1.42, 2.80, 4.16, 6.90, and 11.19 nm^{-1}. Since these are in the order of 1, 2, 3, 5, and 8, the ordered accumulation of lamellar layers with repeating distance of 4.4 nm is formed in the as-prepared membrane. While meaningful variation on XRD pattern of lamellar structure was not observed until 4 min, it occurred at 5−6 min. In addition to lamellar structure with a repeating distance of 4.4 nm, new lamellar structures with repeating distances of 3.8 and 3.6 nm appeared. However, such structures disappeared at 7 min exposure and remained only lamellar structure with 4.4 nm repeating distance. On the other hand, the existence of a Bragg peak at Q= 15.2 nm^{-1} indicates the ordered structure within the lamellar layer. Whereas the accumulated ordered lamellar structure was conserved even after 24 h exposure to the SDS solution, the structure within the lamellar layer became disordered after 1 min because the 15.2 nm^{-1} peak weakened gradually at the start of the exposure procedure and disappeared by 1 min exposure.

The variation in array structure and composition of lipid membrane after the exposure to the SDS solution can be pursued by spectroscopy. Figures 4 and 5 show IR absorption spectra at various exposure periods and the wavenumber or intensity (against intensity of 1470 cm^{-1} band) variation of various IR absorption bands, respectively. Although IR absorption spectra of as-prepared lipid membrane display characteristic bands of component lipids, the variation during the exposure to the SDS solution depends on the absorption bands. Common characteristic bands of three lipids at 2917 and 2869 cm^{-1}, which are assigned to vibration modes of alkyl chain, shifted to slightly high wavenumber after 1 min exposure, although their intensities are

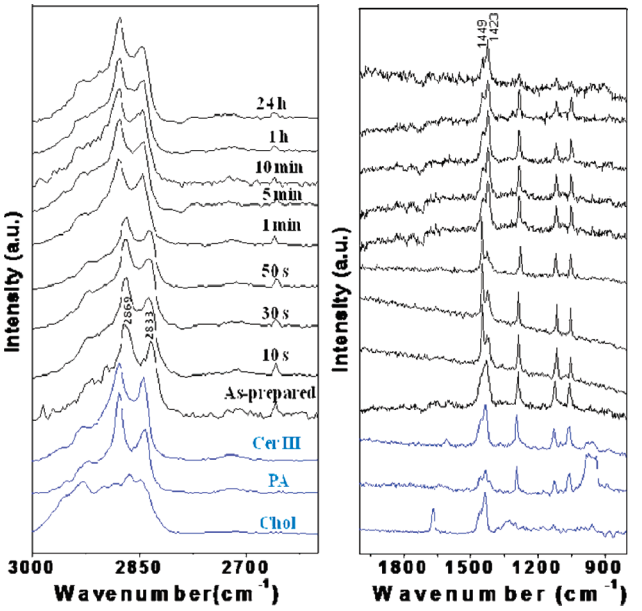

Figure 6. Raman spectra of as-prepared lipid membranes exposed to an aqueous SDS solution at different periods.

invariable. This supports slight configurational change of the alkyl chain. Meanwhile, it should be noticed that an amide C═O stretching vibration band drastically varied from 1640 to 1614 cm^{-1} after 4 min exposure, indicating configurational change of the amide head group in ceramide III.

Moreover, the intensities of 1708 and 1378 cm^{-1} bands, which are assigned a carbonyl C═O stretching vibration mode of palmitic acid and a COH in plane bending mode of cholesterol, respectively, decreased after certain exposure period, although the wavenumbers remained constant. The former decreased after 5 min exposure, and the latter did after 1 min. At the same time, the intensity of C−O stretching band of cholesterol at 1057 cm^{-1} decreased, but two C−O bands of ceramide III at 1076 and 1050 cm^{-1} remained. These results confirm the removal of palmitic acid after 5 min and cholesterol after 1 min from the lipid membrane. Finally, the IR spectrum after 24 h exposure resembled that of neat ceramide III with a trace of palmitic acid.

Raman spectra and wavenumber or intensity variation (against intensity of the 2869−2878 cm^{-1} band) during exposure period are shown in Figures 6 and 7, respectively. Two stretching vibration bands of alkyl chains at 2869 and 2833 cm^{-1} shifted abruptly to 2878 and 2847 cm^{-1}, respectively, without clear intensity variation, after 1 min exposure. At the same time, the sharp intensity conversion of a deformation band of alkyl chains was observed between 1447 and 1423 cm^{-1}, keeping constant intensity. These variations indicate the configurational change of alkyl chains with exposure to the SDS solution, consistent with the behavior of the corresponding IR spectrum. Raman spectrum after 24 h exposure was again mostly similar to that of ceramide III.

Recovery of Damaged Lipid Membrane. In the cosmetics market, great efforts are made to recover the undesirable damages on human skin. The use of fat oil additives can at least partially restore the lipid film.[18] Petrolatum brings about an immediate partial restoration for the skin barrier properties of the stratum corneum, whereas physiological lipid mixtures slowly penetrate

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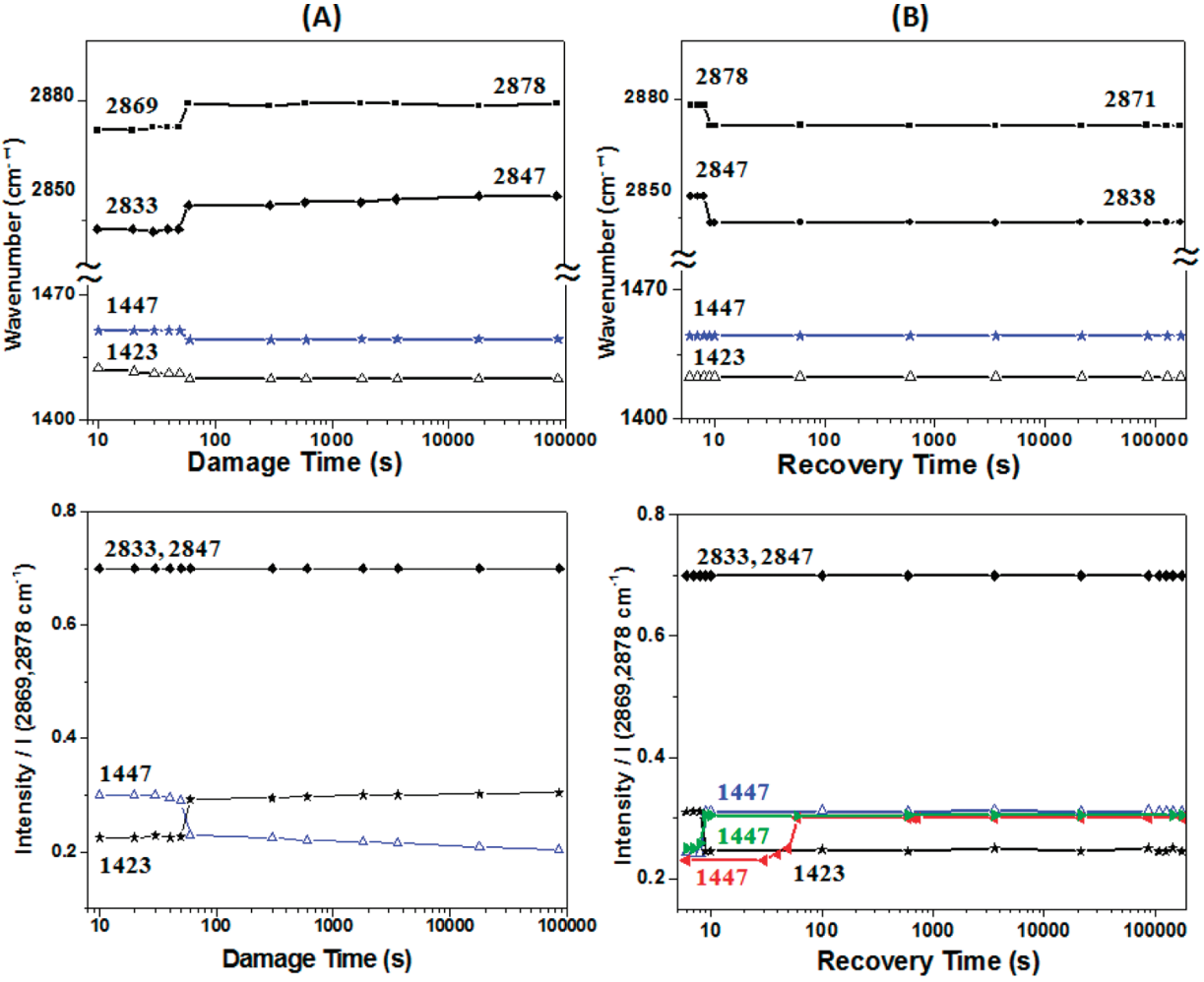

Figure 7. Wavenumber and intensity plots of Raman bands of lipid membranes as a function of exposed period. (A) Damage process of as-prepared lipid membrane exposed to an aqueous SDS solution at different periods. (B) Recovery process of damaged lipid membrane exposed to an ethanol solution of lipids at different periods: (black, blue) palmitic acid and cholesterol, (red) palmitic acid, (green) cholesterol. Numerical values in the figure denote IR band positions.

into the epidermis, build up the skin barrier again, and help surfactant-induced scaly skin to recover by naturally accelerating the process of metabolization in the lamellar bodies.[19] Then, in the present work, evaluating the recovery of lipid membrane, which incurred SDS-induced damage, was investigated by additives of two lipids, palmitic acid and cholesterol, because these lipids were elucidated through damaging examination to be mainly eluted from lipid membrane after the exposure of as-prepared lipid membrane to an aqueous SDS solution.

DSC was measured after the damaged lipid film (by SDS for 24 h) was exposed to an ethanol solution of palmitic acid and cholesterol. As seen in Figure 2, the weak T_1 peak corresponding to the phase transition along alkyl chains[18] appeared within 10 s exposure, indicating the recovery of the structure. The T_3 peak of the transition from gel to liquid crystalline weakened during 5 min exposure, suggesting the penetration of lipid components eluted. The behavior of both endothermic peaks during the exposure to the lipid solution confirms the structural recovery of damaged lipid membrane to the as-prepared one. The peak at T_x, however, almost disappeared after 6 h exposure, being equivalent to the disappearance of the crystalline structure. In Figure 2B, the DSC profile after the exposure for 6 h and more approaches the DSC thermogram of enough hydrated human skin.[20]

The structural recovery of lipid membrane was also evaluated from XRD by exposing the damaged lipid membrane to ethanol solutions of palmitic acid and cholesterol (mixture and each neat lipid), as shown in Figure 8. It should be noticed that the meaningful variations of peaks were observed in the small angle region ($Q < 12$ nm^{-1}) of XRD. In addition to the gradual decrease in the intensity of the first-order peak of lamellar structure, repeat distance of lamellar structure changed from 4.5 to 4.9 nm after 16 min exposure to the mixed solution. The similar meaningful variations in the small-angle region were also observed, even when the membrane was treated with a palmitic acid solution or a cholesterol solution (Figure 8). A Bragg peak at Q= 15.2 nm^{-1} in the XRD pattern arose after 16 min exposure to a mixed solution of palmitic acid and cholesterol and gradually strengthened (Figure S2 in the Supporting Information), indicating the appearance (recovery) of ordering in the lamellar layer, although the recovery was less on the exposure to a solution of neat palmitic acid or neat cholesterol. As seen in the wide-angle region ($Q > 12$ nm^{-1}) in Figure S2, there is no distinctive Bragg peaks except a peak at Q= 15.2 nm^{-1} irrespective of as-prepared or the recovered one (see Figure 3, too). This is completely different from component lipid membranes with ordered lateral

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structure because of the mutual incorporation of component lipids in the lipid mixture membranes.

The time course of IR spectra on the exposure process to lipid solutions and the plots of wavenumber or intensity versus exposure time are shown in Figures 5 and S3 in Supporting Information, respectively. While 2919 and 2852 cm^{-1} bands of alkyl chains returned to 2916 and 2849 cm^{-1}, respectively, a 1614 cm^{-1} band of amide (ceramide III) recovered to 1640 cm^{-1} immediately after 8 s exposure to a mixed solution of palmitic acid and cholesterol, although the intensities of these bands scarcely changed. These indicate that the structure of ceramide III recovered. Even if damaged membrane was exposed to a cholesterol solution, the structural recovering of ceramide III happened, as well. However, the exposure to a palmitic acid solution did not recover the structure of amide. This confirms that the coexistence of cholesterol affects the structure of ceramide III.

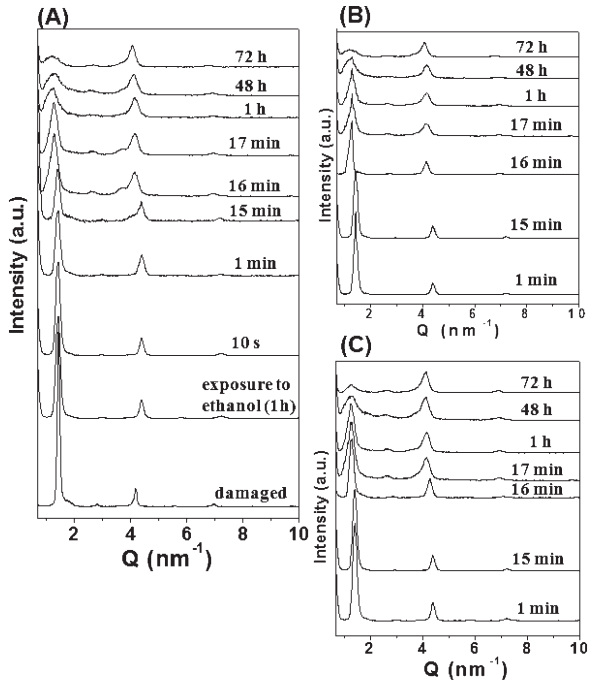

Figure 8. X-ray diffraction patterns of damaged lipid membranes exposed to an ethanol solution of lipids at different periods: (A) palmitic acid and cholesterol, (B) palmitic acid, and (C) cholesterol.

A 1708 cm^{-1} band of C=O stretching mode (palmitic acid) gradually began to intensify after 3 min exposure to an ethanol solution of palmitic acid and cholesterol. On the other hand, the intensification of a 1378 cm^{-1} band (COH in-plane bending mode of cholesterol) increased after 8 s exposure to the same solution. However, the former increased in intensity after 11 min exposure to an ethanol solution of palmitic acid, while the latter did after 8 s exposure to cholesterol in ethanol. These results suggest that the penetration rate of cholesterol is faster than that of palmitic acid.

It is apparent from Raman spectra in Figures 7 and S4 in Supporting Information that two bands at 2878 and 2847 cm^{-1}, stretching vibration mode of alkyl chains, abruptly shifted to 2871 and 2838 cm^{-1} within 9 s exposure to a mixed solution of palmitic acid and cholesterol. At the same time, the intensity of bending bands of alkyl chains at 1447 and 1423 cm^{-1} dramatically changed. These results indicate that alkyl chains recover ordering partially toward that in as-prepared lipid membrane. The Raman band shift of the stretching vibration band of alkyl chains occurred after 11 min on the membrane exposure to a solution of palmitic acid, while the exposure to a solution of cholesterol allowed the shift of these two bands after 9 s. The corresponding time of bending vibration bands of alkyl chains occurred at 40−50 and 9 s, respectively. The gap between a solution of palmitic acid and a solution of cholesterol is along with IR results.

Process and Mechanism of Damage/Recovery on Lipid Membrane. It is particularly intriguing to elucidate the successive

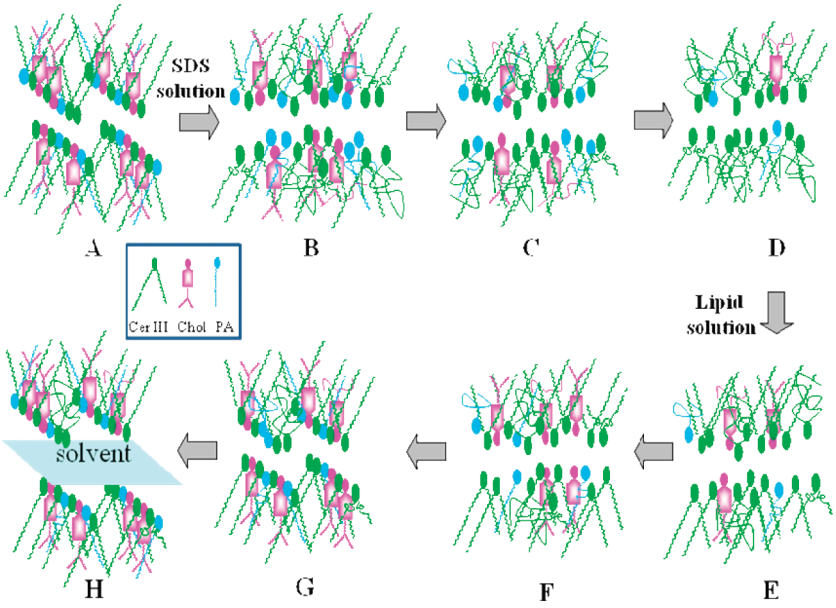

Figure 9. Schematic diagram of the damage/recovery process by SDS on lipid membrane. Damage process: (A) as-prepared lipid membrane; (B) 1 min exposure, detectable elution of cholesterol, disappearance of intralayer ordering of lamellar structure, and partial structural change of alkyl chain); (C) 5−6 min exposure, structural change of hydrophilic head (amide) group of ceramide III, and detectable elution of palmitic acid; (D) 24 h exposure, remanence of majority ceramide III. Recovery process: (E) 8−9 s exposure, start of cholesterol penetration, recovery of amide structure of ceramide III, and recovery of alkyl chain structure; (F) 3 min exposure, start of palmitic acid penetration; (G) 16 min exposure, start of recovery of intralayer structure in lamellar layer, and increase of lamellar repeat distance; (H) 48 h exposure, considerable recover of intralayer structure, and recovery of membrane component.

influence of SDS on three lipid components in mimic SC membrane and the recovery process by additive lipids. The plausible process is illustrated in Figure 9. Through the qualitative analyses described above, it is evident from IR results that cholesterol gets to elute detectably from the lipid mixture membrane after 1 min exposure, palmitic acid does after 5 min exposure, and ceramide III remains in the lipid membrane as a less soluble component. It can be noted that cholesterol is eluted easier than palmitic acid. However, it is also mentioned that the intralayer ordering of lamellar structure disappears at the early stage before 1 min exposure, as elucidated from XRD results. Additionally, partial structural variation of the alkyl chain occurs at 1 min exposure. These results indicate that even extremely small elution of lipids influences the structure within the lamellar layers of lipid membrane at an early step of the exposure to a SDS solution.

However, as seen from the behavior of an amide C═O band and XRD peaks, the configurational structure of the hydrophilic domain of ceramide III and the ordered structure within the lamellar layer vary after 4−6 min exposure, where lipids are meaningfully eluted. These results can be explained by the penetration of SDS and solvent (water) in the lipid membrane, the coupling of SDS with cholesterol and palmitic acid, and the elucidation of coupled molecules. The structure of ceramide III as one of the components in the mixed lipid membrane varies with the formation and elucidation of molecular coupling and approaches that of neat ceramide III. Incidentally, neat ceramide III exhibits a main IR amide C═O band at 1615 cm^{-1} and Raman alkyl vibration bands at 2878, 2844, and 1434 cm^{-1}. The configurational change along the alkyl chains and the componential change (elution) in the lipid membrane also appeared in the DSC results and microscopic observation. It must be noticed that, although SDS is assumed to be penetrated into lipid membrane, the characteristic IR bands (SO_3 stretching) of SDS at 1251 and 1222 cm^{-1} were not observed. Since penetrated SDS molecules do not strongly (or specifically) interact with lipid membrane (or lipids), they should be washed out during the procedure of rinse.

In term of the recovery process, at 8−9 s exposure of the damaged lipid membrane to a mixed solution of palmitic acid and cholesterol, cholesterol starts to penetrate into the lipid membrane. At the same time, the amide and alkyl chain structures of ceramide III recover. At 3 min exposure, palmitic acid starts to penetrate into the membrane. Moreover, at 16 min exposure, the structure within the lamellar layer starts to recover. After 48 h exposure, the intralayer structure considerably recovers its ordering and the component of the membrane gets close to that of original (as-prepared) membrane.

It must be noted that the recovery of amide structure is contributed by cholesterol but not palmitic acid and that the structure of alkyl chains is recovered at 8−9 s exposure by cholesterol but at 11 min exposure by palmitic acid. Accordingly, it can be mentioned that cholesterol is dominant on the recovery process. Moreover, it may be focused that, at 16 min exposure, repeat distance of lamellar layers increases from 4.5 to 4.9 nm. It is assumed that the small amount of molecules, like solvent, is intercalated between the lipid layers in the head group regions of the lipids, resulting in an increase in the distance of lamellar layers. This should be related to the disappearance of the T_x peak in DSC that occurs under the condition of enough hydration.

Conclusions

Mimetic lipid membrane used in this investigation consists of three lipids, which are the main constitutional substances in SC, the tissue with a complex structure. However, the simplified model membrane used has shown to be useful for evaluation of the relation among ceramide III, palmitic acid, and cholesterol against the action of the anionic surfactant, SDS. Furthermore, in the situation where only limited information is available on the effects of surfactants on lipid membrane,[13−15] there is a need to explore this research field in more detail and to further understand the recovery of skin damaged by surfactants. In the present work, the effective approach for monitoring structural reorganization in the damaged membrane was used on the recovery investigation following a series of treatments. In the outcome, the effective approach ascertained recovering the damaged membrane by eluted lipids. The present results provided important information on the SC lipid profile in healthy and diseased human skin with relation to the SC lipid organization and to the skin barrier function. The methodology can be transposed to in vivo studies to evaluate the degree of damage and the duration for recovery of skin barrier function.

Supporting Information Available: Additional characterization by optical microscopic images, XRD patterns, IR absorption spectra, and Raman spectra are provided. This material is available free of charge via the Internet at http://pubs.acs.org.