Dielectric Study Concerning the Dynamics of Water in Artificial Stratum Corneum Lipids

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The dielectric properties were studied on an artificial stratum corneum lipids–water system, consisting of a pseudo-ceramide, stearic acid, cholesterol, and water, by using time-domain reflectometry. Two relaxation processes were found in the lipid mixtures of pseudo-ceramide/stearic acid (1/1 mole ratio)/cholesterol–water (10 wt%), over a frequency range of 10^7—10^{10} Hz. The high-frequency relaxation peaks, located at around 7—16 GHz, were attributed to free water relaxation. The low-frequency processes were observed around 500—900 MHz, and were concluded to be due to bound water relaxation in the lipid matrix, judging from the relaxation time. Upon increasing the cholesterol concentration, a progressive increase of the relaxation strength of free water appeared, whereas that of bound water decreased. In this system, the incorporation of cholesterol was found to decrease the entropy changes by melting, which reflected the disorder of the arrangement of the lipid molecules. Therefore, changes in the dielectric properties, due to an increase in cholesterol, would be associated with the structural changes of lipids.

The stratum corneum lipids (SCL) are known to provide an epidermal barrier against water loss through skin.[1,2] The effective barrier function has been attributed to a bilayer-forming capacity of SCL in the intercellular space of the stratum corneum (SC).[3—5] Therefore, the mechanism for the self-assembly of a lipid bilayer have been a subject of considerable interest.[6—10] The SC lipid (SCL) lamellae is predominantly made up of ceramide, free fatty acids, cholesterol, and cholesteryl esters; the presence of ceramides has been suggested to be the basis for the structural organization of SCL in bilayers.[3,11] Moreover, it has been reported that ceramides play an important role in the water-retaining properties of SC.[12—15]

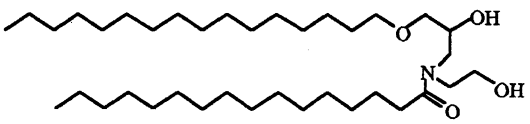
We have investigated the thermotropic behavior and structural characteristics of SCL using a pseudo-ceramide, named sphingolipid E (SLE). The pseudo-ceramide has a molecular structure analogous to that of the natural ceramide of type 2,[16] and exhibits water-retaining properties similar to those of natural lipids.[12—15] We have found that an equimolar mixture of SLE and long-chain saturated fatty acids (stearic and/or palmitic acid) form a stable lamellar structure.[17—19] Furthermore, a previous study showed that the incorporation of cholesterol into an anhydrous and/or hydrated equimolar mixture of SLE and stearic acid, causes a marked decrease in the melting entropy as well as the apearance of a liquid lateral packing of hydrocarbon chains.[20] The effect of cholesterol in this α-gel phase could be attributed to the disorder of the molecular arrangement in the lamellar planes. However, there is no information available concerning the dynamics of water in such artificial SC lipids. It would be very important to study the dynamics and structure of water in the lipid matrix, which is responsible for the barrier properties of SC lipids.

In the present study, we report on the dielectric properties of water at 25 °C in the SLE/stearic acid (1/1 mole)–cholesterol system, which is a simplified model for natural SCL. The dielectric measurements were performed by employing a time-domain reflectometry (TDR) method which covers 10^7—10^{10} Hz. This TDR method has been developed for high-precision dielectric measurements in the gigahertz region, especially for measurements of aqueous solutions[21—25] and of adsorbed water on solid surfaces.[26,27] The technique can observe the relaxation processes of free water and bound water, directly and separately, whereas the other classical methods, such as differential scanning calorimetry and nuclear magnetic resonance cannot.

Thus, the main object of the present study was to elucidate the dynamic behavior of water in simplified SCL containing synthetic pseudo-ceramide. Furthermore, we discuss the relationship between the dynamic properties of water in the lipid matrix and the aggregation structure of the lipids as a function of the cholesterol concentration.

Experimental

Materials. The pseudo-ceramide (Scheme 1), *N*-(3-hexadecyloxy-2-hydroxypropyl)-*N*-(2-hydroxyethyl) hexadecanamide (sphingolipid E; SLE) was obtained from Kao Co. (Tokyo, Japan), and the purity was greater than 99%, as measured by a high-performance liquid-chromatographic analysis. Stearic acid and cholesterol of reagent grade were purchased from Wako Pure Chemical Industry (Osaka, Japan). Water and chloroform were of spectrophotometric grade.



Scheme 1. Molecular structure of Sphingolipid E.

Preparation of Samples. Appropriate amounts of lipids containing SLE (5—10 g) were weighed into glass test tubes with a Teflon®-sealed screw cap, and were melted at 90—100 °C to ensure homogenous mixing. Then, the samples were cooled to 25 °C. Hydration of samples was achieved by heating the lipid mixtures and water (water concentration = 10 wt%) at 80 °C for 30 min with vigorous agitation. The samples were then cooled to 25 °C and maintained at that temperature for 7 d or more, after which all measurements were started.

Time Domain Reflectometry (TDR). The TDR system employed in this work was nearly the same as that reported by Mashimo et al.[21] A step pulse (35 ps), generated by a sampling oscilloscope (HP54121T, Hewlett Packard), was used. The complex permittivity ($\varepsilon^*(\omega)$) of an unknown sample is given as a function of the known permittivity ($\varepsilon_s^*(\omega)$) of the reference sample as

$$\varepsilon^*(\omega) = \varepsilon_s^*(\omega)\frac{1+\{(c_{fs})/[j\omega(\gamma_d)\varepsilon_s^*(\omega)]\}\rho f_x}{1+\{[j\omega(\gamma_d)\varepsilon_s^*(\omega)]/(c_{fs})\}\rho f_s}, \quad (1)$$

where

$$\rho = \frac{r_s - r_x}{r_s + r_x},$$

$$f_x = Z_x \cot Z_x,\ Z_x = (\omega_{d/c})\varepsilon^*(\omega)^{1/2},$$

and

$$f_s = Z_s \cot Z_s,\ Z_s = (\omega_{d/c})\varepsilon_s^*(\omega)^{1/2}.$$

Here, d is the cell length, γd is the electric length, r_s and r_x are the Fourier transform of the reflected pulse from the reference sample $R_s(t)$ and that from the unknown sample ($R_x(t)$), j is the imaginary unit, ω is the angular frequency, and c is the speed of propagation in vacuo. Therefore, the permittivity ($\varepsilon^*(\omega)$) of the unknown sample is given by measuring $R_s(t)$ and $R_x(t)$.[24] In the experiments, we used an electrode with an electric length (γ_d) of 0.29 mm, and chose chloroform as the reference sample. All of the measurements were performed at 25 °C.

Results and Discussion

Two relaxation peaks were found in hydrated mixtures of SLE/stearic acid (1/1 mole)–cholesterol (0—43 mol%), where the water contents were 10 wt% over a frequency range of 10^7—10^{10} Hz. Typical dielectric spectra at 25 °C are shown in Fig. 1. The absorption of ε'' comprised of two parts: A high-frequency part (peak A in Fig. 1) observed at f=10^9—10^{10} Hz, and a low-frequency part at f=10^7—10^9 Hz (peak B in Fig. 1). The complex permittivity was expressed by the sum of the Debye-type relaxation processes[23] (Eq. 2),

$$\varepsilon^*(\omega) = \varepsilon_\infty + \frac{\Delta\varepsilon_l}{1+j\omega\tau_l} + \frac{\Delta\varepsilon_h}{1+j\omega\tau_h}, \quad (2)$$

where ε_∞ is the constant extrapolated to $\omega=\infty$, $\Delta\varepsilon$ is the relaxation strength, and τ is the relaxation time. These parameters in Eq. 2 were determined by a least-square fitting procedure, and are given in Table 1. An example of the best-fitted curve to the experimental data (cholesterol=43.1 mol%) is shown in Fig. 2. The relaxation strength for both the high-relaxation and low-relaxation process showed a great dependence on the cholesterol concentration, as can be seen in Fig. 3. With increasing the cholesterol concentration, the relaxation strength ($\Delta\varepsilon_h$) of the high-frequency process progressively increased (see Fig. 3 and Table 1) and the relaxation time (log τ_h) shifted to a higher frequency region (see Table 1). The literature value of the relaxation time for pure water at 25 °C is 8.7 ps (18 GHz); therefore, the high-frequency process at around 10—20 ps (7—16 GHz) was concluded to be due to the orientation of free water, judging form the value of the relaxation time (τ_h). However, the relaxation strength ($\Delta\varepsilon_l$) of the low-frequency process at around 0.2—0.3 ns (0.5—0.8 GHz) decreased due to the incorporation of cholesterol. At a cholesterol content of 33 mol% (30 wt% except for water), an approximate composition in the natural SC lipids without cholesteryl esters, cholesteryl sulfate, and other minor components,[6,20] the relaxation strength ($\Delta\varepsilon_l$) of the low-frequency process was reduced by 30% from that of the cholesterol-free lipids, and that of the high-frequency relaxation ($\Delta\varepsilon_l$) increased by 21%. Since changes in the intensity for both at the relaxation processes correlate with each other, and the relaxation intensity of low-frequency process decreased with increasing the intensity of free water relaxation, the low-frequency process could be attributed to

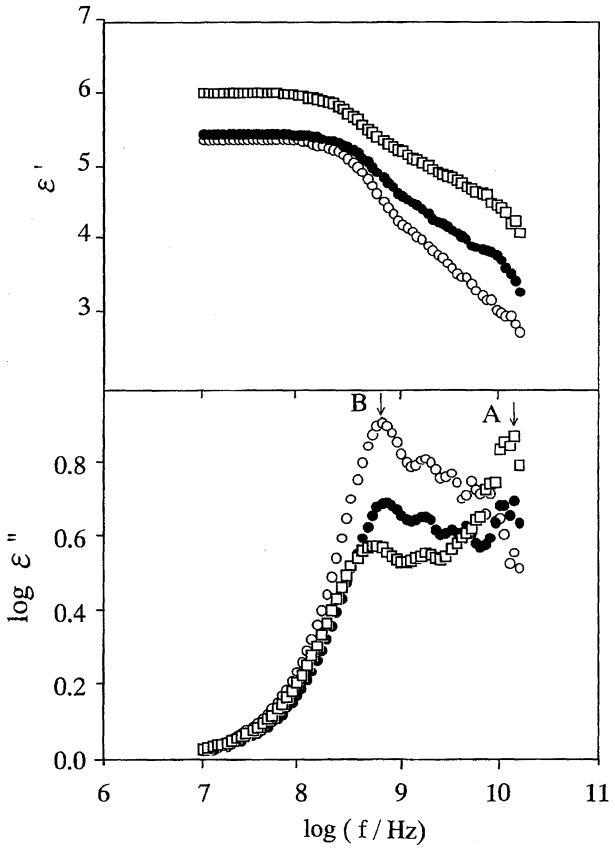


Fig. 1. Dielectric dispertion (ε') and absorption (ε'') curves at 25 °C for hydrated sphingolipid E (SLE)/stearic acid/cholesterol mixtures (SLE/stearic acid=1 : 1 mole, water content=10 wt%) with mol% of cholesterol being 0 (○), 22.1 (●), and 43.1 (□).

Table 1. Dielectric Relaxation Parameters in Eq. 2 Determined for Sphingolipid E (SLE)/Stearic Acid/Cholesterol and Water (10 wt%) System[a] at 25 °C

	Low-frequency relaxation		High-frequency relaxation		
Cholesterol/mol%	$\log(\tau_l/s)$	$\Delta\varepsilon_l$	$\log(\tau_h/s)$	$\Delta\varepsilon_h$	ε_∞
0	−9.71	1.71	−10.68	1.02	2.63
11.3	−9.74	1.47	−10.66	0.96	3.19
22.1	−9.67	1.26	−10.80	1.01	3.14
32.8	−9.69	1.18	−11.00	1.23	3.29
43.1	−9.55	1.11	−11.01	1.65	3.22

a) SLE/stearic acid=1/1 mole ratio.

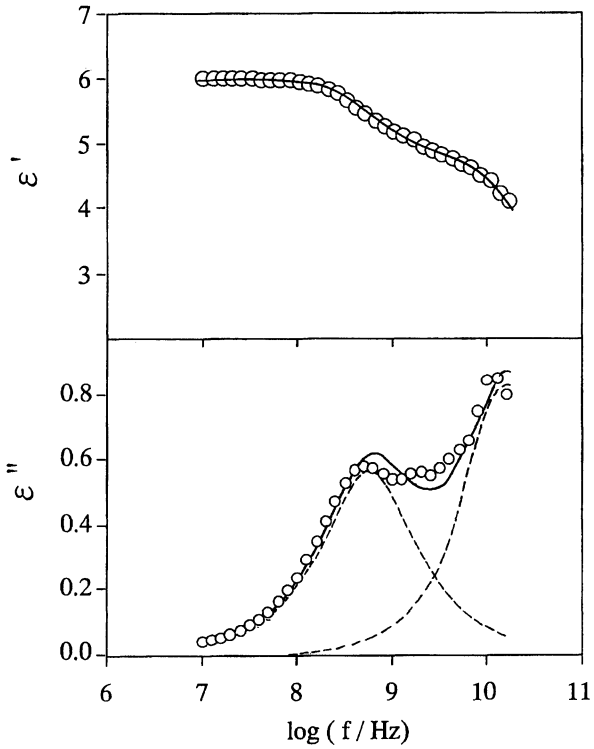


Fig. 2. The best fit curve to the dispersion (ε') and absorption (ε'') at 25 °C for hydrated sphingolipid E (SLE)/stearic acid/cholesterol mixtures (SLE/stearic acid=1 : 1 mole, water content=10 wt%) with 43.1 mol% of cholesterol.

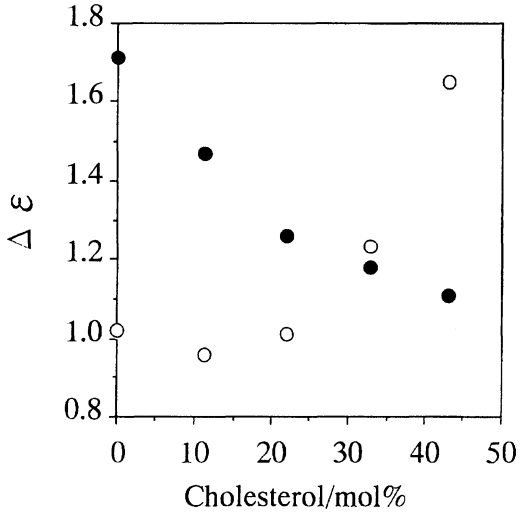


Fig. 3. Variation of relaxation strengths $\Delta\varepsilon_l$ (●), and $\Delta\varepsilon_h$ (○) for hydrated sphingolipid E (SLE)/stearic acid/cholesterol mixtures (SLE/stearic acid=1 : 1 mole, water content=10 wt%) as a function of cholesterol concentration.

the orientation of bound water molecules in the lipid matrix. The dielectric strength ($\Delta\varepsilon$) is a rough measure of the water content multiplied by the square of its dipole moment. If the polarity is not much changed upon absorption, the ratio $\Delta\varepsilon_l/\Delta\varepsilon_h$ would be equal to the ratio of the water contents, bound to free.[22] Figure 4 clearly shows that there is a good linear relationship between $\Delta\varepsilon_l/\Delta\varepsilon_h$ and the cholesterol concentration. Also, the total water content is known to be 0.1 g/0.9 g of lipids, from which we can estimate the bound water and free water content. The amounts of the bound and free water content were calculated, and are summarized in Table 2.

Upon increasing the cholesterol content, the amount of free water progressively increased, whereas that of bound water in the lipid matrix decreased. The numbers of bound water per one lipid molecule were also calculated, and are given in Table 3. At a cholesterol content of 33 mol%, the approximate composition in the natural SC lipids, the artificial SC lipids bound 1.3 molecules of water per one lipid molecule. In a previous study, cholesterol was shown to decrease the entropy changes by the melting (ΔS_m) of an equimolar mixture of SLE and stearic acid, and to enhance the mobility of the hydrocarbon chains of the constituent lipids.[20] Moreover, a linear relationship was found between the ΔS_m and the mole fraction of cholesterol over the range of 0—33 mol% cholesterol (see Table 2). The magnitude of the melting entropy (ΔS_m) reflects the degree of order (or disorder) of molecular packing below the melting point (55—60 °C); therefore, the effect of cholesterol in this lamellar α-gel can be attributed to the disorder of the molecular packing of lipids.[20] Changes in the dielectric properties of water in these artificial SC lipids seem to be related to those of the aggregation structure and molecular packing of the lipid molecules in bilayers. To clarify the relation between the dielectric properties of water in this artificial SCL and the lipid molecular packing, we examined the relationships between the amount of bound (free) water and the ΔS_m in this hydrated lipid system. The estimated amount of bound and free water is plotted against the logarithm of the ΔS_m in

Table 2. Estimated Amount of Bound Water and Free Water, and Entropy Changes by Melting for Sphingolipid E (SLE)/Stearic Acid/Cholesterol and Water System[a)]

Cholesterol/mol%	Bound water[b)]/g%	Free water[b)]/g%	ΔS_m[c)]/J K^{-1} mol^{-1}
0	7.0	4.2	118.9
11.3	6.7	4.4	71.7
22.1	6.2	4.9	38.5
32.8	5.4	5.7	5.7
43.1	4.5	6.6	0.6

a) SLE/stearic acid=1/1 mole ratio. b) Weight of water per 1 g of lipids (25 °C). c) Entropy calculation is based on the weight of lipids and that of water neglected (from Ref. 20).

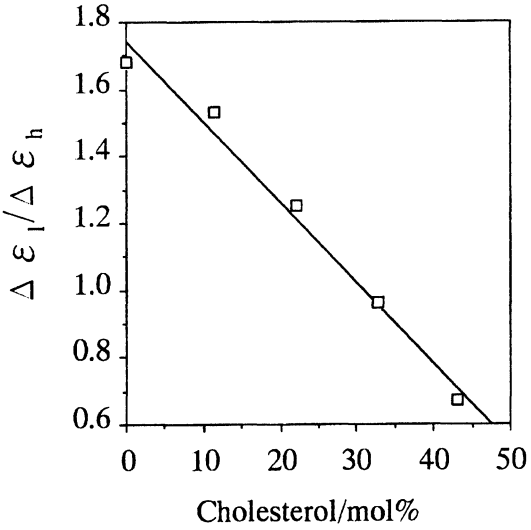


Fig. 4. Variation of the ratio of relaxation strengths $\Delta\varepsilon_l/\Delta\varepsilon_h$ for hydrated sphingolipid E (SLE)/stearic acid/cholesterol mixtures (SLE/stearic acid=1 : 1 mole, water content=10 wt%) as a function of cholesterol concentration.

Table 3. Numbers of Bound Water Molecule in the SLE/Stearic Acid/Cholesterol Mixtures[a)] at 25 °C

Cholesterol/mol%	Numbers of bound water[b)]
0	1.7
11.3	1.6
22.1	1.5
32.8	1.3
43.1	1.0

a) SLE/stearic acid=1/1 mole ratio.

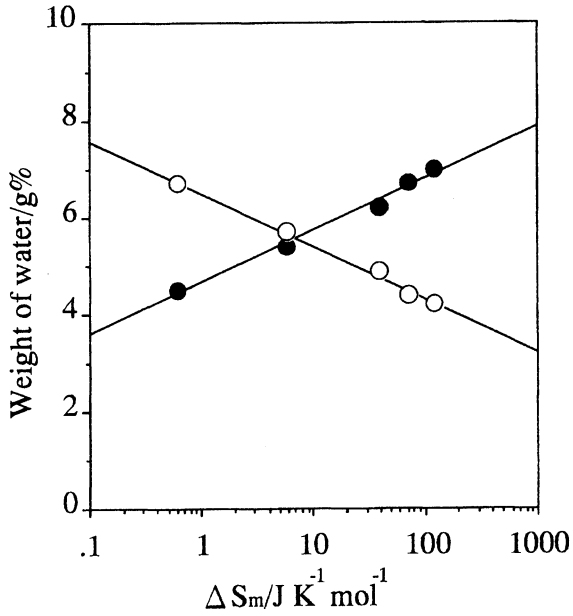


Fig. 5. Relationships between the amount of bound water (●) and of free water (○) at 25 °C for hydrated SLE/stearic acid/cholesterol system (SLE/fatty acid=1/1 mole, total water content=10 wt%), and melting entropy (ΔS_m, from Ref. 21).

Fig. 5. The figure clearly shows a good linear relationships between the amount of both bound and free water, and the logarithm of ΔS_m for the SLE/stearic acid (1/1 mole)–cholesterol–water system. These results successfully show that the dynamic properties of water molecules in lipid bilayers are strongly affected by the lipid molecular packing, and that an enhancement of the molecular motion of the lipids increases the amount of free water. Moreover, the relaxation time of free water decreased along with the cholesterol content (see Table 1); thus, the molecular motion of free water was believed to be increased by the incorporation of cholesterol. Suzuki and co-workers[17)] measured the amount of non-freezing water at around 0 °C of the hydrate SLE/stearic acid (1/1 mole)–cholesterol system using of differential scanning calorimetry (DSC). The reported values for the amount of non-freezing water for lipids containing 0, 22, and 43 mol% cholesterol were 6.8, 6.6, and 8.3 g%, individually. Although the amount of non-freezing water measured by the DSC technique for cholesterol-free samples was nearly equal to that of bound water (7.0 g%) by the TDR method, data for 22 and 43 mol% cholesterol were inconsistent with each other (see Table 2). In addition, the difference between the amount of non-freezing water and that of bound water increased with increasing the cholesterol concentration. One interpretation of these results would suggest that water molecules in the cholesterol-free samples were tightly confined to the polar region of the lipids, and existed as interlamellar water, even at an elevated temperature (25 °C). The incorporation of cholesterol into the bilayer loosens the molecular packing of lipids and decreases the interaction between water and hydrophilic part of lipids at higher temperatures, although these water molecules are loosely bound to the lipid matrix, and are not frozen at around 0 °C. Therefore, by increasing

the cholesterol content, loosely bound water between the bilayers may be released outside the bilayers by a temperature increase (0→25 °C).

In conclusion, the dynamic properties of water in a hydrated equimolar mixture of psuedo-ceramide and stearic acid, forming a lamellar gel, were drastically changed by the incoporation of cholesterol. Judging from the relaxation strength and time of the dielectric relaxation at around 7—16 GHz, upon increasing the cholesterol content, the amount of free water was found to increase progressively, and the molecular motion of free water was also enhanced. However, the amount of bound water confined in the bilayers decreased with the cholesterol content. Furthermore, the dynamic properties of water were closely related to the molecular motion or arrangement of lipids in bilayers, and an enhancement in the mobility of lipids resulted in a decreased amount of bound water in the interlamellar layers. The results presented here strongly support the idea that cholesterol can regulate the mobility of a natural SC lipid bilayer so as to affect the water-holding properties of SCL, which would also be responsible for the SC barrier properties.

Abbreviations: ΔS_m, melting entropy; TDR, time domain reflectometry; SCL, stratum corneum lipids; SLE, sphingolipid E; $\Delta\varepsilon_h$, relaxation strength of high-frequency process; $\log \tau_h$, relaxation time of high-frequency process $\Delta\varepsilon_l$, relaxation strength of low-frequency process; $\log \tau_l$, relaxation time of low-frequency process.

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