Cholesterol-Induced Formation of Liquid Ordered Phase–Like Structures in Non-Phospholipid Systems

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Abstract: The formation of liquid ordered (L0) phase–like structures in stearyltrimethylammonium chloride/cholesterol/1,3-butane diol/water and hepta(oxyethylen) octadecyl ether/cholesterol/1,3-butane diol/water systems was investigated. Differential scanning calorimetry and X-ray scattering measurements confirmed that L0 phase–like structures were formed in both surfactant/cholesterol systems, similar to the lysophospholipid/cholesterol system. It was revealed that the concentration of cholesterol at which only L0 phase–like structures are formed increases in the order lysophospholipid < hepta(oxyethylen) octadecyl ether. In addition, for both surfactants, the interlayer spacing, d, was larger for L0 phase–like structures than for α-gel structures. These results suggest that the ionicity and structure of the hydrophilic group of each surfactant play important roles.

Key words: liquid ordered phase–like structure, cholesterol-induced structure, phase transition, gel

1 INTRODUCTION

The gel-liquid crystal intermediate state, reported in 1976 by Demel and Kruyff4), has been called the liquid-ordered (L0) phase in more recent years. The L0 phase demonstrates intermediate properties between a hydrated solid (gel) and a liquid crystal and forms a lamellar structure. There are numerous studies involving the L0 phase, and examples include evaluation of the L0 phase by nuclear magnetic resonance (NMR)3, a study of model biomembranes6, a study of thermodynamic models7, and a study of L0 phase separation in giant unilamellar vesicle (GUV)8.

α-Gel, which has a lamellar structure and is often used in cosmetics, is composed of a surfactant, higher alcohol, and water. Its packed (sub-cell) structure of hydrophobic groups is a hexagonal sub-lattice. In other words, its structure is a hydrated crystalline phase with hexagonal packed lipophilic groups forming network structure resulting in gelling excessive solvent. A number of studies have been carried out from the standpoint of evaluating the skin moisturization, safety, and sensory characteristics of α-gels. For example, it was reported that α-gel dispersion consisting of cetyl alcohol has a high moisturizing effect. It was also reported that an emulsion surrounded by a lamellar layer displays excellent moisturizing effects on the skin compared with an ordinary emulsion which was surrounded by surfactant monolayer. Other studies have examined the skin effects of high-water-content α-gel consisting of pseudo-ceramide13, and α-gel formed with sodium methyl stearoyl taurate/behenyl alcohol/water12. A cationic surfactant was shown to form an α-gel with a higher alcohol and water11, and there are numerous related studies in the rheology field16-19. The gel-liquid crystal transition can negatively affect the stability of cosmetics. Accordingly, in the field of cosmetics, in which stability over a wide temperature range is desirable, controlling the temperature dependence of the α-gel structure is an important task.

Because the L0 phase is unique in that a phase transition does not take place, it has been utilized in cosmetics, which require stability under a variety of conditions. By repeated occurrences of melting-solidification, the generated crystals irritate the skin at the time of use, and the texture becomes markedly worse. The typical example is a mixed system of phospholipid and cholesterol (Chol). Numerous studies have been conducted involving liposomes, and there are also applications as products. The effect of Chol on cationic liposomes consisting of phospholipid and alkylammonium chloride was recently investigated. However, few studies have examined mixed systems composed of Chol and a surfactant other than a phospholipid.

It was hypothesized that lysophospholipid (LPL), which...
is obtained by the hydrolysis of a phospholipid, can solubilize a larger amount of Chol in the molecular assembly than phospholipid, due to the shape of the molecule\(^{25}\). Therefore, we investigated the formation of an \(L_0\) phase as a potential cosmetic base material in cosmetics by mixing Chol with lysophospholipid, which can dissolve a large amount of Chol and which exhibits a high moisturizing effect\(^{26, 27}\). We found that an \(L_0\) phase is also formed with the lysophospholipid, as is the case with phospholipids\(^{28}\).

In cosmetics, a cationic surfactant or nonionic surfactant as well as a phospholipid can be used. Cationic surfactants are widely used in hair conditioners to give softness, smoothness, and antistatic properties to the hair after shampooing. Poly(oxyethylene)-type nonionic surfactants are widely used as emulsifiers in emulsion formulations such as milky lotions, creams, and essence products.

In this study, the formation of an \(L_0\) phase was investigated by mixing Chol with either stearyltrimethylammonium chloride (STAC) or polyoxyethylene stearyl ether (\(C_{18}EO_{7}\)). We chose these non-phospholipid surfactants because they have a hydrocarbon chain with a similar length as that of a phospholipid. Also, hydrophilic surfactants were selected to compare results with hydrophilic lysophospholipids, which we reported previously\(^{29}\).

2 EXPERIMENTAL

2.1 Materials

STAC was obtained from Tokyo Chemical Industry Co., Ltd., Tokyo, Japan, and its purity of STAC is greater than 99.0%. Hepta(oxyethylene)octadecyl ether (\(C_{18}EO_{7}\)) was purchased from Nikko Chemicals Co., Tokyo, Japan, and its purity of \(C_{18}EO_{7}\) is greater than 98.0%. Chol was obtained from Nippon Suisan Kaisha, Ltd., Tokyo, Japan, and its purity of Chol is greater than 93.0%. 1,3-Butanediol (1,3-BD) was obtained from Daicel Corporation, Tokyo, Japan, and its purity of 1,3-BD is greater than 99.8%. 1,3-BD was added to increase the solubility of cholesterol in the system. If the content of 1,3-BD is about 5 wt\%, there is almost no influence on phase behavior and assembled structure\(^{29}\). Water used was purified using an RO system (Elix 3, Millipore).

2.2 Sample preparation

Required amounts of reagents contained in screw-cap test tubes were homogenized at 110°C and then cooled at 25°C over 1 day. The samples were used for X-ray scattering and differential scanning calorimetry (DSC) measurements. The measurements were carried out after a certain period of time (within 1 week after sample preparation) in order to avoid the influence of structural change by time.

2.3 DSC measurements

DSC was performed on a differential scanning calorimeter (DSC6200, SII Nano Technology Inc., Ciba, Japan). Samples of 10-20 mg were weighed in aluminum capsules, and an empty aluminum capsule was used as the reference. The samples were cooled to \(-80^\circ\text{C}\) before DSC to avoid a super-cooled state. The samples were heated from \(-10\) to 110°C at a rate of 10°C/min.

2.4 X-ray scattering measurements

X-ray scattering was performed using a Kratky-type small-angle scattering camera (SAXSess, Anton Paar, Austria). Cu-K\(\alpha\) (\(\lambda = 0.1542\) nm) radiation was used. An image plate was used as the detector, and X-ray scattering intensity was measured using a Cyclone image plate reader (Perkin Elmer Co. Waltham, MA, USA).

3 RESULTS

3.1 DSC measurements in STAC/Chol/1,3-BD/water and \(C_{18}EO_{7}/\text{Chol}/1,3\)-BD/water systems

DSC measurements of the surfactant/Chol/1,3-BD/water system were carried out, and the results are shown in Fig. 1. The concentration of highly polar solvent (1,3-BD + purified water) in the system was fixed at 50 wt\%, and the concentration of 1,3-BD in the highly polar solvent was fixed at 10 wt\%.

Figure 1 shows the results of DSC measurements for the

![Fig. 1](image)

(a) STAC

(b) \(C_{18}EO_{7}\)

DSC curves as a function of \(X_1\) for (a) STAC/Chol/1,3-BD/water and (b) \(C_{18}EO_{7}/\text{Chol}/1,3\)-BD/water systems. \(X_1\) indicates the mole fraction of Chol in the STAC + Chol mixture. The 1,3-BD aqueous solution concentration was fixed at 50 wt\% for both systems, and 1,3-BD concentration was fixed at 10 wt\% in 1,3-BD aqueous solution.
STAC and C_{18}EO_{7} systems. In the STAC system, a large endothermic peak was observed in the vicinity of 30°C when $X_{1} = 0$, where $X_{1}$ represents the mole fraction of Chol/(surfactant + Chol). This endothermic peak indicates dissolution of the hydrocarbon chain of the surfactant molecule and is called the "gel-liquid crystal transition." With an increase in the amount of Chol added, the peak due to the gel-liquid crystal transition became gradually smaller and disappeared when $X_{1} = 0.38$. By contrast, in the C_{18}EO_{7} system, a large endothermic peak was observed in the vicinity of 50°C when $X_{1} = 0$. As in the case with STAC, this endothermic peak was due to a gel-liquid crystal transition. With an increase in the amount of Chol added, the peak gradually shifted to the lower temperature side, and it disappeared when $X_{1} = 0.60$. For both systems, the phase transition temperature gradually decreased with increasing concentration of Chol. The main phase transition enthalpy calculated from Fig. 1 was then plotted with respect to the mole fraction $X_{1}$ of Chol, and the result is shown in Fig. 2.

Linear function approximation was performed for the plots shown in Fig. 2, and the value $X_{1}^{\Delta H = 0}$, at which the phase transition enthalpy becomes zero, was determined. The data for the LPL system are from our previous report^{28}. The value for the STAC system was 0.34, whereas the value for the C_{18}EO_{7} system was 0.58, and the value for the LPL system was 0.42. Thus, the STAC system exhibited a lower value than the LPL system. The C_{18}EO_{7} system, however, indicated a higher value than the LPL system.

3.2 X-ray scattering structural analysis in the STAC/Chol/1,3-BD/water and C_{18}EO_{7}/Chol/1,3-BD/water systems

X-ray scattering analyses at 15°C were carried out for two surfactant/Chol/1,3-BD/water systems. Wide-angle and small-angle X-ray spectra for both surfactant systems are shown in Fig. 3. In both surfactant systems, when $X_{1} = 0$, a sharp peak was observed at $d = 0.42$ nm ($q = 15$ nm$^{-1}$) on the wide-angle side, indicating a hexagonally arranged sublattice^{30,31}. In both surfactant systems, the peak intensity decreased with increasing values of $X_{1}$, and the peak disappeared when $X_{1} = 0.38$ in the case of STAC and when $X_{1} = 0.50$ in the case of C_{18}EO_{7}.
In the small-angle region, peaks (a) and (c), which represent a lamellar structure, were observed in the respective surfactant systems without added Chol. Following the addition of Chol to the respective surfactant systems, peaks (b) and (d), which represent another lamellar structure, were observed on the small-angle side. In the STAC system, peak (a) was observed up to $X_1 = 0.28$, and peak (b), with a wider interlayer spacing, appeared when $X_1 = 0.09$ or higher. By contrast, peak (c) was observed in the $C_{18}$EO$_7$ system up to $X_1 = 0.27$, and peak (d), with a wider interlayer spacing, appeared when $X_1 = 0.14$ or higher.

### 3.3 Interlayer spacing, $d$, of the $L_n$ Phase–Like Structure in the STAC/Chol/1,3-BD/water and $C_{18}$EO$_7$/Chol/1,3-BD/water systems

The interlayer spacing, $d$, calculated from the results of small-angle X-ray scattering analyses, is shown in Fig. 4. The data for the LPL system are from our previous report [34]. In the $C_{18}$EO$_7$ system, the interlayer spacing, $d$, of the $L_n$ phase–like structure formed by the addition of Chol was approximately 8.31 nm. Thus, in the $C_{18}$EO$_7$ system, as in the LPL system, the interlayer spacing, $d$, of the $L_n$ phase-like structure formed by the addition of Chol was larger than that of the $\alpha$-gel structure. Similarly, the interlayer spacing, $d$, due to the $\alpha$-gel structure was also smaller in the STAC system compared with the $L_n$ phase-like structure formed by the addition of Chol. With an increase in the amount of Chol added, the interlayer spacing, $d$, of the $L_n$ phase-like structure increased from approximately 6.98 nm to approximately 8.45 nm. That is, the interlayer spacing, $d$, of the $L_n$ phase for the LPL system and that of the $L_n$ phase-like structure for the $C_{18}$EO$_7$ system was nearly constant; however, that for the STAC system was found to be dependent on $X_1$.

### 4 DISCUSSION

#### 4.1 Formation of $L_n$ Phase–Like Structures in the STAC/Chol/1,3-BD/water and $C_{18}$EO$_7$/Chol/1,3-BD/water systems

In the phospholipid systems examined, the $L_n$ phase was identified based on the results of DSC measurements in which the endothermic peak due to the gel-liquid crystal transition disappeared [34] and the presence of a lamellar structure in which the ordering of the alkyl chain is reportedly between the crystalline and liquid-crystalline states [35].

As shown in Fig. 1, disappearance of the gel-liquid crystal transition due to the addition of Chol was observed in the DSC data for both the STAC and $C_{18}$EO$_7$ systems, which was concluded that this is because Chol increases the flexibility of the lipid bilayer. Specifically, the gel-liquid crystal transition temperature did not significantly change with the addition of Chol, and the endothermic quantity continually decreased and ultimately reached zero.

As shown in Fig. 3, for both surfactants, peaks (a) and (c) were derived from the $\alpha$-gel structure by considering the peak on the wide-angle side. With increasing concentration of Chol, the intensities of peaks (a) and (c) on the small-angle side, as well as the peak on the wide-angle side, decreased, indicating that the $\alpha$-gel structure was gradually disappearing. By contrast, peaks (b) and (d) indicated a lamellar structure, as was the case with peaks (a) and (c), and the intensity increased with increasing concentration of Chol, while the peak on the wide-angle side broadened and disappeared. In other words, the formation of a loose lamellar structure occurred in both surfactant systems following the addition of Chol. These data suggest that an $L_n$ phase-like structure was formed in the STAC/Chol/1,3-BD/water and $C_{18}$EO$_7$/Chol/1,3-BD/water systems.

As shown in Fig. 2, the $X_1^{L_n} = 0.34$ in the case of STAC and 0.58 in the case of $C_{18}$EO$_7$, indicating that peaks (b) and (d) were derived from the $L_n$ phase-like structure. That is, both surfactants form an $L_n$ phase-like structure in a mixed system with Chol, and the value of $X_1$ at which the phase transition enthalpy becomes zero is the concentration of Chol at which only the $L_n$ phase-like structure is formed.

The reported value for a mixed system of dimyristoylphosphatidylcholine (the main component of phospholipids) and Chol is 0.30-0.37 [34], whereas the value for a...
mixed system of decaglyceryl dilaurate (a nonionic surfactant) and Chol is 0.34. Compared with other surfactants often used in cosmetics, the value for C18EO7 was particularly high. The $X_{\text{el}}^{100}$ value for the C18EO7 system was greater than that for the STAC or LPL systems, perhaps due to an inability of the hydrophobic group in the crystal line state to be fluidized in the absence of a large amount of Chol because of hydrogen bonding attraction between the hydrophilic groups. Conversely, the STAC system formed an L0 phase–like structure when the $X_1$ value was less than that of the C18EO7 or LPL systems. Thus, the membrane likely becomes fluidized by mixing less Chol because of increased repulsion between neighboring STAC hydrophilic groups.

4.2 A mechanism of the effect of Chol on the lipid bilayer in the STAC/Chol/1,3-BD/water and C18EO7/Chol/1,3-BD/water systems

As shown in Fig. 4, the interlayer spacing, $d$, of the L0 phase–like structure for the C18EO7 system was nearly constant, but that for the STAC system was found to be dependent on the addition of Chol. A possible mechanism of the effect of Chol on the lipid bilayer in the STAC/Chol and C18EO7/Chol mixed systems is illustrated schematically in Fig. 5. STAC is a cationic surfactant in which the hydrophilic group is a dissociated quaternary ammonium salt. In the system without added Chol, chloride ion, which is the counter-ion of STAC, is also subjected to interionic attraction by the surrounding STAC molecules, restricting dissociation. When Chol is added, some domains with more Chol are formed in the membrane due to the non-uniform distribution of Chol. In that domain, the charge density on the surface of the hydrophilic side of the lipid bilayer decreases, and the dissociation of the counter-ion of STAC increases. As a result, the interlayer spacing, $d$, of the L0 phase–like structure, which appeared following the addition of Chol, increases due to the increase in electrostatic repulsion. There is also a possibility that the increase in the lipophilicity of the membrane due to the solubilization of Chol decreases the interlayer spacing. However, the data in this paper cannot judge which is correct. By contrast, C18EO7 is a nonionic surfactant, and it has a polyoxyethylene chain as the hydrophilic group. In the system without added Chol, hydration between the polyoxyethylene chains is restricted. When Chol is added, some domains with more Chol are formed in the membrane, as is the case with STAC, due to the non-uniform distribution of Chol. In that domain, the concentration of the polyoxyethylene chain decreases, and as a result, the area occupied by a surfactant molecule and the degree of hydration increase, resulting in marked expansion into the water. As a result, the interlayer spacing, $d$, of the L0 phase–like structure, which appeared following the addition of Chol, increases. As also seen from Fig. 4, the interlayer spacing, $d$, of the C18EO7 system was larger than that of the LPL or STAC systems, probably because the hydrophilic group of C18EO7 is a polyoxyethylene chain, which expands significantly in water between the lipid bilayers compared with the hydrophilic group of the ionic surfactant. The interlayer spacing, $d$, of the L0 phase–like structure of the C18EO7 systems or the L0 phase of the LPL systems is constant regardless of the increase in $X_1$. By contrast, the interlayer spacing, $d$, of the L0 phase of the STAC system becomes larger with increasing values of $X_1$. The dissociation of the counter-ion of STAC likely increases according to the concentration of Chol, and the electrostatic repulsion between the lipid bilayers gradually increases; thus, the interlayer spacing, $d$, also increases with increasing values of $X_1$.

5 CONCLUSION

As is the case with phospholipid and LPL, STAC and C18EO7 readily form an L0 phase–like structure upon mixing with Chol. We also found that the concentration of Chol at which only the L0 phase–like structure is formed increases in the order STAC < LPL < C18EO7. These data suggest that the ionicity and structure of the hydrophilic group of each surfactant play important roles.

For both surfactants, the interlayer spacing, $d$, was larger for the L0 phase–like structure than for the α-gel structure. Furthermore, the interlayer spacing, $d$, gradually increased with increasing Chol concentration only in the case of the cationic surfactant, STAC. The degree of ionization of STAC increases with increasing Chol concentration.

As a result, both the ionic strength in the lipid bilayer and the electrostatic repulsion increase.

The results of this study suggested that the reported fragmentary formation of an L0 phase in a non-phospholipid system is a phenomenon that occurs ubiquitously with both ionic and nonionic surfactants. Due to its lack of a phase transition between 0°C to 80°C, the incorporation of an L0 phase–like structure is useful in cosmetics because stability under various temperature conditions is important. Furthermore, the L0 phase–like structure can stably retain Chol, which is an important component for maintaining healthy skin and hair. Accordingly, the L0 phase–like structure shows promise as a constituent of cosmetic formulations designed to moisturize the skin or repair damaged hair.

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Fig. 5  Schematic illustration of the structural change from $\alpha$-gel to liquid ordered phase-like structure due to incorporation of Chol. (a)STAC/Chol system, (b)C$_{18}$EO$_7$/Chol system.

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