Effects of Ethanol and Cholesterol on Thermotropic Phase Behavior of Ion-Pair Amphiphile Bilayers

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Abstract: Ion-pair amphiphiles (IPAs, also known as catanionic surfactants) are lipid-like double-chained molecules potentially used for fabricating liposome-like vesicular drug and gene carriers. Frequently ethanol and cholesterol are added to modulate the properties of their bilayer membranes. Effects of ethanol and cholesterol on the fundamental properties of IPA bilayers such as thermotropic phase behavior, however, is not known. In this work, the bilayer phase transition behavior of two IPAs (decyltrimethylammonium-tetradecyl sulfate, DeTMA-TS, and dodecyltrimethylammonium-dodecyl sulfate, DTMA-DS) in tris buffer with various amounts of ethanol was studied by using differential scanning calorimetry (DSC). Effect of cholesterol (CHOL) addition on bilayer phase transition of IPAs with 20 vol% ethanol was thereafter systematically investigated. The experimental results showed that the main phase transition temperature ($T_m$) was monotonously decreased with the increase of ethanol concentration up to 30 vol%. The degree of $T_m$ depression by ethanol is essentially the same for the two IPAs regardless of different symmetry in the hydrocarbon chains. Further addition of CHOL, however, caused a slight decrease in $T_m$ on the one hand and a significant decrease in the enthalpy of phase transition on the other hand. When the added CHOL exceeded a specific amount, the phase transition disappeared. More hasty disappearance of phase transition was found for IPA with asymmetric structure than the symmetric one. Possible mechanisms of ethanol effect based on binding in the headgroup region of the bilayers and CHOL effect based on opposite (condensing and disordering) interactions with IPA molecules in bilayers, respectively, were proposed.

Key words: ion-pair amphiphile (IPA), catanionic surfactant, bilayer phase transition, ethanol effect, cholesterol effect, differential scanning calorimetry

1 INTRODUCTION

"Double long-chain salt" as distinguished from the usual "single long-chain salt" was prepared for the first time in 1943 by Scott et al.\(^1\). The term "catanionic" was introduced to designate this class of surfactant by Jokela et al. in 1987.\(^2\). Catanionic surfactants (also known as ion-pair amphiphiles, IPAs) are formed by pairing two single-chained oppositely charged surfactants with equimolar ratio. After removing the inorganic counterions, the resulting surfactants are thus uncharged and can be considered as pseudodouble-chained surfactants, in the sense that the two chains are not covalently bonded to the same headgroup. One book chapter\(^3\) and two comprehensive reviews\(^4,5\) on catanionic surfactants (or IPAs) are available in the literature. More recently, nine homologous IPAs were synthesized and the thermotropic transition behavior of their bilayers in excess water was systematically investigated\(^6\).

Furthermore, effects of three sterol-like additives on the thermotropic transition behavior of four IPA bilayers in excess water were also studied\(^7\). Bilayers are the basic building blocks of a number of amphiphilic structures. They are formed in dilute solution by amphiphilic molecules with critical packing parameters between 0.5 and 1\(^8\). This implies that bilayers are favored by amphiphiles with large volume of the tail(s), such as those with double hydrocarbon chains. Typical examples include the double-chained cationic surfactants and zwitterionic phospholipids. IPAs surely fall into this category. In reality, bilayers tend to avoid interaction between the hydrocarbon chains and solvent at their edges by closing up on themselves to form enclosed structure known as a vesicle.

Owing to their benefits of abundant sources, low cost, chemical stability, and chemical structure designability
among others, lipid-like IPAs have emerged as attractive materials for preparing vesicular carriers of drug and gene delivery systems\textsuperscript{8–13}. Catanionic vesicles have been fabricated from IPAs by the classic mechanical dispersion method through the preparation of thin films, which is frequently used for preparing conventional liposomes from lipids\textsuperscript{8–13}. The fabricated catanionic vesicles were investigated and regarded as feasible replacements of liposomes to serve as DNA delivery carriers. At the same time, ethosome-like catanionic vesicles, which are composed of IPA, water, and a relatively high content of ethanol, have also been fabricated by a simple semi-spontaneous process\textsuperscript{14–19}. As a counterpart of ethosomes\textsuperscript{20–26}, which are composed of lipid, water, and a relatively high content of ethanol, ethosome-like catanionic vesicles were developed by using ethanol as a permeation enhancer in aid of transdermal drug delivery. Furthermore, cholesterol (CHOL) effects on the physical stability, oil-soluble drug encapsulation behavior, and gelation of the ensuing ethosome-like catanionic vesicles were also studied.

It is noteworthy that an examination on the results of catanionic vesicle and ethosome-like catanionic vesicle studies mentioned above\textsuperscript{8–19} revealed the importance of ethanol and CHOL in determining the performance of catanionic vesicles and ethosome-like catanionic vesicles. The mechanisms by which ethanol and cholesterol may induce effects, however, is poor understood. It is, therefore, most desirable that ethanol and cholesterol effects on the fundamental properties of IPA bilayers such as thermotropic phase behavior can be systematically studied.

In this work, two double-chained IPAs with the same total number of carbon atoms in the hydrocarbon chains but with different hydrocarbon chain symmetry were designed and synthesized from single-chained cationic and anionic surfactants by the precipitation method. By using differential scanning calorimetry (DSC), the thermotropic transition behavior from gel phase (L\textsubscript{g}) to liquid-crystalline phase (L\textsubscript{c}) was studied for bilayers of these two lipid-like IPAs in tris buffer solution with various amounts of ethanol. Moreover, effect of CHOL addition on bilayer phase transition of IPAs with 20 vol\% ethanol was thereafter systematically investigated.

### 2 EXPERIMENTAL

#### 2.1 Materials

Two cationic surfactants used in this work were decyltrimethylammonium bromide (DeTMAB) and dodecyltrimethylammonium bromide (DTMAB). Two anionic surfactants used were sodium dodecylsulfate (SDS) and sodium tetradecylsulfate (STS). All above-mentioned surfactants with purity higher than 99\% were purchased from Sigma and used as received without further purification. Ethanol (99.8\% pure) was purchased from Sigma-Aldrich. The buffer saline Trizma base (Tris [hydroxymethyl] amino methane, 98\% pure) and cholesterol (99\% pure) were purchased from Sigma.

#### 2.2 Synthesis of IPAs

Double-chained IPA as precipitate will come out when aqueous solutions of single-chained cationic and anionic surfactants with sufficiently high concentrations are mixed together. In this work, such precipitate was prepared by mixing equal volume (500 mL) of 20 mM aqueous solutions of cationic and anionic surfactants. A concentration of 20 mM is well beyond the corresponding critical micelle concentrations (CMCs) of the surfactants. After settled for 1 h, the precipitate was separated from the aqueous phase by repeated centrifuging and washing, and the collected precipitate was then dried for 36 h under vacuum and ground into fine powder. Lipid-like IPAs by pairing of two oppositely charged surfactants were thereby obtained. All experiments were conducted with pure water that was passed through a Milli-Q plus purification system (Millipore, USA) with a resistivity of 18.2 M\Omega-cm.

#### 2.3 Methods

Samples for DSC measurements were prepared by combining water, IPA at the concentration of 40 mM, buffer at 15 mM, ethanol at the indicated content (vol\%), and extra addition of CHOL at the indicated concentrations (mM). It should be noted that high concentration (40 mM) of IPA was used for DSC measurements due to the sensitivity of the calorimeter. The solutions containing various components were kept at 70°C and passed through a homogenizer at the speed of 11000 rpm for 15 minutes. Defoaming was then carried out by using a sonicator. All dispersions were let to stand for 1 day at 25°C before any measurement was performed. By careful sampling, the suspensions were placed into small aluminum pans. The pans were sealed and scanned in calorimeter (DSC 7, PerkinElmer, USA) using a reference pan containing buffer solution at 15 mM. The heating rate and scanning range were 5°C/min and 15°C - 60°C, respectively, in all the experiments.

When an IPA bilayer at gel phase is heated, it undergoes a phase change that can lead to the formation of a liquid-crystalline phase. DSC measures the excess heat capacity of the bilayer system as a function of temperature. The heat capacity exhibits maxima at the main phase transition temperature, T\textsubscript{m}. The integration of the areas under the transition enthalpies ΔH\textsubscript{m}, to be calculated from the measured transition enthalpies\textsuperscript{27}.

A transmission electron microscope (TEM, model H-7500, Hitachi) was also used to obtain the structure.
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Images of 40 mM IPA in excess water by the negative staining technique. For the sample preparation, a few drops of IPA dispersion were applied to carbon-coated Cu grid and dried. A drop of 1 wt% uranyl acetate in water-ethanol (1:1) solution was then added as the staining agent.

For X-ray diffraction experiments, sample dispersions (100 μL) were sealed in quartz glass X-ray capillary tubes. The capillary tubes were then mounted in a temperature-controlled sample holder at 30°C. Lamellar samples were characterized by a NANOFLUX U system (Bruker AXS GmbH, Karlsruhe, Germany) coupled with the IP-S-type microfocus X-ray tube operated at 50 kV and 600 μA. The wavelength was 0.154 nm. All samples were swept by small angle X-ray scattering (SAXS) for 2 hours. Detector calibration was realized using silver behenate.

3 RESULTS AND DISCUSSION
3.1 IPAs

Two IPAs were prepared: decyltrimethylammonium-tetradecylsulfate (DeTMA-TS, CH₃(CH₂)₉N(CH₃)₃-CH₂(CH₂)₁₀SO₄) and dodecyltrimethylammonium-dodecylsulfate (DTMA-DS, CH₃(CH₂)₁₁N(CH₃)₃-CH₂(CH₂)₁₁SO₄). The compositions of the resulting pure IPAs were analyzed by elemental analysis (EA), mass spectrum (MS) determination, and nuclear magnetic resonance (NMR) spectroscopy. Details of analyses on the as-synthesized IPAs have been described elsewhere. It was confirmed that each IPA could be considered as a pseudodouble-chained amphiphilic compound containing amphiphilic cation and amphiphilic anion in an equimolar ratio. Figure 1 shows the molecular structures of the two IPAs which are formed from their corresponding moieties, ethanol, and CHOL.

Figure 2 shows, for example, TEM micrographs of 40 mM DTMA-DS IPA in excess water. It is noteworthy that coexisting planar and vesicular bilayer structures were observed. As shown in Fig. 3, the SAXS spectra reveal that a sharp peak at around q = 0.19 Å⁻¹ (d = 32.98 Å⁻¹) appears for X_CHOL = 0 - 0.15, indicating the formation of Lβ phase.

![Fig. 2 TEM micrographs of 40 mM DTMA-DS in excess water showing the coexistence of planar and vesicular (inset) bilayer structures. Magnification: 100000x.](image)

![Fig. 3 Effects of CHOL on SAXS spectra of DTMA-DS/CHOL binary mixtures in excess water.](image)

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This peak, however, disappears at higher concentrations \(X_{\text{CHOL}'} = 0.33\) and \(0.43\). At \(X_{\text{CHOL}'} = 0.05\), a new peak at around \(q' \approx 0.14\ \text{Å}^{-1}\) begins to appear, indicating the formation of liquid-ordered phase (\(L_{\text{o}}\)). This latter peak grows and coexists with the former one for \(X_{\text{CHOL}'} = 0.05 – 0.15\) on the one hand, and becomes the single peak at higher concentrations \(X_{\text{CHOL}'} = 0.33\) and \(0.43\) on the other hand. The findings of cholesterol effects on the reciprocal spacing, phase structure, and repeat spacing of IPA bilayers by SAXS are summarized in Table 1. IPA bilayers with interlayer spacing from 3.3 nm to 4.6 nm can then be confirmed.

### 3.2 Ethanol effect on the thermotropic phase transition of IPA bilayers

The DSC thermograms for the two IPA bilayers at 40 mM in tris buffer are shown in Fig. 4. The measured \(T_m\) values are slightly lower than that reported previously for the same IPAs in excess water\(^6\). This indicated that buffer could not affect the phase transition behavior significantly. As shown in Fig. 4(a), a tiny endothermic peak was detected at 34°C when the 30 vol% ethanol was added to the DeTMA-TS system. On the other hand, the similar peak was not observed in the DTMA-DS system as shown in Fig. 4(b). This is possibly due to the partial collapse of bilayers with low packing in the DeTMA-TS system, which is IPA composed of hydrocarbon chains with less symmetry.

It is noteworthy the relatively large enthalpy changes of pure IPAs as compared to those of phospholipids such as phosphatidylethanolamines (PEs) with the same hydrocarbon chains suggested that the phase transition of IPA bilayers includes the contribution of the hydration changes among head groups in addition to that of the conformational change. The identification of the two contributions, however, is beyond the scope of this work.

With increasing ethanol concentration, the phase transition temperatures were monotonously decreased. For IPA bilayers with 20 vol% ethanol, 5.75 and 5.60°C decrease in \(T_m\) were observed for DeTMA-TS and DTMA-DS, respectively. Figure 5 shows the variations of \(T_m\) and \(\Delta H_m\) as functions of ethanol concentration. Nearly the same degree of ethanol effect on \(T_m\) lowering and \(\Delta H_m\) was found for both DeTMA-TS and DTMA-DS, which are IPAs composed of the same headgroups and hydrocarbon chains with different symmetry. This is due to the fact that ethanol interacts with IPA bilayers at the IPA-water interface rather than in the hydrocarbon core.

In contrast to some typical phosphatidylcholines (PCs\(^{28-35}\)), however, IPAs do not exhibit a biphasic dependence of the main phase transition temperature on ethanol concentration. In the concentration range studied in this work, only a lowering but not a reversal of \(T_m\) was observed as the ethanol concentration increased. A lack of biphasic dependence on ethanol concentration suggests that, as a class of lipid-like amphiphiles, IPA does not favor interdigitation of hydrocarbon chains in the presence of ethanol. Furthermore, phase transition experiments were also conducted in this work by an increasing-decreasing-increasing temperature mode. The results (not shown here) indicated that the main transition temperature appeared to be completely re-

### Table 1  Effects of CHOL on the reciprocal spacing, phase structure, and repeat spacing of IPA bilayers.

| \(X_{\text{CHOL}}\) | 0 | 0.05 | 0.1 | 0.15 | 0.33 | 0.43 |
|-----------------|---|-----|-----|-----|-----|-----|
| \(q (\text{Å}^{-1})\) | 0.1905 | 0.1913 | 0.1906 | 0.1913 | 0.1906 | 0.1913 |
| \(d (\text{Å})\) | 32.98 | 32.83 | 32.94 | 32.83 | 32.94 | 32.83 |

Reciprocal spacing: \(q = (4\pi \sin \theta)/\lambda\), repeat spacing: \(d = 2\pi/q\), where \(\theta\): diffraction angle, \(\lambda\): wavelength. \(L_p\): gel phase, \(L_{o}\): liquid-ordered phase

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Fig. 4 DSC thermograms for IPA bilayers with various amounts of ethanol. (a) DeTMA-TS and (b) DTMA-DS.
versatile. This reconfirmed the absence of interdigitation of hydrocarbon chains in the presence of ethanol. It should be noted that the lack of biphasic dependence on ethanol concentration was also reported in the literature for Pes\(^{37}\), phosphatidylserine (PS)\(^{36}\), and PCs with large \(\Delta C\) values\(^{32}\), where \(\Delta C\) is the effective acyl chain length difference between the sn-1 and sn-2 acyl chains.

On the other hand, ethanol binding to the lipid-water interface of phospholipid bilayers may significantly altered the orientation of the bilayer headgroups and disordered the entire lengths of the hydrocarbon chains\(^{37}\). Pronounced increase in the area per lipid\(^{37,38}\), decrease in the ordering of the chains\(^{37,39}\), and higher fluidity and permeability of the bilayers\(^{38,39}\) were attributed to ethanol and other short-chain alcohols. The monotonous lowering of \(T_m\) exhibited by IPA bilayers, therefore, is possibly due to the same disordering effects of ethanol on lipid bilayers.

### 3.3 Cholesterol effect on the thermotropic phase transition of IPA bilayers

Table 2 summarizes results of CHOL effect on the \(T_m\), \(\Delta H_m\), and \(\Delta S_m\) of DeTMA-TS and DTMA-DS bilayers with 20 vol\% ethanol. As shown in Fig. 6(a), \(T_m\) was nearly unaffected for DeTMA-TS bilayers and was decreased slightly for DTMA-DS bilayers with increasing CHOL concentration. Figure 6(b), however, shows that the enthalpy of phase transition was decreased accordingly for both DeTMA-TS and DTMA-DS bilayers. When the addition of CHOL exceeded a specific amount, the phase transition disappeared. Moreover, a larger decrease in \(\Delta H_m\) and hasty disappearance of phase transition was found for the asymmetric IPA than for the symmetric one.

Lots of investigations have been devoted to the effects of CHOL, which is incorporated in lipid bilayers for modulating their characteristics, on the phase transition behavior, vesicular bilayer rigidity, and other properties by using various techniques such as DSC, fluorescence polarization...
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Among them, the well-known condensation effect of CHOL on lipid bilayers at a temperature above its $T_m$ (that is, lipid bilayers in the liquid-crystalline phase, $L_{α}$), making the bilayers more rigid was evidenced and documented. On the other hand, the disordering effect of CHOL, which worked on lipid bilayers at a temperature below its $T_m$ (that is, lipid bilayers in the gel phase, $L_{β}$), making the bilayers more fluidic was also evidenced and documented. The interesting opposite effects of CHOL, therefore, lead to the formation of a single liquid-ordered phase, $L_{α}$, and resulted in gradual disappearance of lipid bilayer phase transition with increasing CHOL concentration as observed in DSC experiments. Actually, this is due to the fact that cholesterol interacts with IPA bilayers mainly in the hydrocarbon core rather than only at the IPA-water interface.

4 CONCLUSIONS

Differential scanning calorimetry was used to study the bilayer phase transition behavior of two catanionic surfactants (DeTMA-TS and DTMA-DS), which are ion-pair amphiphiles with the same total number of carbon atoms in the hydrocarbon chains but with different hydrocarbon chain symmetry, in tris buffer with various amounts of ethanol. The experimental results revealed that the main phase transition temperatures were monotonously decreased with increasing ethanol concentration for both IPAs. This is attributed to the disordering effects of ethanol on IPA bilayers. The degree of $T_m$ depression by ethanol, which interacts with IPA bilayers in the headgroup region rather than in the hydrocarbon core, is essentially the same for the two IPAs regardless of different symmetry in the hydrocarbon chains. Furthermore, the lack of biphasic dependence on ethanol concentration suggests that IPA does not favor interdigitation of hydrocarbon chains in the presence of ethanol.

Effect of CHOL addition on bilayer phase transition of IPAs with 20 vol% ethanol was thereafter systematically investigated. The experimental results revealed that addition of CHOL, which is a highly nonpolar additive and was incorporated into IPA bilayers, caused a slight decrease in $T_m$ on the one hand and a significant decrease in the enthalpy of phase transition on the other hand. When the added CHOL exceeded a specific amount, the phase transition disappeared. More hasty disappearance of phase transition was found for IPA with asymmetric structure than the symmetric one. The general trend and disappearance of IPA bilayer phase transition with increasing concentration of CHOL can be explained by the opposite (condensing and disordering) CHOL effects on the molecular ordering in hydrocarbon chains of IPA bilayers at temperatures above and below, respectively, the main phase transition temperature in line with that of lipid bilayers.
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