A Preferable Method for the Formation of Vesicles from Lamellar Liquid Crystals Using Chemical Additives

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Abstract: We present a method for vesicle formation from lamellar liquid crystals (LCs) using a cationic amphiphilic substance, namely 2-hydroxyethyl di(alkanol)oxyethyl methylammonium methylsulfate (DEAE). Vesicle formation from the DEAE lamellar dispersion occurred via a two-step chemical addition. This method required neither additional mechanical energy nor the use of special solvents. The transition was solubilized using an organic substance (e.g., limonene) in the lamellar DEAE LC, after which, a small amount of inorganic salt was added to the solubilized lamellar LC dispersion with gentle stirring. The viscosity of the DEAE dispersion following salt addition decreased sharply from 10^5 mPa·s to 10^2 mPa·s, and the DEAE dispersion was converted into a high fluidity liquid. Several organic substances were examined as potential solubilizers to initiate the lamellar-vesicle transition. Inorganic salts were also examined as transition triggers using various types of electrolytes; only neutral salts were effective as trigger additives. Dissociation of inorganic salts yielded anions, which inserted between the DEAE bilayer membranes and induced OH⁻ ion exchange. In addition, a number of cations simultaneously formed ion pairs with the DEAE counter ions (CH₃SO₄⁻ ions). However, as the amount of solubilized organic substances in the DEAE bilayer membrane decreased over time, the vesicles were transformed into lamellar LCs once again. The DEAE states in each step were measured by monitoring the zeta potential, pH, viscosity, and by examination of scanning electron microscopy and atomic force microscopy images. A possible molecular mechanism for the lamellar-vesicle transition of DEAE was proposed.

Key words: lamellar liquid crystal, vesicle formation, lamellar-vesicle transition, cationic vesicle, cationic amphiphilic compound

1 INTRODUCTION

The first attempt to confirm the formation of a biomimetic membrane structure from a simple cationic organic compound was carried out by Kunitake in 1977¹⁾. He concluded that double-tailed ammonium salts could form vesicles when dispersed in water under sonication²⁾. Subsequent research revealed that various organic compounds also formed vesicles in water. For example, Sudhoelter reported that the novel synthetic double-chained amphiphiles, 17-(4-*N*-methylpyridinium)tritriacontane iodide and 3,5-di-carbo-*n*-hexadecyloxy-1-methylpyridinium iodide, formed uni- and multilamellar vesicles in aqueous solutions, as indicated by electron microscopy³⁾. However, the

vesicles prepared using these cationic compounds were unable to maintain their structures over long periods due to the gradual relaxation of the energy exerted for vesicle formation. Therefore, although the application of vesicles to drug delivery systems has been attempted using a variety of techniques, no practical vesicle implementation methods exist^{4,5)}, as the vesicles would be in a metastable thermodynamic state.

In vesicle preparation, various procedures have been proposed earlier. For the reason that spontaneously formed equilibrium vesicles would be preferred for these applications, reports of spontaneous formation of vesicles from aqueous surfactants can be found⁶⁻¹³⁾. As a typical

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example, the positive and negative electrostatic interactions were regarded as the main driving forces for the vesicle formation in aqueous cationic and anionic surfactant mixtures. However, it is easy to imagine that the vesicles prepared using these procedures cannot maintain their structure by infinite dilution and change in ionic strength. Unlike an association equilibrium of micelles, in essence, vesicles must be in thermodynamic non-equilibrium with the molecules monodispersed in water, and sparingly water-soluble amphipathic substances are used as vesicle forming agents. Therefore, the external energy (mechanical power) has been used for the vesicle formation in aqueous system^{14–24}. Moreover, calculation of the external energy exerted on the liquid crystals (LCs) to form vesicles using mechanical or ultrasonic power is difficult; hence, the energy required for the formation of vesicles from lamellar LCs has yet to be estimated. Indeed, no suitable studies have determined the transition energy in cationic vesicles.

We herein describe a preferable method for the formation of vesicles from the lamellar LCs of cationic compounds using chemical additives as opposed to conventional mechanical methods or favorable solvents. We also propose a possible mechanism for the lamellar-vesicle transition stage. Furthermore, the thermodynamic parameters for vesicle formation will be provided assuming that the lamellar-vesicle transition can be controlled by the amount of chemical additives alone.

2 EXPERIMENTAL PROCEDURES

2.1 Materials

2-Hydroxyethyl di(alkanol)oxyethyl methylammonium methylsulfate (DEAE, MW: 667.98) was chosen as the cationic double-tailed amphiphilic compound (Scheme 1). DEAE is a quaternary ammonium salt that dissolves sparingly in water. As outlined below, various organic and inorganic substances were employed as chemical additives to achieve the DEAE bilayer transition.

The following organic substances were used as solubilizers for the hydrophobic groups of the DEAE bilayers: 7 normal paraffins (i.e., C_nH_{2n+2} , $n = 6, 8, 10, 12, 14, 16$, and

18), 1-methyl-4-(1-methylethenyl)-cyclohexene (limonene), 2 perfluorocarbon compounds (i.e., perfluorohexane and perfluorononane), and 2 surfactants (i.e., cetyltrimethylammonium bromide and octaoxyethylene monohexadecyl ether). All compounds (special grade reagents) were purchased from Wako Pure Chemical Industries and were used without further purification.

The following inorganic salts were used: eight neutral salts (i.e., calcium chloride, magnesium chloride, sodium chloride, potassium chloride, sodium bromide, calcium bromide, sodium sulfate, and calcium nitrate), an acidic salt (i.e., ammonium chloride), and four basic salts (i.e., sodium acetate, calcium acetate, sodium carbonate, and sodium borate). These salts may affect the zeta potential and hydrophilic groups in DEAE LCs. All salts (special grade reagents) were taken from available stock reagents that were purchased from Wako Pure Chemical Industries.

2.2 Apparatus

X-ray diffraction (XRD) patterns of the DEAE LC samples were obtained using a Rigaku RU-200B X-ray diffractometer (Rigaku, Japan). A specially designed aluminum cell was used to mount the DEAE LCs to avoid overlapping effects between the XRD peaks from the standard glass cell and the bilayer membrane structures in the low angle regions. The measurements were performed at 40 kV and 50 mA using the Cu $K\alpha$ line. The divergence slit was $1/2^\circ$, the scattering slit was $1/6^\circ$, and the receiving slit was 0.15 mm. The scanning range was 0.2 – 5.00° and the scanning speed was $1.00^\circ/\text{min}$.

The viscosity of the DEAE LC dispersions was measured using a Brookfield viscometer (Shibaura Systems, VS-A1). The viscosity measurements were repeated five times for each sample at a given temperature (25 – 60°C) to obtain an average value for the DEAE LC dispersions.

The pH values for the DEAE LC dispersions were measured at 30°C using a desktop type pH/ion meter (HORIBA, F-23), and the zeta potentials for the LC dispersions were monitored using an electrophoretic light scattering photometer (Otsuka Electronics, ELS-8000) from 25 – 60°C . The DEAE dispersions were diluted with water to measure the zeta potentials, while the dispersions containing inorganic salts were diluted with the same salt solutions used for the

Scheme 1 2-Hydroxyethyl di(alkanol)oxyethyl methyl ammonium methylsulfate (DEAE).

measurements. The LC particle size was measured at 30°C using a dynamic light scattering (Otsuka Electronics, FPAR-1000).

The LC shapes were observed at each stage by scanning electron microscopy (SEM, Hitachi, S-4000). Prior to observation, the LC samples were pretreated according to the following procedure: a sample was mounted on a glass wafer, spread widely and thinly, dried in the desiccator overnight, and then coated with carbon vapor to diminish electrostatic perturbations. The surface shapes of the LCs were observed at each stage by atomic force microscopy (AFM, SII, SPA 400). A sample was mounted on a silicon wafer, and then shapes of the LCs were observed with the cantilever (SI-DF40P) by DFM mode, which the cantilever taps the surface shapes of the LCs at fixed intervals.

The amount of solubilized limonene in the LC bilayers was computed from the peak area ratio between the solubilizing substance and the dodecane internal standard (a special grade reagent purchased from Wako Pure Chemical Industries), as measured by gas chromatography (Yokogawa Electric Corporation, HP5890 SERIES II). The formation of LCs in each sample was confirmed by polarizing microscopy (Olympus, BX-51).

2.3 Determination of the a suitable DEAE dispersion concentration for the lamellar-vesicle transition study

The concentration of the DEAE dispersions in water was fixed at 20 wt% (the weight ratio of DEAE to water was 1:4) as this is the concentration prepared industrially for fabric softeners. Several properties such as the LC shape, viscosity and crystallinity of the DEAE dispersion were investigated to confirm whether DEAE is a suitable model substance to verify our hypothesis. The DEAE dispersion was investigated by XRD and polarizing microscopy to determine if it successfully formed a lamellar LC.

2.4 Procedure for the chemical additive route to the lamellar-vesicle transition

Our hypothesis for the lamellar-vesicle transition was examined with the addition of (1) inorganic salts, (2) organic substances, and (3) both organic substances and inorganic salts.

2.4.1 Addition of inorganic salts

The addition of inorganic salts to an aqueous solution changes the dissolution state of a solute. Therefore, various acidic salts, neutral salts, and basic salts (as sources of monovalent and divalent cations) were examined in relation to the dispersed state of DEAE LCs. Each inorganic salt was added to a 20 wt% DEAE dispersion (the concentration of each salt in water varied between 0.1 mmol kg⁻¹ and 1500 mmol kg⁻¹). Each mixture was shaken by hand and fully mixed; the state of each DEAE dispersion was then carefully investigated.

2.4.2 Addition of organic substances

DEAE was completely dissolved in an appropriate amount of 2-propanol (IPA), and the organic substance (DEAE mole fraction = 0.1 to 0.3) was added to the DEAE solution. The IPA was then removed from the solution by evaporation to give a molecularly mixed substance. The addition of water to the resulting precipitate gave a weight ratio of 1:4 between the mixed substance and water. The mixture was shaken by hand and fully mixed, and the DEAE dispersion solubilized with organic substances was examined to determine the dispersed state of the LCs.

Solubilization of the organic substance into the DEAE bilayer membrane may be promoted to slow the progression of bilayer gelation. Limonene, which is commonly used as a fragrance, was selected as the solubilized substance, as its bulky and slightly volatile nature were expected to result in characteristic effects, such as a change in the molecular sequence or packing state on the hydrophobic groups of the DEAE LCs.

2.4.3 Addition in order of organic substance and successively inorganic salt

As DEAE is an amphiphilic compound, if both organic substances and inorganic salts are added to the DEAE dispersion, synergetic effects would be expected, as these compounds would simultaneously affect the hydrophobic and the hydrophilic groups of DEAE. Therefore, we attempted to add both types of compounds to the lamellar LCs and monitor the effect.

The DEAE dispersion (20 wt%) was initially solubilized with organic substances according to the previously discussed procedure (see Section 2.4.2), and each inorganic salt was added to the dispersion at the desired concentration (0.1–750 mmol kg⁻¹). Each mixture was shaken by hand and fully mixed. The properties of the DEAE dispersions (20 wt%) containing the various organic substances and inorganic salts were then investigated.

3 RESULTS

3.1 Effects of chemical additives on lamellar LCs

Concentrated cationic surfactants with long hydrocarbon chains form high viscosity lamellar LCs in water^{25–28}. The 20 wt% DEAE dispersion in water (viscosity ~10⁵ mPa·s) was observed by polarizing microscopy and XRD to investigate its self-assembled structure. A number of Maltese cross patterns were observed by polarizing microscopy, as shown in **Photo 1**. This indicates that the DEAE dispersion formed a lamellar phase, similar to conventional amphiphilic compounds. Moreover, the dispersion exhibited two XRD peaks, which could be attributed to structure of regularity, as shown in **Fig. 1**. From these observations, the 20 wt% DEAE dispersion was confirmed to form lamellar LCs in water, thus experimentally verifying our concept for the la-

Photo 1 Polarized image of the 20 wt% DEAE dispersion at 30°C.

mellar-vesicle transition.

Due to the large particle size of the DEAE LCs, it was not possible to distinguish each particle surface (the surface appeared flat in the AFM data, as shown in Photo 2A). Similarly, the shape of the lamellar LCs was not observed in the SEM images, as can be seen in Photo 3A. Information concerning shapes of the LCs is obtained by AFM observation. The distance of the bilayer membrane is examined by XRD measurement. Though nothing was confirmed by SEM observation, the lamellar LCs having a thickness of 50–100 μm surely exist. We therefore speculate that when the DEAE LC dispersion was mounted and dried on the glass cell, the LC particles coalesced to create a single flat surface.

3.2 Effects of inorganic salt addition to DEAE LCs

The addition of inorganic salts to the DEAE dispersion was investigated to determine the effects of interaction

Fig. 1 XRD pattern for the 20 wt% DEAE dispersion.

with the hydrophilic groups of the DEAE structure. We assumed that this addition transferred the lamellar LCs into the vesicles. Figure 2 shows the variation in viscosity of the 20 wt% DEAE dispersion with calcium chloride concentration. Inorganic salt addition to DEAE LCs degenerate the diffuse double layers. In the results, the viscosity of the DEAE dispersion decreased, because the apparent particle size is reduced and the bound water attached to the particle is transformed into free water. Upon increasing the salt

Photo 2 AFM images of the 20 wt% DEAE dispersion containing (A) no additives, (B) 500 mmol kg⁻¹ CaCl₂, (C) limonene (molar fraction for DEAE = 0.1), and (D) both limonene (molar fraction for DEAE = 0.1) and 500 mmol kg⁻¹ CaCl₂.

Photo 3 SEM images of the 20 wt% DEAE dispersion containing (A) no additives, (B) 500 mmol kg⁻¹ CaCl₂, (C) limonene (molar fraction for DEAE = 0.1), and (D) both limonene (molar fraction for DEAE = 0.1) and 500 mmol kg⁻¹ CaCl₂.

▼: DEAE 20%
◇: DEAE 20% + Limonene (molar fraction for DEAE = 0.1)

Fig. 2 Variation in viscosity with salt concentration in the 20 wt% DEAE dispersions.

concentration, the viscosity of the DEAE dispersion decreased less than expected based on the additive salt effect for the diffuse double layers of charged colloid particles. Thus, to confirm the effects of inorganic salt addition on the LC shapes, SEM and AFM images were examined with respect to the DEAE dispersion (500 mmol kg⁻¹ calcium chloride). The viscosity of this dispersion was slightly lower than for the dispersion containing no additives. The images displayed in **Photos 2B** and **3B** show that the dispersion retained its lamellar LC shape, even in the presence of higher calcium chloride concentrations. Similar results were obtained for the other inorganic salts. Therefore, although some effects on the hydrophilic group of

DEAE were observed upon the addition of inorganic salts to the dispersion, these salts were unable to transfer the lamellar LCs into the vesicles.

3.3 Solubilizing effects of organic substances on the DEAE LCs

The addition of organic substances was expected to cause a transition of the lamellar LCs into the vesicles due to the solubilizing effects on the DEAE LC bilayer structures. **Figure 3** shows the change in viscosity over time of a 20 wt% DEAE dispersion containing limonene (DEAE mole fraction = 0.1). Limonene was solubilized in the bilayer region of the DEAE LCs, with little change in viscosity past ~400 days. Moreover, the individual shapes of the dispersed LCs could not be observed in the SEM and AFM images, as shown in **Photos 2C** and **3C**, respectively. Similar results were obtained for the other organic substances studied herein. It therefore appeared that the addition of organic substances did not promote the transfer of lamellar LCs into the vesicles. Similarly, the DEAE LCs did not form vesicles with the addition of inorganic salts alone.

Incidentally, we found that when non-electrolyte organic substances were added to the DEAE LCs, the zeta potential of the solubilized DEAE dispersion increased compared to that prior to the addition of the 20 wt% DEAE dispersion. Indeed, a similar trend was observed for all solubilized DEAE LCs examined, as shown in **Table 1**. For example, the zeta potential increased by +64.05 mV upon solubilization with limonene, while an increase in only +36.51 mV was observed for the non-additized DEAE dispersion. These phenomena will be examined in detail later and are

○: DEAE 20%
■: DEAE 20% + Limonene (molar fraction for DEAE = 0.1)

Fig. 3 Variation in viscosity over time for the 20 wt% DEAE dispersions in the presence and absence of additives.

Table 1 Zeta potentials of the 20 wt% DEAE dispersions both in the presence and absence of additives.

Additive (molar fraction for DEAE = 0.1)	Zeta potential/mV
None	+36.51
Limonene	+64.05
Cyclohexane	+59.02
Hexane	+54.24
Octane	+66.71
Decane	+62.79
Dodecane	+76.33
Tetradecane	+74.83
Hexadecane	+66.94
Octadecane	+58.68
Perfluorohexane	+62.28

particularly important for estimating the energy of the lamellar-vesicle transition.

Examination of the zeta potential results shown in **Table 1** indicated that solubilization of organic substances in the bilayer region was promoted by increasing the flexibility of the hydrophobic regions and by the dissociation of the DEAE counterions (i.e., CH_3SO_4^- ions). This trend was also observed for other organic materials, such as the hydrocarbons and perfluorocarbons mentioned previously.

3.4 Lamellar-vesicle transition

Upon solubilization with the organic additives, it was assumed that the hydrophobic regions of DEAE expanded and became flexible due to fewer lateral interactions between the double-tailed chains of the DEAE molecules. Furthermore, we assumed that the addition of calcium chloride to the limonene-solubilized lamellar LC would result in the coexistence of organic substances and inorganic salts in the DEAE dispersion, thus leading to various synergetic effects. The bilayer properties of the 20 wt% DEAE dispersion were therefore carefully investigated in the presence of various combinations of organic and inorganic additives.

The change in viscosity with calcium chloride concentration in limonene-solubilized 20 wt% DEAE dispersions is shown in **Fig. 2**. The viscosity of the DEAE dispersion began to decrease at calcium chloride concentrations > 10 mmol kg^{-1} , decreasing rapidly from 10^5 to 10^2 mPa·s when the calcium chloride concentration reached ≥ 100 mmol kg^{-1} . Indeed, the apparent properties of the DEAE dispersion containing both limonene and calcium chloride were distinct from the other dispersions examined. SEM and AFM images of the fluid dispersion revealed spherical structures, such as the vesicles shown in **Photos 2D** and **3D**. The

Table 2 Zeta potentials and pH values for the 20 wt% DEAE dispersions both in the presence and absence of additives.

Additive	Zeta potential/mV	pH
None	36.51	2.8
Limonene	64.05	2.8
Limonene and CaCl_2	17.47	3.4

number average particle diameter corresponding to the vesicles observed by SEM was also measured by DLS. Comparable results were obtained for the structural transition of the DEAE bilayers for similar combinations of the non-electrolyte organic substances and the neutral salts.

3.5 Molecular mechanism of the transition process

We determined that the vesicles were formed by a synergetic effect based on the addition order of the organic substance and the inorganic salt. To elucidate the effect of the chemical additives on the DEAE LCs, the zeta potentials and the pH values of the various dispersions were measured, as shown in **Table 2**. Upon the addition of limonene to the DEAE dispersion, no change in pH was observed, although the zeta potential increased with an increase in limonene concentration. In contrast, the addition of 500 mmol kg^{-1} calcium chloride to the limonene-solubilized DEAE dispersion gave a decrease in zeta potential and an increase in pH.

Subsequently, the water was removed from the limonene-containing 20 wt% DEAE dispersion by centrifugation, and the limonene content in the LCs was measured by gas chromatography. In the result, the DEAE solution (mole fraction = 0.081) was found to contain limonene. Therefore, an increase in the zeta potential accompanied by the addition of organic substances results in changes to the DEAE properties. These observations are important when considering the effects of chemical additives on the lamellar LCs.

3.6 Reversibility of the lamellar-vesicle transition

Limonene is often used as a perfume in cosmetics and foods because it is somewhat volatile. We periodically measured the DEAE dispersion viscosity over time to further examine the effect of limonene solubilization. After 250 days, the viscosity of the high fluidity DEAE dispersion (containing both limonene and calcium chloride) began to increase, as shown in **Fig. 4**. After 670 days, the viscosity reached similar levels to the DEAE dispersion containing no additives. SEM was then used to determine whether the vesicle structure was maintained; however, we were unable to observe the spherical vesicle-like structures.

In contrast, following aging in a sealed container, the above dispersion (i.e., the dispersion containing limonene and calcium chloride) maintained a low viscosity, suggest-

Fig. 4 Variation in viscosity over time for the 20 wt% DEAE dispersions containing limonene (mole fraction for DEAE = 0.1) and $500 \text{ mmol kg}^{-1} \text{ CaCl}_2$.

ing that the amount of chemical additives in the dispersion may have changed. As it was unlikely that the amount of the non-volatile calcium chloride salt decreased, we measured the limonene content using gas chromatography. Indeed, the limonene content had decreased significantly (the corresponding DEAE mole fraction was 0.006), indicating a connection between the limonene content and the viscosity.

It was unclear whether this dispersion maintained its vesicle structure following 330 days in a sealed container. However, the vesicles prepared by this novel procedure were expected to retain their structure upon limonene solubilization in the palisade layers of DEAE.

4 DISCUSSION

4.1 Transition mechanism

Upon the addition of either calcium chloride or limonene to the DEAE dispersion to form lamellar LCs, the viscosity remained relatively constant and the initial structures were maintained. This confirmed that the vesicles could not be formed from lamellar LCs using these procedures. However, the synergetic effect resulting from the addition of both a non-electrolyte organic substance and a neutral salt did indeed transform lamellar LCs into vesicles. Our observations for vesicle formation from lamellar LCs upon employing chemical additives are outlined below.

The mechanism for the lamellar-vesicle transition is outlined in Scheme 2. When DEAE was added to water, from the results of pH increase, the CH_3SO_4^- counterions split the water into OH^- and H^+ ions. Due to the increased OH^- concentration, the OH^- ions likely replaced the CH_3SO_4^- ions on DEAE, thus giving the DEAE dispersion an acidic pH (i.e., pH 2.8). Furthermore, if the dissociation of CH_3SO_4^- is not promoted, the zeta potential of the DEAE LCs was predicted to be negative because the permittivity of DEAE was lower than that of water. Unexpectedly, the presence of free DEAE led to a positive zeta potential. In other words, a positive zeta potential of the DEAE LCs can be an evidence for the dissociation of CH_3SO_4^- . Thus, three con-

Scheme 2 Mechanism for lamellar-vesicle transition.

ditions are possible for the formation of lamellar LCs from DEAE: (1) Free DEAE, (2) DEAE associated with OH^- ions, and (3) DEAE associated with CH_3SO_4^- ions. We defined the coexistence of these three states as state 0, the model for which is shown on the left of **Scheme 2** (lamellar LC).

We then moved on to examine the state following the addition of limonene to state 0. After the addition of limonene, the zeta potential of the DEAE LCs increased from +36.51 to +64.05 mV, although no difference in pH was observed. This change occurred as the presence of limonene widened the distance between the molecules in the DEAE bilayers. In addition, even if the DEAE molecules became positively charged through ionic dissociation, the bilayers would be able to maintain their initial structures due to the reduction in electrical repulsions between the DEAE molecules. Thus, the dissociation of CH_3SO_4^- ions and subsequent association with OH^- ions could be promoted, as it was not necessary for these ions to attach to DEAE. However, as no change in pH was observed, it appears that only the dissociation of CH_3SO_4^- ions was promoted. We defined this state as state 1, the model for which is shown in the center of **Scheme 2** (lamellar LC, limonene solubilization).

Finally, we examined the state where calcium chloride was added to state 1. Upon the addition of 500 mmol kg^{-1} calcium chloride to the limonene-solubilized lamellar LCs, the zeta potential decreased from +64.05 to +17.47 mV and the pH increased from 2.8 to 3.4. In state 1, two possible conditions exist for the DEAE molecules in the lamellar LCs: (1) Free DEAE, and (2) DEAE associated with OH^- ions following CH_3SO_4^- release. Each condition was examined following calcium chloride addition. When calcium chloride was added to free DEAE, it appeared that Cl^- ions resulting from the dissociation of calcium chloride were attached to the cationic polar groups of the DEAE molecules. As a result, the zeta potential of the DEAE LCs decreased. In contrast, when calcium chloride was added to DEAE associated with OH^- ions, the increase in pH indicated that some Cl^- ions were exchanged for OH^- ions. In both cases, these results indicate that Cl^- ions entered between the bilayer membranes of DEAE. We defined this state as state 2, the model for which is shown on the right of **Scheme 2** (lamellar LC associated with Cl^- ions).

We made the following hypothesis. The bilayer membranes were then separated from the lamellar LCs by Cl^- ions. When only the anti-symmetric vibration occurred in these bilayer membranes, it was possible to induce inter-membrane aggregation locally, with contact points forming between the bilayers. The particle then was formed because the bilayers were torn off at the contact points. Spherical structure was most stable for the particle shape, in the results, it was assumed that more stable vesicles were formed easily. Therefore, our mechanism can be used

to interpret vesicle formation from lamellar LCs. However, when the mole fraction of limonene in the DEAE vesicles decreased from 0.081 to 0.006, the vesicle structures changed upon the introduction of chemical additives.

In contrast, **Fig. 2** shows that vesicles were formed when the calcium chloride concentration was $\geq 100 \text{ mmol kg}^{-1}$, indicating that the lamellar-vesicle transition can be controlled by the quantity of chemical additives employed. Moreover, as shown in **Fig. 2**, an intermediate region existed between the high viscosity lamellar region and the low viscosity vesicle region. These results suggest that lamellar-vesicle transition is an equilibrium where the continuous function Gibbs free energy can be controlled by calcium chloride addition. Such findings allowed us to discuss the thermodynamics of this transition for the first time.

4.2 Requirements for vesicle stabilization

The addition of both organic substances and inorganic salts to lamellar LCs led to synergetic effects and vesicle formation. However, the structure of the vesicles formed in the presence of chemical additives changed upon decreasing the mole fraction of limonene in the DEAE vesicles, as shown in **Fig. 4**. This indicates that it was important to prevent aggregation between the long hydrocarbon chains. Furthermore, it was necessary to melt the hydrophobic group of the DEAE structure constantly to maintain vesicle structural stability.

In addition, Tajima et al. reported that a stable emulsion could be prepared using phospholipid vesicles, naming this phenomenon three-phase emulsification²⁹⁾. They confirmed that the vesicles used in this three-phase emulsification were stable for long periods, although the stability mechanism was not clarified.

The results of our study could therefore explain the stabilization of the vesicles used in the three-phase emulsification process due to the constant melting of the hydrophobic group in the amphiphilic compound. In other words, vesicle stabilization is required for the coexistence of vesicles and oily materials.

5 CONCLUSION

The following points summarize the main achievements and conclusions from the study described herein:

- 1) Vesicles were prepared with the addition of various chemical additives to lamellar LCs, with the oily materials being added to the lamellar LCs prior to the neutral salts.
- 2) The mechanism of action of the various additives on the lamellar LCs was clarified, with results indicating that the addition of these compounds to lamellar LCs provided a synergetic effect that resulted in vesicle

formation.

- 3) Vesicles must be stabilized by the coexistence of both vesicles and oily materials if the vesicles are to be suitable for use in industrial fields.
- 4) The liquid crystalline state of the DEAE could be controlled only by the amount of chemical additives employed. We could therefore estimate the energy required for the lamellar-vesicle transition for the first time, as this transition is an equilibrium system whose continuous function is the Gibbs free energy.

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