In situ fluorescence microscopy enabled the observation of a phase transition from the ordered liquid-condensed (LC) phase to the disordered liquid-expanded (LE) phase for dipalmitoyl phosphatidylcholine (DPPC) monolayers prepared at alkane/water interfaces, which depended on the DPPC interfacial concentration, temperature, and the length of the alkane alkyl chains. Some experimental conditions were examined, and it was found that the LC–LE phase transition was an intrinsic feature of the DPPC monolayers. The dependence of the LC–LE phase transition temperature (T_m) on the DPPC interfacial concentration was analyzed using a common thermodynamic relation. The penetration of alkane molecules into the DPPC monolayer was suggested, and the matching of alkyl groups of DPPC with organic phase solvents was found to be an important factor for the phase transition. At the toluene/water and limonene/water interfaces, the phase transition of the DPPC monolayer was not observed because of poor matching.

1. Introduction

Biological bilayer membranes consist of various components, such as phospholipids, cholesterol, membrane proteins, and they exist in membranes inhomogeneously. The main component is phospholipid; common phospholipids have two hydrophobic aliphatic groups and a hydrophilic part, and they are amphiphilic. The bilayer membranes act as a wall that separates the internal aqueous phase from the external aqueous phase. There are no chemical bonds between the components, but the membranes exist stably through their attractive interactions. Furthermore, they can hinder the passing of many substances and channels existing in the membranes can pass only required substances. Therefore, they provide an ideal model of separation.

Much research in physical chemistry has paid attention to biological bilayer membranes and has studied the natures of artificial bilayer membranes using mainly vesicles in aqueous solutions [1-5]. Some studies employed phospholipid monolayers prepared on aqueous solution surfaces [6-8]. These studies
showed that a phase transition occurred for phospholipid layers. For example, a gel–liquid crystalline phase transition occurs at 41.6 °C for dipalmitoyl phosphatidylcholine (DPPC) bilayers [5]. For phospholipid bilayers, the phospholipid molecules are packed closely, and thus the area occupied by a single phospholipid molecule is almost constant. On the other hand, the surface concentration of the phospholipid can change on aqueous solution surfaces, and another transition of an ordered liquid-condensed (LC) phase to a disordered liquid-expanded (LE) phase was observed depending on the surface concentration and temperature [8]. The gel or LC phase has a lower fluidity, and the liquid crystalline or LE phase has a higher fluidity. These phase transitions are quite interesting because they may occur by the lateral interactions of phospholipid molecules and they are unique features of the phospholipid layers. There are few studies of phospholipid monolayers prepared at liquid/liquid interfaces [9,10], and little information is available for phospholipid monolayers at liquid/liquid interfaces.

The liquid/liquid interface is a key field in solvent extraction; substances pass through the interface during extraction processes and various reactions occur there [11,12]. Amphiphilic substances are known to be highly adsorbed at liquid/liquid interfaces, and they sometimes form microdomains [13-15] resembling the LC phase of phospholipids. In situ optical microscopic observation is necessary for the study of the microdomain formation. We already reported many microscopic studies of reactions and phenomena occurring at liquid/liquid interfaces [13-20]. On the other hand, liquid/liquid interfaces are sometimes regarded as models of biological membranes, because they contain both an organic surface wall and an aqueous surface wall. However, real biological membranes mainly consist of phospholipids.

The present study focuses on the phase transition behavior of phospholipid monolayers at liquid/liquid interfaces with two purposes. (1) If a stable phospholipid monolayer is prepared, it would show a unique interface in solvent extraction. (2) If a stable phospholipid monolayer is prepared and it shows features similar to phospholipid bilayers, it will be a better model of biological membranes than bare liquid/liquid interfaces. DPPC monolayers prepared at some hydrocarbon/water interfaces were investigated by in situ fluorescence microscopy in detail; DPPC is employed because its phase transition temperature is around room temperature.

2. Experimental

2.1 Reagents

As a phospholipid, dipalmitoyl phosphatidylcholine (1,2-diheptadecanoyl-sn-glycero-3-phosphocholine, DPPC, purity > 99%, Avanti) was used. As a fluorescent phospholipid, Texas Red® 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt ((C2H5)3NH⁺·TR-DHPE– (T-1395), Molecular Probes) and 2-(12-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-amino)-dodecanoyl-1-hexadecanoyl-sn-glycero-3-phosphocholine (NBD-PC (N-3787), Molecular Probes) were examined. Their chemical structure is shown in Fig. 1. The excitation and emission maxima for the fluorescence of TR-DHPE– are 583 and 601 nm, respectively, and those of NBD-PC are 465 and 534 nm, respectively [21]. A cyclohexane–chloroform mixture (9:1 in volume) was the main solvent for the phospholipid solutions, and cyclohexane and another cyclohexane–chloroform mixture (7:3 in volume) were also examined in some cases. Cyclohexane (purity 99.5%) and chloroform (purity 99.7%) were purchased from Kanto Chemical. The total concentration of the phospholipids was set at $1.0 \times 10^{-5}$ mol dm$^{-3}$. As the organic
phase solvent, dodecane (Sigma-Aldrich, purity ≥ 99%), tetradecane (Sigma-Aldrich, purity ≥ 99%), hexadecane (Sigma-Aldrich, purity 99%), toluene (Kanto Chemical, purity 99.7%), (R)-(+)-limonene (Sigma-Aldrich, purity 97%) were examined, but tetradecane was mainly employed. Water was purified with a Milli-Q system (Milli-Q SP, Millipore). Sodium chloride was of analytical reagent grade.

2.2 Preparation of the DPPC monolayers at hydrocarbon/water interfaces

For the preparation of the phospholipid monolayers at the hydrocarbon/water interfaces, a homemade thin-layer two-phase microcell was used [13], which is shown in Fig. 2. First pure water (or an NaCl aqueous solution) was put into the lower part of the microcell. Then an aliquot (4 – 20 mm³) of the phospholipid solution was put on top of the water and left for about 5 min. The organic solvents of the phospholipid solution (cyclohexane and chloroform) vaporized completely, and a phospholipid monolayer was formed at the air/water interface. Finally, an organic phase solvent was gently put on the phospholipid monolayer.

2.3 Fluorescence microscope

A fluorescence microscope consisting of an inverted microscope (IX-51, Olympus), an objective lens (UPlanFl, 20×, NA 0.50, Olympus), two mirror units (homemade, U-MWIB2 (Olympus)), and a light source (Hg discharge lamp (100 W), Olympus) was used. Excitation light was irradiated through the objective lens (epi-illumination), and fluorescence images were observed with the same objective lens. The homemade mirror unit contained an excitation filter (BP520-550, Olympus), a dichroic mirror (DM565, Olympus), and an emission filter (XF3083, Omega Optical). It was used for the observation of TR-DHPE.
fluorescence (excitation 520 – 550 nm, observation 565 – 625 nm). U-MWIB2 was used for the observation of NBD-PC fluorescence (excitation 460 – 490 nm, observation 510 – 900 nm). Fluorescence microscope pictures were obtained with a monochromatic CCD camera (WAT-100N, Watec Co., Ltd.), and were transferred as digital pictures to a personal computer for analysis. An automatic thermo-controlled plate (MATS-555MRO, Tokai Hit) was used to control the temperature of the microcell. To know the accurate temperature around the interface, a digital thermometer with a sensor (CT-1200D, Custom) was used. The end of the sensor was soaked in the organic phase and was located at 300 µm above the interface, as shown in Fig. 2. After the temperature had been kept constant for about 10 min, it was changed by 0.5 °C. The temperature range in the present study was 10 – 45 °C.

3. Results and Discussion

3.1 Thermotropic phase separation of the DPPC monolayer prepared at the tetradecane/water interface

DPPC is only slightly soluble in alkanes and water, and thus almost all of the added DPPC remained at the interface. Therefore, the interfacial concentration of DPPC \((\Gamma_{\text{DPPC}})\) was calculated from the added amount of DPPC and the interfacial area (19.6 mm²). At a fixed \(\Gamma_{\text{DPPC}}\) value at the tetradecane/water interface, the temperature around the interface was changed. Figure 3 shows an example of the results. The picture was homogeneous at 40.0 °C, but dark dots appeared at 34.0 °C. The dots grew and many round dark microdomains were observed at 29.0 °C. The size of the microdomains increased with cooling. On the other hand, the size of the microdomains became small on heating and finally disappeared at 34.5 °C (Fig. 3f).

As mentioned above, a gel–liquid crystalline transition is observed for phospholipid bilayers, and the transition of the ordered liquid-condensed (LC) phase to the disordered liquid-expanded (LE) phase is observed for phospholipid monolayers. When the DPPC molecules of the monolayer are closely packed, the LC–LE phase transition corresponds to the gel–liquid crystalline transition of the bilayers.

When the temperature was kept at 29.0 °C, the shape and size of the dark microdomains in Figs. 3c did not change but their position randomly changed. These facts mean that the round microdomains have low fluidity and the bright continuous region has high fluidity. Therefore, the dark microdomains are in the LC phase and the bright region is in the LE phase, as shown in Fig. 4.
The temperature of Fig. 3b is the same as that of Fig. 3e, but the former is in a heating process and the latter is in a cooling process. Both the pictures seem to be quite similar, meaning that the present LC–LE phase transition is reversible. The formation of the LC phase may correspond to the two-dimensional crystallization occurring at the liquid/liquid interface. The darkness of the LC phase means that the fluorescent phospholipid (TR-DHPE–) is excluded from the LC phase, which is also similar to the crystallization situation, as shown in Fig. 4. The critical temperature at which the LC phase disappeared is now defined as the LC–LE phase transition temperature \( T_{m} \). From Fig. 3, \( T_{m} \) was determined to be 34.5 °C. This temperature corresponds to the fusion temperature (melting point) of the LC phase. Similar heating and cooling processes were repeated with the same DPPC monolayer and the same \( T_{m} \) values were obtained. This fact also shows the reversible phase transition and no loss of DPPC molecules from the interface during heating or cooling.

3.2 Effects of fluorescent phospholipids

Two fluorescent phospholipids (TR-DHPE–, NBD-PC) were examined with the present microscope system. TR-DHPE– has a fluorescent moiety around the polar group, whereas NBD-PC has a fluorescent moiety around the hydrophobic group. TR-DHPE shows a higher fluorescence intensity and weak photobleaching, whereas NBD-PC has a lower fluorescence intensity and strong photobleaching.

To examine the effects of the amount of TR-DHPE– on \( T_{m} \), TR-DHPE– was added to DPPC in 0.002 and 0.01 mole fraction amounts at the tetradecane/water interface with \( \Gamma_{\text{DPPC}} = 4.1 \times 10^{-6} \text{ mol m}^{-2} \). As a result, 34.5 and 32.7 °C were obtained as \( T_{m} \) values, respectively. These values were almost the same.

When NBD-PC was used, a similar LC–LE phase transition was observed but the exact \( T_{m} \) value could not be obtained due to the lower fluorescence and strong photobleaching. The phase transition was observed in the temperature range 35–37 °C at the tetradecane/water interface with \( \Gamma_{\text{DPPC}} = 8.2 \times 10^{-6} \text{ mol m}^{-2} \) (a mole fraction amount of 0.01), which approximately agreed with the \( T_{m} \) value with TR-DHPE– (37.5 °C, shown below).

These facts mean that the fluorescent phospholipids do not cause the LC–LE phase transition of the DPPC monolayer. In other words, the LC–LE phase transition is an intrinsic feature of the DPPC monolayer. TR-DHPE– is more suitable for the present study, and a mole fraction amount of 0.002 TR-DHPE– was added in the following sections.

3.3 Selection of the solvent of the phospholipid solution

Generally, phospholipids are soluble in methanol and chloroform. However, both these solvents are not suitable for the present system, because methanol is miscible with water and the density of chloroform is higher than that of water. Therefore, three other types of solvent were examined that were immiscible with water, were volatile, and had a lower density than water, that is, cyclohexane and two mixtures of cyclohexane–chloroform (9:1 and 7:3 in volume).

The results are shown in Fig. 5. This figure displays the dependence of \( T_{m} \) on the added volume of the DPPC solutions. The DPPC concentration was set at \( 1 \times 10^{-5} \text{ mol dm}^{-3} \) for all of the solutions. The \( T_{m} \) value increases with an increase in the amount of DPPC for each solvent. The \( T_{m} \) values for the two mixtures almost agree with each other, but the \( T_{m} \) values for cyclohexane are 15–17 °C lower than those
for the mixtures. The increase in the $T_m$ values with increasing amounts of DPPC implies that the DPPC concentration of the cyclohexane solution was lower than those of the mixed solutions. In fact, DPPC is barely soluble in cyclohexane at room temperature, and it was only dissolved by heating. Therefore, a fraction of DPPC would be precipitated at room temperature and the concentration of dissolved DPPC would be lower than the expected concentration. On the other hand, DPPC readily dissolves in the cyclohexane–chloroform mixtures at room temperature. Therefore all of the added DPPC was dissolved in these mixtures. In the above and following sections, the cyclohexane–chloroform mixture (9:1 in volume) was employed.

3.4 Effects of the DPPC interfacial concentration

The DPPC interfacial concentration ($\Gamma_{DPPC}$) was varied by changing the added volume of the DPPC solution, and $T_m$ was measured in the same way. As shown in Fig. 6, $T_m$ increases with an increase in $\Gamma_{DPPC}$, and becomes almost constant in the range of $(6 \sim 10) \times 10^{-6}$ mol m$^{-2}$. The cross-sectional area per one DPPC molecule was reported to be 0.39 nm$^2$ [4], from which the saturated interfacial concentration of DPPC was calculated to be $4.3 \times 10^{-6}$ mol m$^{-2}$. The $\Gamma_{DPPC}$ value at the intersection of the two lines in Fig. 6 is $5.0 \times 10^{-6}$ mol m$^{-2}$, which almost agrees with the saturated value. This fact means that the interface was almost saturated with DPPC at the higher values of $\Gamma_{DPPC}$. The excess DPPC may leave the interface by adsorption on the inner wall of the microcell. The $T_m$ value of the saturated DPPC monolayer (37.5 °C) is about 4 °C lower than the temperature of the gel–liquid crystalline phase transition ($T_b$) of the DPPC bilayer (41.6 °C), implying that both transitions are similar but somewhat different.

As shown in Fig. 3, the LE phase remained below $T_m$, meaning that the number of components was 2 according to the Gibbs’ phase rule. One component is obviously DPPC and the other is the organic phase solvent, tetradecane. Therefore, a mixture of two components, A and B, is considered as a model of the LC–LE phase transition of the DPPC monolayer; the components A and B having high and low fusion temperatures, respectively. When the mixtures of A and B are an ideal solution or a regular solution, the following relation is valid [22].

![Figure 5. Effects of the DPPC solution solvent. The DPPC monolayer was prepared at the tetradecane/water interface. The lines were drawn for clarification.](image)

![Figure 6. Dependence of $T_m$ on the DPPC interfacial concentration ($\Gamma_{DPPC}$) prepared at the tetradecane/water interface.](image)
\[
\ln x_A = \frac{\Delta h_f}{R} \left( \frac{1}{T_m} - \frac{1}{T_m^o} \right)
\]

where \( x_A \) is the mole fraction of component A, \( T_m^o \) is the reference fusion temperature at \( x_A = 1 \), \( R \) is the gas constant, \( \Delta h_f \) the molar enthalpy of fusion at \( x_A = 1 \). In Eqs (1) and following (2), \( T_m \) and \( T_m^o \) represent their absolute temperature. The fusion temperature (melting point, \( T_f \)) of tetradecane is 5.5 °C [23], which is lower than the \( T_b \) value of the DPPC bilayer (41.6 °C). Thus the components A and B correspond to DPPC and tetradecane, respectively. The nature of the mixing of the alkyl groups of DPPC with alkanes is expected to be that for a regular solution.

\[
T_m = \frac{T_m^o \Delta h_f}{\Delta h_f + RT_m^o \ln x_{DPPC}}
\]

where \( x_{DPPC} \) is the mole fraction of DPPC in the monolayer just before the phase transition. Before comparing the result in Fig. 6 with Eq. (2), \( x_{DPPC} \) should be estimated. It is known that the cross-sectional area of substances at liquid/liquid interfaces slightly depends on their size, and therefore the area of tetradecane was assumed to be approximately equal to that of DPPC in the LE phase. On this assumption, \( x_{DPPC} \) was calculated [24]. Figure 7 shows the plot of \( T_m \) against \( x_{DPPC} \), where \( 5 \times 10^{-6} \) mol m\(^{-2} \) was treated as the saturated concentration. The line calculated by Eq. (2) with \( \Delta h_f = -78 \) kJ mol\(^{-1} \) and \( T_m^o = 37.5 \) °C agrees well with the observed \( T_m \) values. The obtained \( \Delta h_f \) value will be discussed in the following section.

### 3.5 Effects of organic phase solvents

Four other organic phase solvents were examined, that is, dodecane, hexadecane, toluene, and limonene. Their \( T_f \) values are dodecane –9.6 °C [25], hexadecane 18.1 °C [23], toluene –95.0 °C [25], and limonene –96.6 °C [25]. All of them are lower than the \( T_b \) value of the DPPC bilayer, and thus the LC phase was mainly formed by DPPC molecules.

The DPPC monolayer prepared at the dodecane/water and hexadecane/water interfaces showed similar LC–LE phase transitions. Figure 8 gives the effects of alkanes on \( T_m \). \( T_m \) increases with an increase in \( \Gamma_{DPPC} \) for all of the solvents, and it also increases with an increase in the length of the alkane alkyl chains. The \( T_m \) values for dodecane is about 6 – 10 °C lower than those for tetradecane and hexadecane. \( T_m \) at the saturated \( \Gamma_{DPPC} \) value is different for each alkane, and the \( T_m^o \) value in Fig. 7 does not
agree with the $T_b$ value for the DPPC bilayer. These facts suggest that the LC phase is similar to but not quite the same as the gel phase of the bilayer. One possibility for the difference is penetration of alkane molecules into the DPPC molecules in the LC phase. The penetration of foreign but similar substances leads to a lowered fusion temperature such as for mixed crystals. The carbon number of one of the alkyl groups of DPPC is 15, which is between those of tetradecane and hexadecane, and the $T_f$ value of hexadecane is closest to the $T_b$ value of the DPPC bilayer. Therefore, hexadecane is the best match for the alkyl groups of DPPC and the $T_m$ value is highest. The second is tetradecane and the third is dodecane.

The fluorescence microscope pictures of the DPPC monolayers at alkane/water interfaces are shown in Fig. 9. Each picture was obtained under similar conditions, at 10 °C below each $T_m$ value, but the microdomains are darkest and largest at the hexadecane/water interface. The second darkest is tetradecane again. This picture also suggests the matching order; the LC phase is most easily formed at the hexadecane/water interface, next at the tetradecane/water interface. Thoma et al. also showed that alkane molecules penetrated the DPPC molecules in the LC phase and that the mole ratio of DPPC to alkane was 1:1 in the LC phase [10]. Figure 10 shows a modified illustration of the LC and LE phases of the DPPC monolayer prepared at alkane/water interfaces.

From direct thermodynamic measurements for aqueous solutions of DPPC bilayers (vesicles), the $\Delta h_f$ value was determined to be about $-35$ kJ mol$^{-1}$ [4,5]. On the other hand, Thoma et al. estimated that $\Delta h_f$ for the DPPC monolayer prepared at the hexadecane/water interface was in a range from $-85$ to $-73$ kJ mol$^{-1}$ at 30 – 35 °C by another indirect method [10]. Our $\Delta h_f$ value is close to the values of the DPPC monolayer, but it is about double the value of the DPPC bilayer. The DPPC bilayer contains only DPPC molecules, but the DPPC monolayer contains DPPC and tetradecane molecules. Therefore, $\Delta h_f$ for the bilayer corresponds to the enthalpy of fusion of 1 mol of DPPC, whereas $\Delta h_f$ for the monolayer corresponds to the enthalpy of fusion of 1 mol of DPPC and 1 mol of tetradecane if the mole ratio in the LC phase is 1:1. This consideration supports the double $\Delta h_f$ values for the monolayer.

In the cases of toluene and limonene, the microdomains of the DPPC LC phase were not observed in the temperature range of 10 – 45 °C. Two reasons are possible. As for the relation between the DPPC alkyl groups and non-chained toluene or limonene molecules, the structural matching is poor and the difference in the fusion temperature is large. Therefore, if toluene or limonene molecules penetrated the DPPC alkyl groups in the LC phase, $T_m$ should be lowered considerably. This is one possibility. If penetration does not
occur, a higher $T_m$ value is expected. Thoma et al. also did not observe the microdomains of the LC phase for the DPPC monolayer prepared at the non-chained bicyclohexyl/water interface. They considered that the DPPC molecules in the LC phase excluded the bicyclohexyl molecules and that tiny microdomains were formed that were not observable by optical microscopy [10]. This is the other possibility.

3.6 Effects of NaCl concentration

DPPC is a zwitterion, and thus there is a weak electrostatic interaction between DPPC molecules, such as dipole–dipole interaction. Therefore, ions in the aqueous phase were expected to have a small influence on the DPPC monolayer. However, a large amount of salts exists in biological cells and fluid. Figure 11 shows the effect of the NaCl concentration on $T_m$. $T_m$ somewhat decreases with an increase in the NaCl concentration. There are ionic attractive interactions between Na\(^+\) and the anionic site of DPPC in the disordered LE phase and between Cl\(^-\) and the cationic site, whereas DPPC molecules in the ordered LC phase tend to exclude ions. This leads to a slight stabilization of the DPPC molecules in the LE phase, resulting in a decrease in $T_m$ [22,26].

![Figure 11. Effects of NaCl concentration on the $T_m$ value of the DPPC monolayer prepared at tetradecane/aqueous NaCl interfaces with $\Gamma_{DPPC} = 4.1 \times 10^{-6}$ mol m\(^{-2}\). The line was drawn for clarification.](image)

Conclusion

The stable DPPC monolayer prepared at alkane/water interfaces showed a LC–LE phase transition depending on the DPPC interfacial concentration, temperature and the length of the alkane alkyl chains. The alkane molecules penetrate the alkyl groups of DPPC, which is different from the bilayer, but the similarity of the LC–LE phase transition to the gel–liquid crystalline transition suggests that these transitions are mainly caused by the lateral interaction between the single layer phospholipid molecules. The phospholipid monolayers can hinder the passing of substances through the liquid/liquid interface. This ability could lead to the future construction of a highly-selective interface with a channel that can pass only a specific substance. On the other hand, the total-internal reflection (TIR) of light can be carried out at the liquid/liquid interface because common organic solvents have a higher refractive index than water. The TIR technique is one of the useful methods for the study of interfacial chemistry but is not applicable to phospholipid bilayers in aqueous solutions. The dynamic behaviors of biological substances, such as proteins or DNA, near phospholipids will be investigated more precisely by using the TIR technique and phospholipid monolayers at liquid/liquid interfaces.

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References and footnotes

24) \[ x_{\text{DPPC}} = \frac{n_{\text{DPPC}} + n_{\text{tdn}}}{\Gamma_{\text{DPPC}} + \Gamma_{\text{tdn}}} \] where \( n \) is the amount of substance (in mol) at the interface and the subscript tdn means tetradecane. The assumption means that \( \Gamma_{\text{DPPC}} + \Gamma_{\text{tdn}} \) corresponds to the saturated interfacial concentration (\( \Gamma_{\text{sat}} \)). Therefore, \( x_{\text{DPPC}} = \frac{\Gamma_{\text{DPPC}}}{\Gamma_{\text{tdn}}} \).
26) \( \frac{\partial \mu}{\partial T} = -s \), where \( \mu \) is chemical potential and \( s \) is molar entropy. \( s \) of the LE phase is considered to be larger than that of the LC phase.