A mechanism of pressure-induced interdigitation of lipid bilayers

N Tamai, M Goto, H Matsuki, S Kaneshina
Department of Life System, Institute of Technology and Science, The University of Tokushima, 2-1 Minamijosanjima-cho, Tokushima 770-8506, Japan
E-mail: matsuki@bio.tokushima-u.ac.jp

Abstract. The bilayer phase behavior of di-O-hexadecylphosphatidylethanolamine (DHPE), one of ether-linked phosphoethanolamines (PEs), was investigated under atmospheric and high pressure by means of differential scanning calorimetry (DSC) and high-pressure light-transmittance techniques. The gel-to-liquid-crystalline phase transition was observed at 67.1 °C under atmospheric pressure. The transition enthalpy was estimated to be 31.7 kJ mol⁻¹. Also under high pressure, only the same type of phase transition was observed, which means that DHPE cannot form the interdigitated structure even under high pressure. In general, it is known that the PE headgroup has an inhibitory effect against the interdigitation, whereas the presence of the ether linkage is advantageous to that. The result in this study indicates that the strong interaction by the formation of the hydrogen bonding network between the PE headgroups completely abolishes the capability of the phospholipid to form the interdigitated structure. By comparing the result with the previous results for the ether-linked phosphocholine (PC) and the ester-linked PC and PE lipids, we speculate on the mechanism of the transformation into the interdigitated structure induced by pressure in terms of the molecular interactions between the lipid molecules in the bilayer membrane.

1. Introduction
A biomembrane is a bilayer membrane composed of many kinds of phospholipids, which contains wide variety of biomolecules including proteins and cholesterol. A typical phospholipid is an amphiphilic molecule with a hydrophilic part of a polar headgroup and a hydrophobic part of two hydrocarbon chains. When dispersed in an aqueous medium, the phospholipid molecules self-assemble to form a bilayer membrane of capsule-like closed structure in general, which is called liposome or vesicle. Artificial phospholipid bilayer membranes have long been utilized as mimetic membranes for examining the physical, chemical and biological properties of the biomembrane, because they have been widely recognized as essentially equivalent to the fundamental structure of the biomembrane. Dipalmitoylphosphatidylcholine (DPPC) is most often used in thousands of model membrane studies that have ever been performed [1], although the biomembrane contains a wide variety of phospholipids. This prevalent use of DPPC is not only due to its simple chemical structure which can be featured by a phosphocholine (PC) headgroup with a net neutral charge and two identical linear saturated hydrocarbon chains (i.e., symmetric saturated phospholipid), but also due to the biological basis that typical plasma membranes contain a relatively large amount of phospholipids.

1 To whom correspondence should be addressed.
with a PC headgroup or phospholipids with one or two hydrocarbon chains consisting of 16 or 18 carbon atoms [2]. Such thorough investigations using DPPC have firmly established the scientific basis in this field. It is well known, for example, that the DPPC bilayer membrane exhibits three kinds of structural changes with increasing temperature under atmospheric pressure. The structural changes are generally treated as thermotropic phase transitions: the subtransition from the lamellar crystal (Lc) phase to the lamellar gel (Lg′) phase, the pretransition from the Lg′ phase to the ripple gel (P6′) phase, and the main transition from the P6′ phase to the liquid crystalline (Lα) phase occur in turn with increasing temperature [3,4]. These sub-, pre- and main transitions are known to be attributable mainly to the variations of the hydration state around the polar headgroups, the change in the molecular arrangement from a planar bilayer into a undulating bilayer and the conformation change of the hydrocarbon chains from the all-trans chains to chains containing some gauche bonds (i.e., chain melting), respectively. Before and after all the phase transitions, therefore, the bilayer structure itself is maintained, and only the molecular arrangement and orientation in the bilayer change at the respective phase transitions.

More drastic structural changes can occur under certain conditions, so that the phospholipid molecules forming bilayer membranes eventually reorganize themselves to form other types of molecular aggregates with peculiar structures than the bilayer structure. Interdigitated structure, usually referred to as LdI phase, is one of such non-bilayer structures. When the interdigitation occurs, the hydrocarbon chains of the phospholipid molecules in a monolayer constituting a bilayer extend beyond the region of the bilayer midplane and, as a result, interpenetrate into the other opposing monolayer. The structure is characterized by the comparatively small membrane thickness and the relatively large cross-sectional area subtended by a single headgroup. Asymmetric saturated PC lipids [5–8] and ether-linked PC lipids [9–11] are well known as phospholipids capable of forming the interdigitated structure under atmospheric pressure. The former type of phospholipids have two different saturated acyl chains in length, and the effect of the chain-length asymmetry on the formation of the interdigitated structure has been systematically summarized by Slater and Huang [12] in terms of the parameter of chain inequivalence by classifying the structure into three types, namely, partially interdigitation, mixed interdigitation and fully interdigitation. DPPC is also known as one of them; that is, the DPPC bilayer membrane can be transformed into the interdigitated structure by the addition of short-chain alcohols [13,14] and polyols [15] and by the exertion of pressure [16–18]. The former and the latter types of the interdigitation are sometimes distinctively called chemically induced interdigitation and pressure-induced interdigitation, respectively, in order to clarify what induces the interdigitation. The pressure-induced interdigitation has been less studied as compared to the other types of the interdigitation including partially and mixed interdigitation which can take place under atmospheric pressure. Consequently, it is still unclear how isotropic hydrostatic pressure drives the transformation into the interdigitated structure.

We have investigated the thermotropic and barotropic bilayer phase behavior of various kinds of phospholipids and constructed temperature (T)–pressure (P) phase diagrams for them. In our previous report [19], we demonstrated that the interdigitated structure is not induced by pressure in the bilayer membrane of dipalmitylophosphatidylethanolamine (DPPE). DPPE is a phospholipid that has a phosphoethanolamine (PE) headgroup, and thus is different from DPPC only in the chemical structure of the polar headgroup; that is, DPPE has an amine group at the end of the polar headgroup whereas DPPC has a trimethylamine group at the same part of the headgroup. Therefore, our previous result indicates that the PE headgroup has an inhibitory effect on the transformation into the interdigitated structure. By contrast, it is well known that di-O-hexadecylphosphatidylcholine (DHPC), which is an ether-linked PC lipid, can form the interdigitated structure even at an atmospheric pressure without any inducer. Since DPPC is an ester-linked PC lipid, DHPC and DPPC have the same chemical structure except for the difference in the chemical structure of the ether and ester linkages between the glycerol backbone and the two hydrocarbon chains. This suggests that the ether linkages between the glycerol backbone and hydrocarbon chains are advantageous to the formation of the interdigitated structure as compared to the ester linkages. Taking these facts into consideration, we investigated the
thermotropic and barotropic phase behavior of the bilayer membrane of di-O-hexadecylphosphatidylethanolamine (DHPE) by high-sensitivity differential calorimetry (DSC) and high-pressure light-transmittance techniques to draw the $T$–$P$ phase diagram. In the present paper, we compare the phase behavior of the DHPE bilayer membrane with those of the DPPC, the DPPE and the DHPC bilayer membranes, and discuss the relationship between the chemical structure of the individual phospholipid molecule and its capability of forming the interdigitated structure. Furthermore, we give a speculation on the mechanism of the pressure-induced interdigitation for DPPC in terms of the molecular interaction between the adjacent molecules in the membrane.

2. Experimental

A synthetic phospholipid, 1,2-di-O-hexadecyl-sn-glycero-3-phosphoethanolamine (DHPE) was purchased from Larodan Fine Chemicals AB (Malmö, Sweden), and used without further purification. A weighed amount of lipid powder was suspended in an appropriate amount of double distilled water by vortexing at room temperature for a few minutes and subsequently sonicating at about 70 °C for a few minutes to obtain a homogeneously translucent aqueous dispersion of the DHPE multilamellar vesicle with the lipid concentration of 1.0 mmol kg$^{-1}$.

Light transmittance measurements were performed under atmospheric and high pressure using a U-3010 spectrophotometer (Hitachi Co., Tokyo) equipped with a Model PCI-400 high-pressure cell assembly with two sapphire windows (Tera-Mecs Co., Kyoto). The wavelength of the incident beam is 560 nm. The temperature of a sample dispersion in the high-pressure cell was controlled within the accuracy of 0.1 °C by circulating thermostated water. Pressure was generated by a hand-operated hydraulic pump (Hikari High Pressure Instruments, Hiroshima) and monitored by a Heise gauge with a resolution of 0.5 MPa (Heise Co., Newtown, CT). A transmittance profile was obtained from a heating scan carried out at a constant heating rate (0.33 °C min$^{-1}$) under a pseudo-isobaric condition, and the transition temperature was determined from the midpoint of an abrupt change in the profile (i.e., light-transmittance vs. temperature curve) at each pressure measured. A pressure variation due to the rise of the temperature of the sample dispersion was duly corrected.

DSC measurements were performed on an MCS high-sensitivity differential scanning calorimeter (MicroCal, Northampton, MA) using a heating rate of 0.75 °C min$^{-1}$ in the temperature range of 10–80 °C. In a DSC thermogram, a bilayer phase transition was detected as an endothermic peak, and the transition temperature and the enthalpy change of the transition were determined as the temperature at the peak top and from the area of the endothermic peak in the DSC thermogram, respectively.

3. Results and Discussion

3.1. Phase behavior of DHPE bilayer membrane under atmospheric and high pressure

The transition temperature of the DHPE bilayer membrane was determined as a function of $P$ from an abrupt change observed in the light-transmittance vs. $T$ curve. Also the DSC measurements were carried out to thermodynamically characterize the phase transition of the DHPE bilayer membrane at the atmospheric pressure. The transition temperature at the atmospheric pressure was determined to be 67.6 °C from the light-transmittance measurements, and the transition enthalpy ($\Delta H$) was estimated at 31.7 kJ mol$^{-1}$ from the area of the endothermic peak in the DSC thermogram. These results are consistent with a previous report by Hing et al. [20], which demonstrated that for the aqueous system of DHPE above 20% H$_2$O, the chain-melting transition between the lamellar ($L_\beta$) and the $L_a$ phases occurs at 67.1 °C and the enthalpy change of 32.6–34.3 kJ mol$^{-1}$ accompanies this transition. This agreement means that the transition we detected in this study can be assigned as the $L_\beta/L_a$ transition. Figure 1 shows the pressure dependence of the $L_\beta/L_a$ transition temperature for the DHPE bilayer membrane. The slope of the curve ($dT/dP$) was estimated at 0.232 °C MPa$^{-1}$, which is almost equal to the $dT/dP$ values of the main transitions for the DPPC bilayer membrane (0.220 °C MPa$^{-1}$) [21] and for the DHPC bilayer membrane (0.238 °C MPa$^{-1}$) [22]. The volume change ($\Delta V$) can be obtained by applying these thermodynamic quantities to the Clapeyron equation:
For the DHPE bilayer membrane, the $\Delta V$ value of the $L_\beta/L_{\alpha}$ transition was calculated to be 21.4 cm$^3$ mol$^{-1}$. This $\Delta V$ value is slightly smaller than the corresponding values of the DPPC bilayer membrane (25.4 cm$^3$ mol$^{-1}$) and the DHPC bilayer membrane (25.3 cm$^3$ mol$^{-1}$), but is still reasonable as a volume change arising from the melting of the linear saturated hydrocarbon chains of 16 carbon atoms in the bilayer membrane.

In this study, we could detect only the $L_\beta/L_{\alpha}$ transition in the whole temperature- and pressure-ranges where the measurements were made. This means that the interdigitated structure cannot be induced by pressure at least up to ca. 120 MPa in the DHPE bilayer membrane. Even though still higher pressure is exerted on the DHPE bilayer membrane, the interdigitated structure would not be induced because there is no chain tilt in the DHPE bilayer membrane below the $L_\beta/L_{\alpha}$ transition temperature. In our view, as described later in detail, the chain tilt in the bilayer membrane in the gel state plays an important role in the process of the pressure induced interdigitation. Accordingly, we can conclude that DHPE can be classified as a phospholipid that is inherently incapable of forming the interdigitated structure. Regarding the bilayer phase behavior of DHPE, it should be noted that DHPE can form the inverted hexagonal (H$_{II}$) phase above the $L_\beta$/H$_{II}$ transition temperature. This is obviously inconsistent with our result that no other kinds of phase transitions were found besides the $L_\beta/L_{\alpha}$ transition. This inconsistency is due to the temperature range investigated in this study. It has been reported that the $L_{\alpha}$/H$_{II}$ transition occurs at a very high temperature even under atmospheric pressure (e.g., 92.9 °C reported in [19] and 86 °C in [23]), and moreover, the transition temperature rises as the

![Figure 1. Pressure dependence of $L_\beta/L_{\alpha}$ transition temperature for DHPE bilayer. In the whole temperature and pressure region investigated no other phase transitions were observed.](image-url)
pressure is increased [24]. On the other hand, our primary aim in this study is to examine whether or not DHPE can form the interdigitated structure under high pressure, and thus, much of our attention has been focused on the investigation on the phase behavior below the $L_d/L_{st}$ transition temperature. To observe the $L_d/H_{II}$ transition, therefore, the experimental temperature range has to be expanded to even higher temperatures.

3.2. Molecular interaction and pressure-induced interdigitation

According to thermodynamics, the spontaneous formation of the interdigitated structure at a given temperature and pressure means that the structure must be the most stable state on the condition, and thus that the structure must have the optimal molecular geometry for lower free energy of the system. In this meaning, the interdigitated structure can be interpreted as a state appearing as the result of the molecular rearrangement for thermodynamically more favorable molecular interactions. Considering that the molecular interaction in the aggregate structure is essentially relevant to the chemical structure of the individual constituent molecules, the fact that limited kinds of phospholipids can inherently form the interdigitated structure means that they contain a specific chemical structure that can produce and maintain the molecular interaction favorable to the interdigitated structure, or do not have any chemical structure that produces an inhibitory effect against the interdigitation. From this viewpoint, Table 1 summarizes the result in this study together with those in our previous studies [19,22]. Table 1 clearly shows that the PE lipids, regardless of the type of linkage, cannot form the interdigitated structure. The PE lipids have three hydrogen atoms directly attached to the quaternary nitrogen in the polar headgroup, and thus, the hydrogen atoms of a PE lipid can form hydrogen bonds to negatively charged oxygen atoms in the phosphate groups of its adjacent PE lipids in the bilayer membrane. This formation of the hydrogen-bonding network at the bilayer surface enhances the stability of the bilayer membrane. On the other hand, the PC lipids have no hydrogen atom in the polar headgroup that can form hydrogen bonds, and eventually, the PC headgroups interact with each other in the bilayer membranes by comparatively weak electrostatic attractive force acting between the positively charged quaternary nitrogen of a PC headgroup and the negatively charged oxygen in the phosphate group of its neighboring PC headgroup. The difference between the interactions of the PE headgroups and of the PC headgroups is clearly reflected in the temperature of the gel-to-liquid-crystalline phase transition: the temperature of the bilayer membrane of the PE lipid is much higher than that of the bilayer membranes of its PC counterpart. In addition, as for the difference between the ether and ester linkages in the PC lipids, it has been shown in our previous study [22] that the difference produces the similar effect: the presence of the carbonyl oxygen atoms enhances the attractive interaction between the neighboring lipid molecules in the vicinity of the hydrophilic region of the bilayer membrane. This is probably attributed to the hydrogen bonding capability between the neighboring carbonyl oxygen atoms via a water molecule. All these facts indicate that stronger

|          | ether-linked type | ester-linked type |
|----------|-------------------|-------------------|
| diC16:0-PE | X                 | X                 |
| diC16:0-PC | O                 | Δ                 |

$\times$: incapable of interdigitation  
$\circ$: capable of interdigitation  
$\Delta$: capable of interdigitation only under high pressure

Table 1. Interdigitation capability of four types of phospholipids with two C16:0 chains.
interactions between the polar headgroups, or in the hydrophilic region of the bilayer membrane, tend to hamper the transformation into the interdigitated structure. Needless to say, the above consideration should be applicable to the interdigitated structure of the PC lipids induced by pressure. This means that pressure causes the weakening of the attractive interaction in the process of the transformation into the interdigitated structure. Taking this into consideration, we speculate on the mechanism of the pressure-induced interdigitation as follows. In the $L_{d}'$ phase, the ester-linked PC lipid molecules are arranged with their hydrocarbon chains being tilted from the bilayer normal by about 30°, as shown in Fig. 2-A. This chain tilt originates from the equilibration of the concurrent interactions: the steric hindrance between the stereochemically bulky PC headgroups and the van der Waals interaction between the hydrocarbon chains. In this state, the PC headgroup preferably takes a conformation almost parallel to the bilayer surface [25], which can cause an electrostatic attractive force between a positively charged choline group of a PC headgroup and a negatively charged phosphate group of its neighboring PC headgroup. As hydrostatic pressure is externally applied to the bilayer membrane, the intermolecular distance between adjacent lipid molecules becomes shorter and the molecular packing becomes tighter and denser as a whole. With the advance of the compression, the orientation of the hydrocarbon chain perpendicular to the bilayer surface is progressively induced, which concomitantly forces also a gradual conformational change of the PC headgroup from the parallel into the perpendicular to the bilayer surface because of their steric hindrance (Fig. 2-B). As the conformation of the PC headgroups approaches the state where their orientation is almost perpendicular to the bilayer surface, the positively charged choline groups forcibly move away from the vicinities of the negatively charged phosphate groups of their adjacent PC headgroups and eventually locate themselves relatively closer to each other. This change of the relative location of the choline and phosphate groups inevitably affects the interaction between the PC headgroups; that is, the electrostatic attractive interaction gradually decays with increasing separation of a choline and a phosphate groups, and then an electrostatic repulsive interaction begins to emerge with the approach of neighboring choline groups to each other. This process can explain how high pressure weakens the attractive interaction between the neighboring PC headgroups, and finally the interdigitation occurs at a certain stage during the course of the transition from the attractive to the repulsive interaction (Fig. 2-C). It is unfortunate that we have no experimental evidence that directly supports this speculation on the pressure-induced interdigitation at this stage. Further investigation is needed to establish the mechanism of the pressure-induced interdigitation.

Figure 2. Schematic representation of a possible mechanism of pressure-induced interdigitation for an ester-linked saturated PC lipid. (A) Stable bilayer structure in the $L_{d}'$ phase formed at low pressures. In this state, electrostatic attractive interactions are present between the adjacent polar headgroups. (B) Transient state induced by pressure where the lipid molecules are arranged almost perpendicular to the bilayer surface. At this stage, the interactions between the headgroups are repulsive. As the result of the change in the headgroup–headgroup interaction, (C) interdigitated structure is induced above a threshold pressure.
References

[1] Koynova R, Caffray M, 1998 Biochim. Biophys. Acta 1376 91
[2] Hauser H, Poupart G, 2005 The Structure of Biological Membranes ed Yeagle P L., (New York: CRC Press) chapter 1 pp. 1–52
[3] Lewis R N A H, Mak N, McElhaney R N, 1987 Biochemistry 26 6118
[4] Ichimori H, Hata T, Matsuki H, Kaneshina S, 1998 Biochim. Biophys. Acta 1414 165
[5] Huang C, Mason J T, Levin I W, 1983 Biophys. J. 65 2775
[6] Mattai J, Srivapa P K, Shipley G G, 1987 Biochemistry 26 3287
[7] Lewis R N A H, McElhaney R N, 1993 Biophys. J. 65 1866
[8] Lewis R N A H, McElhaney R N, Osterberg F, Gruner S M, 1994 Biophys. J., 66, 207
[9] Ruocco M J, Siminovitch D J, Griffin R G, 1985 Biochemistry 24 2406
[10] Laggner P, Lohner K, Degovics G, Müller K, Schuster A, 1987 Chem. Phys. Lipids 44 31
[11] Kim J T, Mattai J, Shipley G G, 1987 Biochemistry 26 6592
[12] Slater J L, Huang C-H, 2005 The Structure of Biological Membranes ed Yeagle P L., (New York: CRC Press) chapter 3 pp. 121–146
[13] Rowe E S, 1985 Biochim. Biophys. Acta 813 321
[14] Rowe E S, 1987 Biochemistry 26 46
[15] McDaniel R V, McIntosh T J, Simon S A, 1983 Biochim. Biophys. Acta 731 97
[16] Braganza L F, Worcester D L, 1986 Biochemistry 25 2591
[17] Winter R, Pilgrim W C, 1989 Ber. Bunsenges. Phys. Chem. 93 708
[18] Kaneshina S, Tamura S, Kawakami H, Matsuki H, 1992 Chem. Lett., 1963
[19] Kusube M, Matsuki H, Kaneshina S, 2005 Biochim. Biophys. Acta 1686 25
[20] Hing F S, Maulik P R, Shipley G G, 1991 Biochemistry 30 9007
[21] Ichimori H, Hata T, Yoshioka T, Matsuki H, Kaneshina S, 1997 Chem. Phys. Lipids 89 97
[22] Matsuki H, Miyazaki E, Sakano F, Tamai N, Kaneshina S, 2007 Biochim. Biophys. Acta 1768 479
[23] Seddon J, Cevc G, Marsh D, 1983 Biochemistry 22 1280
[24] Chang E L, Yager P, 1983 Mol. Cryst. Liq. Cryst. 98 125
[25] Yeagle P L, Hutton W C, Huang C, Martin R B, 1976 Biochemistry 15 2121