



# Comprehensive characterization of temperature- and pressure-induced bilayer phase transitions for saturated phosphatidylcholines containing longer chain homologs



Masaki Goto<sup>a,1</sup>, Takuya Endo<sup>b</sup>, Takahiro Yano<sup>b</sup>, Nobutake Tamai<sup>c</sup>, Joachim Kohlbrecher<sup>a</sup>, Hitoshi Matsuki<sup>c,\*</sup>

<sup>a</sup> Laboratory for Neutron Scattering and Imaging, Paul Scherrer Institute, CH-5232 Villigen PSI, Switzerland

<sup>b</sup> Department of Biological Science and Technology, Faculty of Engineering, The University of Tokushima, 2-1 Minamijosanjima-cho, Tokushima 770-8506, Japan

<sup>c</sup> Department of Life System, Institute of Technology and Science, The University of Tokushima, 2-1 Minamijosanjima-cho, Tokushima 770-8506, Japan

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## ABSTRACT

Complete elucidation of the phase behavior of phospholipid bilayers requires information on the sub-transition from the lamellar crystal ( $L_c$ ) phase to the gel phase. However, for bilayers of saturated diacylphosphatidylcholines ( $C_n$ PCs), especially longer chain homologs, equilibration in the  $L_c$  phase is known to be very slow. In this study, bilayer phase transitions of three  $C_n$ PCs with longer acyl chains, C19PC, C20PC and C21PC, were observed by differential scanning calorimetry under atmospheric pressure and by light-transmittance measurements under high pressure. Using lipid samples treated by thermal annealing enabled the observation of the sub-, pre- and main transitions of the C19PC and C20PC bilayers under atmospheric pressure. Only the pre- and main transitions could be observed for the C21PC bilayer due to very slow kinetics of the  $L_c$  phase formation for lipids with long acyl chains. The temperature and pressure phase diagrams constructed and phase-transitions quantities (enthalpy, entropy and volume changes) evaluated for these bilayers were compared with one another and with those of bilayers of the  $C_n$ PC homologs examined in previous studies. These results allowed us (1) to clarify the temperature- and pressure-dependent phase sequence and phase stability of the  $C_n$ PC ( $n = 12$ – $22$ ) bilayers as a function of the hydrophobicity of the molecules, (2) to prove the presence of a shorter and a longer limit ( $n = 13$  and  $21$ ) in the acyl chain length for the pressure-induced bilayer interdigitation and (3) to reveal the chain-length dependence of the thermodynamic quantities of the subtransitions including the volume change.

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## 1. Introduction

Phosphatidylcholines are major components of biological membranes in eukaryotic organisms. Bilayers of saturated diacylphosphatidylcholines ( $C_n$ PCs) containing two identical saturated fatty acids have been frequently used as model membranes for life-science studies and simultaneously their properties have been examined by several physico-chemical techniques [1–3]. One notable feature of  $C_n$ PC bilayers is the gel-phase polymorphism.  $C_n$ PC bilayers undergo a thermotropic phase transition between

the gel phases below the main-transition temperature due to the bulky choline head group of the PC molecule. This transition is known as the pretransition from the lamellar gel ( $L_{\beta'}$ ) phase with flat bilayer sheets composed of tilted PC molecules to the ripple gel ( $P_{\beta'}$ ) phase with undulated bilayer sheets. In addition to these gel phases, another gel phase appears in  $C_n$ PC bilayers. It is well known that the fully interdigitated gel ( $L_{\beta I}$ ) phase of  $C_n$ PC bilayers is induced by various additives such as polyols, short-chain alcohols and anesthetics under atmospheric pressure [4–9]. On the other hand, the  $L_{\beta I}$  phase is also induced by an application of pressure. It was found that bilayers of  $C_n$ PCs, such as dimyristoyl-PC (DMPC: C14PC) and dipalmitoyl-PC (DPPC: C16PC), form the fully  $L_{\beta I}$  phase under high pressure [10–18]. In the  $L_{\beta I}$  phase, hydrocarbon chains of the  $C_n$ PC molecules in one of the monolayers constituting a bilayer extend beyond the region of the bilayer midplane and

\* Corresponding author. Tel.: +81 88 656 7513; fax: +81 88 655 3162.

E-mail address: [matsuki@bio.tokushima-u.ac.jp](mailto:matsuki@bio.tokushima-u.ac.jp) (H. Matsuki).

<sup>1</sup> Visiting Scientist; on leave from The University of Tokushima, Japan.

alternately interpenetrate into the other opposing monolayer. Most cases where the pressure-induced interdigitation can be observed involve phospholipids with saturated linear acyl chains like  $C_n$ PC [19–25].

Another feature of the  $C_n$ PC bilayers is the formation of the lamellar crystal ( $L_c$ ) phase, which is often referred as the subgel phase. It is known that the formation of the  $L_c$  phase of the  $C_n$ PC bilayer is slow [26–30]. The time required to completely form the  $L_c$  phase increases with acyl chain length [31]. Hence, it is difficult to determine the  $L_c$ -phase boundary on a phase diagram for longer-chain  $C_n$ PC bilayer. The slow kinetics of the formation of the  $L_c$  phase markedly affects phase stability (i.e., stable, unstable and metastable) of the  $C_n$ PC bilayers. Although the various phases are observed in the  $C_n$ PC bilayers, the  $L_{\beta'}$ ,  $P_{\beta'}$  and  $L_{\beta I}$  phases are polymorphs of the gel ( $L_{\beta}$ ) phases. The fundamental phases of the  $C_n$ PC bilayers are the  $L_c$ ,  $L_{\beta}$  and liquid crystalline ( $L_{\alpha}$ ) phases. Therefore, the information of the  $L_c$  phase depending on temperature and pressure is indispensable for the construction of the complete phase diagrams of the  $C_n$ PC bilayers, in other words, the full elucidation of their phase behavior. The full description of the phase behavior of the  $C_n$ PC bilayers gives us not only detailed knowledge of lipid molecular interactions in the bilayer but also useful information to understand the environmental adaptation of organisms, food and cosmetics processing, biologically relevant phenomena such as pressure reversal of anesthesia and mechanosensing.

Recently, we have found in the temperature ( $T$ )–pressure ( $p$ ) phase diagrams of the  $C_n$ PC ( $n = 14$ – $18$ ) bilayers that a minimum interdigitation pressure (MIP), which is the minimum pressure required for the bilayer interdigitation, decreases with an elongation of the acyl chains [32–34]. From the acyl chain-length dependence of the MIP values, it is expected that in the bilayer of certain  $C_n$ PC with longer acyl chains the enhanced interaction may induce the bilayer interdigitation under atmospheric pressure by only hydration. Previously we demonstrated this on the  $T$ – $p$  phase diagram of the dibehenoyl-PC (C22PC) bilayer by methods of small angle neutron scattering (SANS) and fluorometry [35]. At the same time, it was suggested that the dihenearachidoyl-PC (C21PC) is the longest chain  $C_n$ PC that requires pressure to induce the bilayer interdigitation. However, the bilayer phase behavior of  $C_n$ PCs between C18PC and C22PC, that is, dinonadecanoyl-PC (C19PC), diarachidoyl-PC (C20PC) and C21PC, have not yet been clarified.

In addition to the pressure-induced bilayer interdigitation for bilayers of longer chain homologs, it is interesting to investigate their  $L_c$  phase formation, the process that seems to require a long time. By investigating all the phase transitions including the subtransition for longer-chain  $C_n$ PC bilayers, we can compare the  $T$ – $p$  phase diagrams and the thermodynamic quantities of the bilayer phase transitions for a series of  $C_n$ PCs to consider the phase transitions of the  $C_n$ PC bilayers as a function of the acyl chain length  $n$ , or hydrophobicity, in a wider  $n$ -range [36–40]. Therefore, in the present study, the bilayer phase transitions of C19PC, C20PC and C21PC are observed under atmospheric and high pressures to comprehensively understand the bilayer properties of  $C_n$ PCs as a function of  $n$ . To construct complete phase diagrams containing all phase transitions (sub-, pre-, main transitions and interdigitation) of the  $C_n$ PCs bilayers, we also identify the boundaries of the  $L_c$  phase of the  $C_n$ PC bilayers ( $n < 18$ ). First, the temperature- and pressure-induced phase transitions of the C19PC, C20PC and C21PC bilayers are discussed in terms of the obtained  $T$ – $p$  phase diagrams and thermodynamic quantities. Next, the effect of the acyl chain length on membrane properties of the fully hydrated  $C_n$ PC bilayers ( $n = 12$ – $22$ ) are considered systematically by comparing the phase diagrams and thermodynamic quantities of the C19PC, C20PC and C21PC bilayers to those of the  $C_n$ PC homolog

(C12PC–C18PC, C22PC) bilayers from previous studies [32–34,41] together with data on their subtransition.

## 2. Experimental

### 2.1. Materials and sample preparation

Synthetic phospholipids, 1,2-dioctadecanoyl-*sn*-glycero-3-phosphocholine (C18PC), 1,2-dinonadecanoyl-*sn*-glycero-3-phosphocholine (C19PC), 1,2-diarachidoyl-*sn*-glycero-3-phosphocholine (C20PC) and 1,2-dihenearechidoyl-*sn*-glycero-3-phosphocholine (C21PC), were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA) and/or Sigma Chemical Co. (St. Louis, MO, USA). Other phospholipids,  $C_n$ PC ( $n < 18$ ), were also obtained from both reagent companies. They were used as received. Water used was distilled twice from a dilute alkaline permanganate solution. The multilamellar vesicle dispersions of  $1.0 \text{ mmol kg}^{-1}$  concentration were prepared for each phospholipid by sonicating for a few minutes with a sonifier at a temperature several degrees above the main-transition temperature. The vesicle dispersions of C19PC, C20PC and C21PC were annealed by at least 180 repeated thermal cycles to form the subgel phase [34]; 1 thermal cycle comprises freezing storage at  $-15^\circ\text{C}$  for 23 h, and at  $-30^\circ\text{C}$  for 1 h and cold storage at  $5^\circ\text{C}$  for one day, each of which corresponds to conditions that the complete freeze of bulk water, the complete freeze of freezable interlamellar water and the complete melting of all kinds of water (freezable and nonfreezable interlamellar water and bulk water), respectively [42,43]. The repeating of these conditions as a freeze and thaw cycle promotes the dehydration around the head group, then results in the formation of  $L_c$  phase. For bilayers of  $C_n$ PCs for  $n < 18$ , we performed the above thermal annealing treatment repeatedly until the constant values of the subtransition enthalpy were obtained: 7 cycles for C13PC, 14 for C14PC and C15PC, 21 for C16PC, 70 for C17PC and C18PC.

### 2.2. Phase-transition observation

The phase transitions of the  $C_n$ PC bilayers under atmospheric pressure were observed by a high-sensitivity differential scanning calorimeter MicroCal VP-DSC (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The heating rate was  $0.75^\circ\text{C min}^{-1}$ . For determination of phase transition below  $10^\circ\text{C}$ , another calorimeter, SSC 5200-DSC 120 calorimeter (SII Nanotechnology Co. Ltd, Chiba, Japan), was used with a lipid concentration of  $10$ – $20 \text{ mmol kg}^{-1}$ . The enthalpy changes of the phase transitions were determined from the endothermic peak areas as average values over several measurements. The DSC thermograms were analyzed by use of software Origin 7.0 (Lightstone Corp., Tokyo, Japan).

The phase transitions of the  $C_n$ PC bilayers under high pressure were observed by a light-transmittance technique using high-pressure cell assemblies PCI-400 and PCI-500 (Syn. Corp., Kyoto, Japan) attached to spectrophotometers U-3010 and U-3900 (Hitachi High-Technology Corp., Tokyo, Japan), respectively. The apparatus can detect light-transmittance changes under an isobaric thermotropic condition. The measurements were repeated at least three times at the same pressure to judge the phase stability. The phase-transition temperatures determined by this technique under atmospheric pressure were in good agreement with those by the DSC. The heating rate was  $0.33$  or  $0.50^\circ\text{C min}^{-1}$  and the pressure range was  $0.1$ – $200 \text{ MPa}$  except the C21PC bilayer, the range of which was  $0.1$ – $100 \text{ MPa}$ . The detailed procedure for the measurements under high pressure was described elsewhere [33,34,44,45].

The phase assignment for each  $C_n$ PC bilayer was done by checking the phase-transition behavior (appearance mode, magnitude, cooperativity, etc.) obtained from both DSC and light transmittance

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