Lamellar-lamellar phase separation of phospholipid bilayers induced by salting-in/-out effects

Mafumi Hishida1, Hideki Seto2*
1 Institute for Integrated Cell-Material Sciences, Kyoto University, Kyoto 606-8501, Japan
2 KENS & CMRC, Institute of Materials Structure Science, High Energy Accelerator Research Organization, Tsukuba 305-0801, Japan
*E-mail: hideki.seto@kek.jp

Abstract. The multilamellar structure of phospholipid bilayers is stabilized by the interactions between bilayers. Although the lamellar repeat distance is uniquely determined at the balance point of interactions between bilayers, a lamellar-lamellar phase separation, where the two phases with different lamellar repeat distances coexist, has been reported in a case of adding a salt to the aqueous solution of lipids. In order to understand the physical mechanism of the lamellar-lamellar phase separation, the effects of adding monovalent salt on the lamellar structure are studied by visual observation and by small-angle X-ray scattering. Further, a theoretical model based on the mean field theory is introduced and it is concluded that the salting-in and -out effects of lipid bilayers trigger the lamellar-lamellar phase separation.

1. Introduction
The phospholipid bilayer is the basic structure of biomembranes. Through the simple mixing of synthesized phospholipids and water, the lipids spontaneously form bilayers and are organized as multilamellar vesicles, where a number of bilayers stack regularly. The characteristic repeat distance between bilayers is uniquely determined by the balance of attractive and repulsive interactions. Four main interactions have been considered; van der Waals attraction, hydration repulsion, steric repulsion caused by the thermal fluctuation of bilayers and electrostatic interaction [1]. In cases of neutral lipids, the electrostatic interaction can be ignored. These interactions equilibrate the multilayer repeat distance at 5 to 10 nm. On the other hand, Rappolt et al. observed that two or three lamellar structures with different repeat distances coexist when monovalent salt is added to the aqueous solution of neutral phospholipid in the liquid-crystalline Lα phase [2,3]. This evidence contradicts the model to explain the lamellar repeat distance explained above, and the mechanism of the lamellar-lamellar phase separation has not yet been clarified, even though the authors suggested that osmotic stress is strongly related to the phase separation.

With respect to the effects of monovalent salt on the phospholipid lamellar phase, several intriguing results have been reported in relation to the ‘salting-out effect’, i.e., solutes aggregate in high salinity by a kind of repulsive force between salt and solute, and the ‘Hofmeister series’, i.e., the ion specificity of the salting-out effect [4-9]. Petrache et al. observed neutral phospholipid suspension,
including KCl or KBr, by visual inspection, and it was revealed that multilamellar vesicles float in the salt solution [6]. This high buoyancy of vesicles indicates that a kind of repulsive force exists between phospholipid bilayers and salt ions, and it changes the relation between specific gravities of multilamellar vesicles and bulk brine. This force could be originated by the salting-out effect, and the strength differences of the repulsive force between KCl and KBr solutions follow the ‘Hofmeister series’ [4,5]. The ion specificity is additionally reported in terms of the Debye screening of the van der Waals attraction [7,8]. Furthermore, Korreman et al. investigated the effect of monovalent salt on the anomalous swelling of multilamellar vesicles [9]; the increase of the lamellar repeat distance when approaching the main transition temperature from the Lα to Lβ phase [10-12]. They demonstrated that the anomalous swelling is amplified by increasing the concentration of monovalent salt, and the anomalous swelling is explained as a critical unbinding with the critical exponent 1. The behaviour of anomalous swelling also followed the Hofmeister series, and the order of the effect of adding monovalent salts is as follows: NaCl < KF < KCl < NaF < NaBr < KBr.

Theoretically, the lamellar-lamellar phase separation of neutral lipid membrane has been discussed in relation to the pre-unbinding of stacked membranes [13,14]. The mean field theory of pre-unbinding has been constructed with the Flory theory of multilayers [13]. In the theory, the free energy is described as

\[ g(\phi) = -\chi \phi^2 + \frac{\phi^4}{2(1-\phi)^2} - \mu \phi, \]

where the order parameter \( \phi \) is the volume fraction of lipid [15]. The first term indicates the direct interaction between membranes, the second one is the entropic term of stacked lipid membranes, and the third one is for conservation of the total volume fraction. In the case of \( \chi > 0 \), the lamellar phase coexists with bulk water. In the theory of pre-unbinding, a secondary order parameter other than the lipid concentration is applied, and the lamellar phase separation to two or three phases is induced in the vicinity of the critical unbinding point [13]. In the theory, salt concentration possibly become the second-order parameter and could be the origin of the lamellar phase separation.

In the present study, we discuss the salting-out effect on the lamellar-lamellar phase separation. As a result, a different salt concentration between the two separated lamellar phases is observed, and the salting-in and -out effects are considered to be a main cause of the lamellar-lamellar phase separation. Furthermore, we suggest a theoretical model of the lamellar-lamellar phase separation with considering salting-in/-out effects, based on the pre-unbinding theory. These results may assist in understanding the salting-out effect and the Hofmeister series, which remains a matter of dispute.

2. Materials and methods

As a neutral phospholipid, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC, Wako Pure Chemical Industries) was obtained in powder form and used without further purification. Sodium chloride (NaCl, Wako Pure Chemical Industries) was first dissolved in pure water at several concentrations. Next, the powder DPPC was mixed in the solutions to be 8 wt% or 20 wt% at above the main transition temperature. The salt concentrations were \([\text{DPPC}]:[\text{NaCl}](\text{mol/mol}) = 1:0\) (Pure DPPC), 1000:1, 100:1, 10:1, 1:1, and 1:10. Because NaCl is categorized as “water-structure-making” salt, it is expected to exhibit a high salting-out effect. We also used NaBr and KBr as a salt that exhibit less salting-out effect.

For measuring the repeat distances of the multilamellar phase of the samples, small angle X-ray scattering (SAXS) was performed at BL-15A, Photon Factory, KEK, Japan. Supplemental experiments were performed with SAXS in the laboratory (RA-Micro7HF, Rigaku). A sharp Bragg peak at \( q \) observed by SAXS corresponds to the characteristic repeat distance of lamellar phase \( d = 2\pi/q \). Because the main transition temperature of DPPC is approximately 42°C, the anomalous
swelling was exhibited $42^\circ C < T < 50^\circ C$ [11]. The buoyancies of multilamellar vesicles were observed by visual observation to evaluate the salting-out effect.

3. Results

Figure 1 (a) presents a typical image of the macroscopic phase separation of multilamellar vesicles and bulk water in NaCl solution at $50^\circ C$. In pure water, the $L_\alpha$ lamellar phase of DPPC sinks slightly (the specific volumes are 1.009 mL/g for DPPC and 1.012 mL/g for H$_2$O at $50^\circ C$ [16]). As we add a little amount of salt ([DPPC]:[NaCl] = 1000:1, 100:1, 10:1), the multilamellar vesicles become heavier than the bulk water (see from 1:0 to 100:1). However, when the ratio of NaCl is more than 1:1, the balance of specific gravities between the multilamellar vesicles and bulk water is reversed, and the lamellar phase floats on the upper part of the test tube at high salt concentration.

![Image of phase separation](image)

**Figure 1.** (a) Picture of 8 wt% DPPC solutions with different NaCl concentrations at $50^\circ C$. The white suspension is phospholipid multilamellar vesicles. (b)(c) SAXS profiles of [DPPC]:[NaCl] = 10:1 and [DPPC]:[NaCl] = 1:10 solutions at $50^\circ C$, respectively. The solid lines indicate fitting results by Lorentzian and double-Lorentzian functions, respectively.

![Image of SAXS profiles](image)

**Figure 2.** Anomalous swelling behaviour of pure DPPC and DPPC/NaCl solutions ([DPPC]:[NaCl] = 1:10, (a)[DPPC] = 8 wt% and (b) [DPPC] = 20 wt%). Closed circles denote the lamellar repeat distance of pure DPPC, whereas open circles and open triangles are the phase-separated lamellar repeat distances of DPPC/NaCl solutions. Solid and dashed lines are the fitting results by an exponential function.

The specific gravity of the phospholipid bilayer should be identical even with salt because the bilayer structure is the same [7]. Thus, the observed behaviour may be caused by the asymmetric distribution of the salt between the interlamellar water and the bulk water. When a small amount of salt is dissolved in the solution, the salt is concentrated in the interlamellar water and the multilamellar vesicles sink: the “salting-in effect.” However, as the amount of salt is increased, the local concentration of salt in bulk water become higher than that in the interlamellar water, and the vesicles...
float as a result of buoyancy: “salting-out effect.” In the system of DPPC and NaCl at 50°C, the criteria from salting-in to salting-out is determined between \([\text{DPPC}]/[\text{NaCl}] = 10\) and 1.

By performing SAXS experiments for these samples at 50°C, we observe the salt concentration dependence of the lamellar-lamellar phase separation. Whereas a single Bragg peak is exhibited under the salt concentration below \([\text{DPPC}]/[\text{NaCl}] = 1\) (figure 1 (b)), evident phase separation is observed as salt is concentrated (figure 1 (c)). The temperature dependence of the repeat distances of the coexisting phases at \([\text{DPPC}]/[\text{NaCl}] = 1/10 \ \text{[mol/mol]}\) just above the main transition temperature (the anomalous swelling region) are depicted in figure 2. The two separated phases exhibit different anomalous swelling behaviours for both samples of \([\text{DPPC}] = 8 \ \text{wt\%}, 20 \ \text{wt\%}; \) the lamellar phase with a larger repeat distance swells slightly as it approaches the transition temperature (42°C), whereas that with a smaller repeat distance swells significantly on cooling. This asymmetric behavior of anomalous swelling could be caused by the heterogeneous distribution of salt ions between the two separated lamellar phases, because the degree of the anomalous swelling depends on the salt concentration between layers [9]. However, we were unable to determine distribution of salt, because our results exhibit an opposite tendency to those of Korreman [9].

From these results, the origins of the lamellar-lamellar phase separation can be considered as a result of the reduction of the van der Waals attraction due to the Debye screening and salting-in and -out effects. The dependence of ion species on the lamellar-lamellar phase separation confirms the salting-in and -out effects as an essential origin of the lamellar phase separation (figure 3). According to the Hofmeister series, the ordering of salt for salting-in and -out effects is \(\text{NaCl} < \text{NaBr} < \text{KBr}\). SAXS results indicate that the solutions with NaCl, NaBr, and KBr exhibit different lamellar phase separation behaviour, even when the salt concentrations are exactly the same. The exchange of a potential of hydration repulsion by monovalent salt ions is also considered, because the “water-structure-making” ions strongly bind water molecules [4] and the water layer on the head group of phospholipids would be broken up. However, the hydration repulsion acts as a very short-range interaction, ranging approximately 2 Å [1,17], whereas our results regarding different phase separations between NaCl and KBr solutions range up to 4 Å. This result is inconsistent with the possibility that the exchange of hydration repulsion is an essential origin of the lamellar-lamellar phase separation.

4. Discussion
Based on the Flory theory of pre-unbinding [13], we discuss the lamellar-lamellar phase separation, considering the salting-in/-out effects and the Debye screening of the van der Waals interaction. In the case of the lamellar phase with salt ions, the concentration of salt is defined as the second-order parameter $\psi$ in the mean field theory. Four contributions of salt ions to the free energy are considered: Debye screening of the van der Waals interaction, salting-in/-out effects, translational entropy of salt, and salt concentration. Thus, the free energy is described as

$$g(\phi, \psi) = -(\chi_0 + B\psi)^2 + \frac{\phi}{2(1-\phi)^2} - \mu\phi + \psi \log \psi + \alpha(\psi - \frac{\beta}{\alpha})\phi - \bar{\mu}\psi.$$ 

The first term indicates the direct interaction between lipid bilayers with the Debye screening of the van der Waals interaction caused by the effect of salt ($B \leq 0$), the fourth term is the translational entropy of salt ions, the fifth term reflects the salting-in ($\psi < \beta/\alpha$) and salting-out ($\psi > \beta/\alpha$) effects, and the third and sixth terms denote the conservation of lipid and salt. By minimizing the free energy with respect to $\psi$

$$\frac{\partial g}{\partial \psi} = 0,$$

the concentration of salt is calculated as

$$\psi_m = \exp(B\phi^2 - \alpha\phi + \bar{\mu} - 1).$$

The substitution of $\psi_m$ to the free energy gives the free energy with respect to the concentration of lipid membrane, $g(\phi)$.

In figure 4 (a), the typical free energy profile with respect to the concentration of lipid membrane is shown. $\phi = 0$ denotes the bulk water phase. In a certain parameter range, three local minima at $\phi = \phi_1, \phi_2, \phi_3$ are observed, which indicates that two separated lamellar phase coexist with bulk water. The salt concentration is found to distribute heterogeneously (figure 4 (b)), as observed in our experiments. This theory suggests that the key factor for the lamellar-lamellar phase separation is not the Debye screening of the van der Waals interaction but the salting-in/-out effect, because the free energy exhibits a double minimum profile even if $B = 0$.

![Figure 4](image_url)

**Figure 4.** Calculated free energy profile (a) and corresponding salt concentration (b) with respect to the lipid concentration $\phi$ at $\chi = 2, \mu = -2, B = -1, \bar{\mu} = -1.5, \alpha = 100, \beta = 1.38$.

5. Conclusion

In this study, we investigated the physical mechanism of the lamellar-lamellar phase separation by adding monovalent salt. The visual observation of the buoyancy of lipid vesicles and SAXS measurements of the anomalous swelling behaviour indicate that salt concentrations are different among each of the separated lamellar phases and the bulk water phase. As a result, it is clarified that a certain interaction exists between lipid bilayers and salt ions, which becomes both attractive and
repulsive depending on the salt concentration, i.e., salting-in/-out effects. The SAXS measurements also indicate that the lamellar phase separation differs by ion specificity. These experimental results suggest that salting-in/-out effects dominate the lamellar phase separation accompanied with the Debye screening of the van der Waals interaction by salt. From the theoretical model based on the mean field Flory theory of the pre-unbinding with consideration of the salting-in/-out effects and the Debye screening of the van der Waals interaction, it is concluded that the salting-in/-out effects are the essential origin of the ion-induced lamellar-lamellar phase separation.

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