# Unbinding Transition Induced by Osmotic Pressure in Relation to Unilamellar Vesicle Formation

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**Abstract.** – Small-angle X-ray scattering and phase-contrast microscopy experiments were performed to investigate the effect of the osmotic pressure on vesicle formation in a dioleoylphosphatidylcholine (DOPC)/water/NaI system. Multi-lamellar vesicles were formed when a pure lipid film was hydrated with an aqueous solution of NaI. On the other hand, uni-lamellar vesicles (ULVs) were formed when a lipid film mixed with an enough amount of NaI was hydrated. To confirm the effect of the osmotic pressure due to NaI, a free-energy calculation was performed. This result showed that the osmotic pressure induced an unbinding transition on the hydration process, which resulted in ULV formation.

*Introduction.* – All biomembranes mainly consist of lipid bilayers, and functional proteins float on them. Such bilayers also appear in aqueous solutions of synthesized phospholipids. Therefore, bilayers in such solutions are intensively studied to understand the physical properties of biomembranes. Moreover, these bilayers form vesicles that attract attention for studying model cells. Such vesicles can be effectively obtained by hydrating dry lipid films on substrates or test tubes. (natural swelling method [1]) For example, dioleoylphosphatidylcholine (DOPC) films, one of the typical phospholipids in biomembranes, provide micrometer-size vesicles. These DOPC vesicles normally grow with a multi-bilayer shell (multi-lamellar vesicle; MLV), although uni-lamellar vesicles (ULVs) are prefered for using as model cells. Therefore, effective methods to create ULVs have been proposed [2–9].

One of the authors has recently designed a novel method for preparing ULVs by hydrating dry lipid films mixed with sugar and salt [10]. As some previous studies have pointed out, an osmotic pressure would promote ULV formation [6, 8]. The mechanism of ULV formation induced by osmotic pressure, however, has not been yet clarified.

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A key phenomenon to understand the mechanism of ULV formation is an "unbinding transition", in which the inter-bilayer distance diverges infinitely and the bilayer stacking is unstabilized. First, this transition was predicted theoretically by Lipowsky [11]. Recently, some experiments on this subject have been performed using small-angle X-ray scattering, small angle neutron scattering, and X-ray reflectivity [12–15]. The authors recently clarified the origin of the unbinding transition in terms of the interaction between bilayers; a collaboration of the long- and short-range repulsive force, for example a steric repulsion due to membrane undulation [16] and electrostatic repulsion, would be the origin of this transition [15]. Therefore, the osmotic pressure would play an important role for the unbinding transition as these repulsions.

In this study, we prepared two kinds of samples to investigate the effect of the osmotic pressure when dry lipid films include an additive before hydration. One is an "NaI in film" sample in which a dry DOPC film mixed with NaI was hydrated with pure water, that is, an "NaI in film" sample is made by using the novel method to create ULVs. The other is an "NaI in solution" sample in which a dry DOPC film was hydrated with an aqueous solution of NaI for a control experiment. In this condition, DOPC bilayers were in the liquid-crystalline phase at room temperature, and vesicles were effectively formed by hydration [17]. NaI is suitable to prepare a homogeneous lipid film for "NaI in film", since it is a good solute for both water and methanol. (The details are described in the Experimental section.) To investigate the dependence of the vesicle formation on the sample preparation, the structure of the multi-bilayer shell were observed using small-angle X-ray scattering (SAXS); also, the shape of vesicles were observed by phase-contrast microscopy (PCM).

*Experiments.* – DOPC was purchased from Sigma Chemical Inc. and NaI from Wako Pure Chemical Industries Ltd. To prepare dry lipid films of "NaI in solution" samples, DOPC solutions of organic solvents, 1:2(v/v) methanol/chloroform, were evaporated under $N_2$ gas flow in a glass test tube, and kept in a vacuum at room temperature overnight. The obtained lipid films were hydrated with an NaI aqueous solution with various concentrations of NaI. For "NaI in film" samples, methanol solutions of various NaI concentrations were prepared before mixing with chloroform. Then, DOPC was dissolved in these organic solvents, the organic solvents were evaporated in a vacuum as the "NaI in solution" samples, and the obtained DOPC/NaI films were hydrated in pure water.

The SAXS experiments were performed at the BL40B2 beam port of SPring8 at Japan Synchrotron Radiation Research Institute (JASRI). The incident X-rays were monochromatized by a double-crystal monochromator, and the wavelength was 1 Å ($\Delta E/E \simeq 10^{-4}$). The detector was an imaging-plate area detector placed at 1 m from the sample position. The samples were prepared to be 1wt.% of DOPC concentration, and to have the molar fractions of DOPC:NaI=10000:1, 1000:1, 100:1, 10:1, and 1:1. Since all of the obtained two-dimensional data had no preferred orientation, they were azimuthally averaged to provide one-dimensional data. All of the experiments were performed at room temperature.

The PCM experiments were performed by using Nikon TE-300 and recorded on S-VHS videotape at 30 frames/sec. The DOPC concentration was 1 mM (about 0.08wt.%) so as to avoid vesicle aggregation, and the molar ratio of DOPC:NaI was 1:1. The PCM experiments were also performed at room temperature as SAXS experiments.

*Result.* – Figure 1(a) shows the SAXS profiles obtained from the "NaI in solution" samples. All profiles have sharp Bragg peaks due to the regular stacking of lipid bilayers, whose repeat distance, $d$, is 63.5 Å. This value is almost the same as that of an aqueous solution of DOPC, $d = 63.1$ Å [18]. This means that NaI molecules in water had no effect on the lamellar structure. Figure 1(b) shows the SAXS profiles obtained from the "NaI in

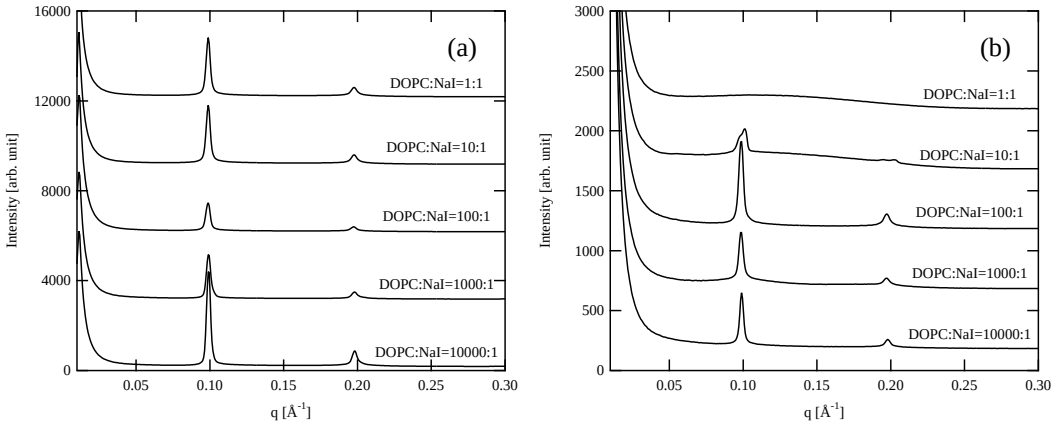


Fig. 1 – Dependence of SAXS profiles on the molar ratio of NaI to DOPC. (a) SAXS profiles of "NaI in solution" samples. The obtained profiles are independent of the molar ratio. (b) SAXS profiles of "NaI in film" samples. The obtained profiles drastically change above DOPC:NaI=10:1. The profiles of higher NaI ratio were shifted for better visualization.

film" samples. Although the SAXS profiles were essentially the same as the "NaI in solution" samples at lower NaI concentration, the Bragg peaks split into two peaks at DOPC:NaI=10:1, and completely disappeared at DOPC:NaI=1:1. This evidence imply that the osmotic pressure due to NaI between bilayers destroys the multi-lamellar structure.

To confirm that lipid bilayers still exist in the sample of DOPC:NaI=1:1, we carried out a model fitting for the obtained SAXS profile. Since the structure factor should be unity because of no correlation between the bilayers, the fitting was performed using

$$I(q) = I_\rho(q) + \frac{|F(q)|^2}{q^2} + B, \tag{1}$$

where $F(q)$ is the form factor of the bilayers, $I_\rho(q)$ the scattering due to the concentration fluctuation of the lipids, and $B$ the constant background. In this model, $I_\rho(q)$ was assumed to be Lorentzian [19],

$$I_\rho(q) \propto \frac{1}{q^2\xi^2 + 1}, \tag{2}$$

where $\xi$ is the correlation length for the concentration fluctuation. $F(q)$ was assumed to be the Fourier transform of three Gaussians, which represents the bilayer electron density, as the following equation [20]:

$$F(q) \propto 2\sigma_H \exp\left[-\frac{(\sigma_H q)^2}{2}\right] \cos\left[\frac{d_{HH} q}{2}\right] + \rho_r \sigma_C \exp\left[-\frac{(\sigma_C q)^2}{2}\right]. \tag{3}$$

Here, $\sigma_H$, $\sigma_C$, $\rho_r$, and $d_{HH}$ are the parameters used to describe the electron density profiles of the lipid bilayers, as shown in fig. 2(b). The fitting was performed with the least-square method for a $q$ range of $0.025 < q < 0.3$ [Å$^{-1}$].

As shown in fig. 2(a), a broad peak corresponding to the correlation between the head groups appeared around $q = 0.12$ Å$^{-1}$. The fitting function reproduced the experimental

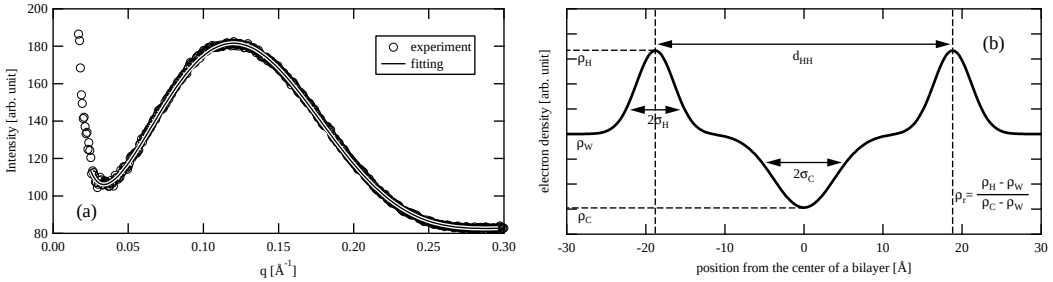


Fig. 2 – (a) Magnified view of the SAXS profile from the "NaI in film" sample of DOPC:NaI=1:1 with the result of fitting. (The same as the uppermost profile of Fig. 1(b).) The fitting model reproduced the experimental result well. (b) Electron density profile obtained from the fitting. $\sigma_H$ is the standard deviation of the electron density of head groups, $\sigma_C$ the standard deviation of the electron density of an end of hydrocarbon tails, $\rho_r$ the ratio of the peaks at the head groups to that at the end of hydrocarbon tails, and $d_{HH}$ the head-to-head distance of a bilayer.

result well, and the fitting parameters were evaluated to be $\sigma_H$=2.38 Å, $\sigma_C$=3.85 Å, $\rho_r$=-1.13, and $d_{HH}$=37.6 Å, which agrees with $d_{HH}$ = 35.3 Å from the literature [18]. (The electron density profile calculated from these parameters is shown in fig. 2(b).) Since the electron density of the phosphate group is much greater than that of the other groups in the DOPC bilayers, $d_{HH}$ is the most characteristic parameter in the electron density profile. Therefore, the agreement of its value confirms that only the scattering due to the form factor was seen in the profile from the "NaI in film" sample at DOPC:NaI=1:1. This suggests that the unbinding transition would occur in the "NaI in film" sample, since the Bragg peak due to the correlation between bilayers disappeared.

Figure 3 shows the vesicles observed by PCM. The difference between the preparation procedures was clear: smaller vesicles with a thick shell formed in the "NaI in solution" sample, whereas larger vesicles with a thin shell formed in the "NaI in film" sample. This is consistent

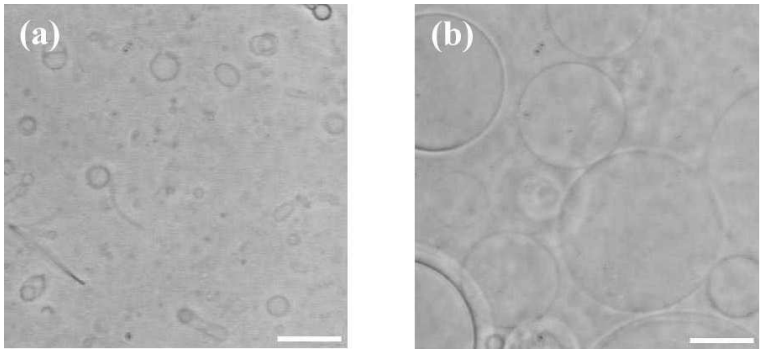


Fig. 3 – Difference of the phase contrast images of the vesicles obtained by the two preparation methods at molar ratio of DOPC:NaI=1:1. (a) Vesicles in the "NaI in solution" sample. The size of the vesicles was below a few $\mu$m and their shells were thick. (b) Vesicles in the "NaI in film" sample. The size of the vesicles was over 10 $\mu$m and their shell thicknesses were thin. The scale bar is 10 $\mu$m.

TABLE I – *Parameters determined by Tristram-Nagle* [18]

| $T$ | $d_l$ | $A$ | $K_c$ | $H$ | $P_h$ | $\lambda$ |
|---|---|---|---|---|---|---|
| 30°C | 45.3 Å | 72.2 Å$^2$ | $1.0 \times 10^{-19}$ J | $4.0 \times 10^{-21}$ J | $5.0 \times 10^{7}$ J/m$^3$ | 2.26 Å |

with the disappearance of the Bragg peak in the "NaI in film" sample. Therefore, we concluded that an addition of NaI to DOPC films before hydration promotes ULV formation [10].

*Discussion.* – To discuss the relation between the unbinding transition and the ULV formation in the "NaI in film" sample, we calculated the free-energy density profile of the outermost bilayer depending on the inter-bilayer distance. In this calculation, we assumed the following situation. First, a lipid bilayer is known to stack on the surface of a substrate before hydration. It is reasonable to assume that NaI molecules in a dry lipid film locate between the bilayers, as shown in fig. 4(a) [21]. Next, only water can penetrate through the lipid bilayers on the hydration process, since the bilayer is known to be semipermeable. Furthermore, the free-energy density caused by osmotic pressure, $f_{osm}$, arises from the concentration difference of NaI between inside and outside of the bilayers [22]. Finally, the inter-bilayer distance increases up to a stable distance, where the free-energy profile has a local minimum. According to previous studies, three other considerable free-energy densities should be considered: the first one is due to the van der Waals attractive force, $f_{vdW}$, the second one is due to the hydration layers, $f_{hyd}$, and the third one is from the steric repulsion due to the membrane undulation, $f_{st}$ [15, 18]. Therefore, the total free energy density $f$ can be described as follows:

$$f = f_{vdW} + f_{hyd} + f_{st} + f_{osm}, \tag{4}$$

$$f_{vdW} = -\frac{H}{12\pi}\left\{\frac{1}{d_w^2} - \frac{2}{(d_w+d_l)^2} + \frac{1}{(d_w+2d_l)^2}\right\}, \tag{5}$$

$$f_{hyd} = P_h\lambda \exp\left[-\frac{d_w}{\lambda}\right], \tag{6}$$

$$f_{st} = 0.42\frac{(k_BT)^2}{K_c d_w^2}, \tag{7}$$

$$f_{osm} = -\frac{2xk_BT}{A}\ln[d_w], \tag{8}$$

where, $H$ is the Hamaker constant, $d_w$ the inter-bilayer distance, $d_l$ the bilayer thickness, $P_h$ the prefactor of $f_{hyd}$, $\lambda$ the decay length of hydration layers, $K_c$ the bending rigidity of bilayers, $x$ the molar ratio of NaI to DOPC, and $A$ the area per DOPC molecule.

Figure 4(b) shows the calculated free-energy densities as a function of $d_w$ by using these expressions with the parameters determined by Tristram-Nagle, as shown in table I. A local minimum is seen at $d_w = 20.3$ Å in the free-energy profile of the lowest NaI content ($x = 0.000025$). The obtained $d$ value ($d = d_l + d_w = 65.6$ Å) agrees with that obtained from the SAXS profiles ($d$ =63.5 Å). Therefore, this local minimum corresponds to the inter-bilayer distance of the bilayer stacking. With increasing the molar ratio of NaI, this local minimum becomes shallow and disappears above $x = 0.0004$ due to an increase of the osmotic pressure. This results in an increase of the inter-bilayer distance up to infinitely, *i.e.*, the unbinding transition takes place. Therefore, we concluded that the osmotic pressure due to the additive induced the unbinding transition on the hydration, which resulted in the ULV formation in the "NaI in film" samples.

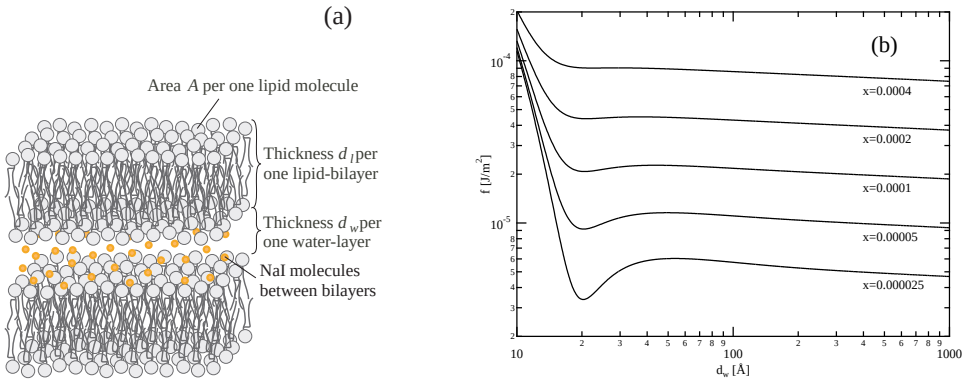


Fig. 4 – (a) Schematic illustration of the assumption in the free-energy calculation [21]. There are two parallel and flat bilayers and NaI molecules located between them. (b) Calculated free energy densities with changing the molar ratio of NaI, $x$. The shift to upside with increasing $x$ is not due to an offset, but to the change of $f_{osm}$.

It should be noted that the calculated critical concentrations to induce the unbinding transition were different from the SAXS result; more than 10% of NaI against DOPC was required to induce the unbinding transition, while only about 0.04% of NaI was required in the model calculation. This would have originated from an over-estimation of the osmotic pressure, since the NaI molecules should locate not only between bilayers, but also at the outside of bilayers in the multi-layer lipid film. This problem is, however, not essential with respect to the mechanism of vesicle formation.

*Conclusion.* – A difference of structure, depending on the process of mixing lipids with salt and hydration ("NaI in solution" and "NaI in film"), was investigated with changing the molar ratio of NaI to DOPC using SAXS and PCM. The SAXS experiment suggested that a large amount of NaI in the "NaI in film" sample induced the unbinding transition, and the PCM experiment showed that NaI in the "NaI in film" promoted the formation of ULVs. The calculation of the free energy for the "NaI in film" sample was also performed, and showed that the osmotic pressure was the origin of the unbinding transition. From these results, we concluded that the osmotic pressure due to much amount of NaI in the lipid film induces an unbinding transition which accelerates the formation of ULVs.

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[22] The osmotic pressure $p_{osm}$ due to the number of the molecule $N$ per volume $V$ is $Nk_BT/V$. In this case, $N$ and $V$ in a unit cell are $2x$ and $Ad_w$, respectively. On the other hand, the relation between $f_{osm}$ and $p_{osm}$ is described as $p_{osm} = -\partial f_{osm}/\partial d_w$ from their definition. Therefore, we obtain eq. (8) under the assumption that any NaI molecules are not ionized.