Unbinding of lipid bilayers 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 osmotic pressure on vesicle formation in a dioleoylphosphatidylcholine (DOPC)/water/NaI system. The multi-lamellar structure of lipid bilayers is unstabilized when a lipid film with a sufficient amount of NaI is hydrated by pure water. It has been confirmed that this phenomenon is due to the effect of osmotic pressure induced by a heterogeneous distribution of NaI molecules. This could be the origin of the unbinding of lipid bilayers to conform large uni-lamellar vesicles.

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Introduction. – All biomembranes mainly consist of lipid bilayers, and functional proteins float on them. Such bilayers also appear in aqueous solutions of synthesized phospholipids; and they are intensively studied to understand the physical properties of biomembranes. Moreover, uni-lamellar vesicles (ULVs) of a few micrometer diameter formed by these lipids attract attention for studying model cells. Such vesicles can be effectively obtained by hydrating dry lipid films on substrates or test tubes. (The natural swelling method [1].) Dioleoylphosphatidylcholine (DOPC) is a typical phospholipid, and provides micrometer-size vesicles. However, DOPC vesicles normally grow as multi-lamellar vesicles (MLVs), and are not suitable for constructing model cells. Therefore, various 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 or salt [10]. The origin to form large ULVs could be due to an effect of osmotic pressure, as some previous studies have already suggested [6,7]. However, the effect of the osmotic pressure of water molecules on vesicle formation has not been investigated either experimentally and theoretically so far.

A key phenomenon to understand the mechanism of the ULV formation is the “unbinding transition”, in which the inter-bilayer distance diverges infinitely and the regular stacking of bilayers is unstabilized. First, this transition was predicted theoretically by Lipowsky and Leibler [11]. Later, Milner and Roux [12] estimated the interaction between bilayers by including an accurate expression of the Helfrich repulsion [13], and gave the following expression:

\[
g(\phi) = \frac{3(\pi k_B T)^2}{128 K_c \delta^3} \phi^3 - k_B T \chi \phi^2 - \mu' \phi,
\]

where \(g(\phi)\) is the excess free energy as the function of the volume fraction of a lipid, \(\phi (\phi = 0 \text{ means unbinding})\), \(\delta\) the thickness of a lipid bilayer, \(K_c\) the bending rigidity, \(\chi\) the correction to the hard-wall result for the virial coefficient, and \(\mu'\) the chemical potential of membrane components. By changing \(\chi\), an unbinding transition is induced as a second-order phase transition.

Recently, some experimental studies have been conducted on the unbinding of lipid bilayers by using small-angle X-ray scattering, small-angle neutron scattering, and X-ray reflectivity [14–17]. The results showed that an unbinding transition could be induced by an increase of the Helfrich repulsion. The authors showed that a collaboration of long- and short-range repulsive
forces, for example the Helfrich repulsion and the electrostatic repulsion, could be the origin of the unbinding transition [17]. This suggests that another repulsive force, for example osmotic pressure, could also induce the unbinding transition.

In this study, we prepared aqueous solutions of DOPC by two procedures. One is a “NaI in solution” sample in which a dry DOPC film was hydrated with an aqueous solution of NaI (see fig. 1). The other is a “NaI in film” sample in which a dry DOPC film mixed with NaI was hydrated with pure water. This procedure follows a novel method to create ULVs [10]. Comparing the structure and the morphology of the obtained vesicles, the effect of osmotic pressure in the hydration process could be clarified. It should be noted that DOPC bilayers are in the liquid-crystalline phase at room temperature, and vesicles were effectively formed by the natural swelling method [18]. The reason for selecting NaI as a salt is that it is a good solute for both water and methanol, and can be distributed uniformly in water and dry lipid films. (Details are described in the Experimental section.) The stacking structure of lipid bilayers was investigated by small-angle X-ray scattering (SAXS), and the macroscopic morphology of vesicles 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 for “NaI in solution” samples, DOPC was dissolved in an organic solvent (1:2(v/v) methanol/chloroform), evaporated by N₂ gas flow in a glass test tube, and kept in a vacuum at room temperature overnight to remove any organic solvent. Dry lipid films without NaI were hydrated by NaI aqueous solutions at various salt concentrations, and left standing for over one day to obtain equilibration. For “NaI in film” samples, methanol solutions of various NaI concentrations was mixed with chloroform, and DOPC were dissolved in these organic solvents. Dry lipid films with NaI molecules were obtained by evaporation with N₂ gas flow in a glass test tube and kept in a vacuum at room temperature. These dry films were hydrated by pure water and kept for over one day.

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 (\(\Delta E/E \approx 10^{-4}\)). The detector was an imaging-plate area detector placed at 1 m from the sample position. The DOPC concentration against water was 1 wt.% for all samples. The molar ratios of NaI to DOPC, \(x\), were 0.0001, 0.001, 0.01, 0.1, and 1, respectively. Since the observed two-dimensional data had no preferred orientation, they were azimuthally averaged to provide a one-dimensional SAXS profile as a function of the momentum transfer, \(q\). All of the experiments were performed at room temperature.

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

**Result.** – Figure 2(a) shows 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 without salt, \(d = 63.1 Å\) [19]. This means that NaI molecules in water had no effect on the lamellar structure. Figure 2(b) shows the SAXS profiles obtained from the “NaI in film” samples. Although the SAXS profiles were essentially the same as the “NaI in solution” samples at lower NaI concentrations, the SAXS profile started to change at \(x = 0.1\), and the Bragg peaks completely disappeared at \(x = 1\). This evidence implies that osmotic pressure due to NaI between bilayers unstabilizes the multi-lamellar structure at \(x = 1\), and lipid bilayers may be isolated to form unilamellar vesicles. The profile at \(x = 0.1\), where a Bragg peak has a shoulder, could be the same as that shown by Rappolt et al. in the liquid crystalline phase of POPC [20]. They suggested that osmotic pressure can induce the splitting of the Bragg peaks. This evidence also support the difference of the SAXS profiles from two types of samples comes from the effect of osmotic pressure.

Under this assumption, the SAXS profile of the “NaI in film” sample with \(x = 1\) is analyzed in terms of the form factor from an unoriented bilayer, because no Bragg peak means no correlation between the bilayers, and the structure factor should be unity. In such a case, the SAXS

**Fig. 1:** Schematic illustration of sample preparation methods. “NaI in film” samples are made by a novel method, and “NaI in solution” samples are used for control.
Unbinding of lipid bilayers induced by osmotic pressure in relation to unilamellar vesicle formation

### Fig. 2: Dependence of SAXS profiles on the molar ratio of NaI to DOPC, $x$. (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 $x = 0.1$. The profiles of higher NaI ratio were shifted for better visualization.

Intensity, $I(q)$, can be described as

$$I(q) = A \frac{|F(q)|^2}{q^2} + B,$$

where $F(q)$ is the form factor of a bilayer with coefficient $A$ and background $B$. The form factor of lipid bilayers in water has been well studied by Nagle and his co-workers [21]. Thus, we used the function of $F(q)$ for DOPC bilayers that they evaluated [22], and only the extrinsic parameters, $A$ and $B$, were determined by a least-squares fitting in a range of $0.05 < q < 0.3 \, [\text{Å}^{-1}]$.

As shown in fig. 3, a broad peak due to the correlation between head groups exists around $q = 0.12 \, \text{Å}^{-1}$, which is the most characteristic feature of the form factor from a lipid bilayer, because the electron density of the phosphate layer is larger than those of the other layers. On the other hand, the steep upturn in the low-$q$ region cannot be explained by this interpretation. (The Guinier radius of the low-$q$ scattering was about $100 \, \text{Å}$.) One possibility of this extra scattering is from large objects, such as small vesicles or aggregations of lipid molecules. Another possibility is the shell thickness of vesicles, since a number of bilayer stacks in a lamellar structure could be the origin of low-$q$ scattering [23]. However, the agreement of the fit function and the observed profile at $q > 0.05 \, \text{Å}^{-1}$ is the most important element to confirm the picture that lipid bilayers in a “NaI in film” sample of $x = 1$ exist solely in water without stacking. Therefore, the result could be interpreted as meaning that the unbinding of lipid bilayers occurs by the effect of NaI molecules in the “NaI in film” sample.

### Fig. 3: Magnified view of the SAXS profile from the “NaI in film” sample of $x = 1$ with the calculated form factor. (The same as the uppermost profile of fig. 2(b).) The calculated form factor is in good agreement with the experimental result.

### Fig. 4: Difference in the phase contrast images of the vesicles obtained by the two preparation methods at molar ratio of DOPC : NaI = 1 : 1. (a) “NaI in solution” sample. The size of the vesicles was about a few µm, and their shapes were miscellaneous. (b) “NaI in film” sample. The sizes of the vesicles were over 10 µm, and the shapes of larger ones were spherical. The images cover $50 \times 50 \, \mu \text{m}^2$.

Figure 4 shows the morphologies of vesicles obtained by the natural swelling method by two procedures. The size and shape of the vesicles are clearly different in these pictures: smaller vesicles were formed in the “NaI in solution” sample, whereas larger vesicles were formed in the “NaI in film” sample. From these results, it is clear...
that the efficiency of vesicle formation is different: only small multi-lamellar vesicles are formed by pouring salt-water to dry lipid films without NaI, while larger vesicles, which would be ULVs according to the SAXS result, are effectively formed by pouring pure water to dry lipid films with NaI. This tendency to improve the efficiency of forming large ULVs is the same as in the case with other salts or sugars [10]. This evidence supports the idea that large ULV formation is promoted by the effect of osmotic pressure.

Discussion. – In order to understand the effect of osmotic pressure on the hydration of lipid bilayers, the chemical potentials of water inside and outside of a bilayer stacking, \( \mu_{\text{in}} \), and that outside of a bilayer stacking, \( \mu_{\text{out}} \), were calculated (see fig. 5). Here, it is assumed that NaI molecules only exist in a bilayer stacking in the case of “NaI in film” samples, as confirmed by the neutron reflectivity measurement [24].

In case of aqueous solutions of NaI molecules, the chemical potential of water, \( \mu \), is

\[
\mu(T, p, x) = \mu_0(T, p) + k_B T \ln[a(x)],
\]

(3)

where \( \mu_0 \) is the chemical potential of pure water, and \( a \) is the activity of water, which is associated with the osmotic pressure (\( a \) is unity in case of pure water), and \( x \) is the same as the definition in the experimental section, that is, the molar ratio of NaI to lipid. For the water molecules in the lamellar phase, the interaction between bilayers should be considered since the inter-bilayer distance changes by the water penetration through bilayers [25]. Therefore, the chemical potential of water molecules is rewritten as

\[
\mu(T, p, x, l) = \mu_0(T, p) + k_B T \ln[a(x)] + \frac{dF(l)}{dn} = \mu_0(T, p) + k_B T \ln[a(x)] + v \frac{df(l)}{dl},
\]

(4)

where \( F \) is the free energy of the system, \( n \) the number of water molecules in the system, \( v \) the volume of a water molecule, \( f \) the free energy density per unit area originating from the interaction between bilayers, and \( l \) the inter-bilayer distance. Since any NaI molecules and bilayers are not in the outside, the difference in the chemical potentials between inside and outside of a bilayer stacking, \( \Delta \mu(= \mu_{\text{in}} - \mu_{\text{out}}) \), can be described as

\[
\Delta \mu(T, p, x, l) = \mu(T, p, x, l) - \mu(T, p, 0, \infty) = k_B T \ln[a(x)] + v \frac{df(l)}{dl}.
\]

(5)

In the present work, the repulsion due to hydration layers and the van der Waals interaction were taken into account as

\[
f(l) = P_h \lambda \exp \left[ -\frac{l}{\lambda} \right] = \frac{H}{12\pi} \left\{ \frac{1}{l^2} - \frac{2}{(l+\delta)^2} + \frac{1}{(l+2\delta)^2} \right\},
\]

(6)

where \( P_h \) is a prefactor for the hydration interaction, \( \lambda \) the decay length of hydration layers, \( H \) the Hamaker constant, and \( \delta \) the bilayer thickness. For simplicity, the Helfrich repulsion [13] was ignored. Under the assumption of an ideal solution, \( \ln[a(x)] \) is described as

\[
\ln[a(x)] \simeq \ln \left[ 1 - \frac{vx}{A_l l} \right] \simeq -\frac{vx}{A_l l},
\]

(7)

where \( A_l \) is the area per one lipid molecule, and the volume of NaI molecules, \( v' \), is assumed to be that of water (\( v = v' \)) to simplify the calculation. Since \( x/A_l l \) indicates the salt concentration, \( c \), this term corresponds to the osmotic pressure, \( \Pi \), as described by van’t Hoff’s law, \( \Pi = c k_B T \). Therefore, the equilibrium condition \( \Delta \mu = 0 \) means that attractive force due to the interaction between bilayers balances with the osmotic pressure. (The solution of \( \Delta \mu = 0 \) is independent of \( v \), since it is canceled in the equation.)

Figure 6 shows the calculated \( \Delta \mu \) as a function of \( l \) from eqs. (5)–(7) with the parameters given in table 1. If there is no NaI molecule (\( x = 0 \)), the equation \( \Delta \mu = 0 \) has two solutions at \( l = 15.3 \AA \) and \( l = \infty \). In the hydration process from a dry lipid film (\( l = 0 \)), a bilayer stacking is swollen by excess water until \( l = 15.3 \AA \), because the region between the bilayers is more preferable for water molecules when \( \Delta \mu < 0 \). Therefore, MLVs with small inter-bilayer distance are stable, when a dry pure lipid film is hydrated. It should be noted here that the repeat distance, \( d = \delta + l = 60.6 \AA \), is little less than that of the experiment, \( d = 63.5 \AA \). Since the Helfrich repulsion we ignored in the calculation, the discrepancy of \( d \)'s will be originated from the insufficient assumption of the free energy. Thus, the discrepancy of \( d \)'s by the calculation and by the experiment is acceptable.

With increasing \( x \), on one more solution \( (l = l'') \) appears between \( l \sim 15.3 \AA \) and \( l = \infty \). However, a bilayer stacking cannot be swollen by excess water above \( l \sim 15.3 \AA \),
Unbinding of lipid bilayers induced by osmotic pressure in relation to unilamellar vesicle formation

Table 1: Parameters used in calculating the chemical potentials. The values given in the literature were determined by the SAXS experiment for DOPC bilayers.

| T        | v     | δ [19] | \(A_L\) [19] | \(H\) [19] | \(P_h\) [19] | \(\lambda\) [19] |
|----------|-------|--------|---------------|-------------|--------------|------------------|
| 30 °C    | 30 Å² | 45.3 Å | 72.2 Å²       | 4.0 × 10⁻²¹ J | 5.0 × 10⁷ J/m³ | 2.26 Å           |

Fig. 6: Calculated chemical potential of water with changing the molar ratio of NaI, \(x\). The profile shift downward with increasing \(x\), which is attributed to an osmotic pressure.

because the region outside the bilayer stacking is more preferable for water molecules when \(\Delta \mu > 0\). Moreover, the derivative of \(\Delta \mu\) is negative at \(l = l_0\). This indicates that the second derivative of the free energy is negative at \(l = l_0\), and the solution is unstable. From this calculation, it could be concluded that MLVs are stable for \(x < 0.003\).

On the contrary, \(\Delta \mu = 0\) has only one solution at \(l = \infty\) for \(x > 0.004\). In this case, \(\Delta \mu\) is negative for all \(l\)'s and a bilayer stacking can include water molecules infinitely, because the osmotic pressure overcomes the attractive force between bilayers. In other words, unbinding due to the osmotic pressure takes place to adjust the extremely lopsided distribution of NaI molecules in the lipid/NaI mixture when hydration starts. Therefore, we concluded that the unbinding induced by the osmotic pressure caused MLVs to be unstabilized, and this could cause the formation of large ULVs.

It should be noted here that the calculated critical concentration to induce the unbinding was different from the SAXS result: more than 10% of NaI against DOPC is required to unbind lipid bilayers from the stack, while only about 0.4% of NaI is required to induce the unbinding in the model calculation. This discrepancy can be understood considering the hydration process of lipid films. In our calculation, it is assumed that one side of a bilayer faces pure water and the other side faces water with salt. However, this assumption works only for the outermost lipid layer, since both sides of a bilayer inside of the stack face water with salt. Therefore, \(\Delta \mu\) can be written as

\[
\Delta \mu = -\frac{v}{A_L} \left\{ \frac{x_{in}}{l_{in}} - \frac{x_{out}}{l_{out}} \right\} + v \left\{ \frac{df(l_{in})}{dl} - \frac{df(l_{out})}{dl} \right\},
\]

where subscript \(in\) indicates the inside of bilayer stacking, and subscript \(out\) the outside of the stacking. Because the differences of \(x\) and \(l\) between both sides of bilayer stacking are the same at equilibrium \((x_{in} = x_{out}, l_{in} = l_{out})\), the unbinding due to osmotic pressure cannot be induced even when \(x > 0.004\). In contrast to such the situation, the hydration of a lipid film is, of course, a kinetic process. In this process, water molecules penetrate into a bilayer stacking from outside, and salt ions transfer from the stack. Thus, water molecules and the salt ions distribute oppositely in a bilayer stacking \((x_{in} > x_{out}, l_{in} < l_{out})\), where the salt concentration of the inside is higher than that of the outside. This could be the realistic origin to induce the unbinding of the bilayers from the stack, since the difference of salt concentration induces the difference of osmotic pressure. In such the situation, the critical concentration for the unbinding should be much larger than that in our calculation. Therefore, the critical concentration in the experiment is several orders of magnitude larger than that in the theoretical consideration.

**Conclusion.** – A difference in the stacking structure of lipid bilayers, depending on the process of mixing lipids, water and salt (“NaI in solution” and “NaI in film”) was investigated by SAXS with changing the molar ratio of NaI to DOPC. The difference in the size and the morphology of vesicles, depending on the mixing process, was also investigated by PCM. The results suggested that a large amount of NaI in the “NaI in film” sample promoted an instability of MLVs. A calculation of the chemical potential of water of the “NaI in film” sample was performed, which confirmed that the osmotic pressure was the origin of the unbinding of lipid bilayers from the stack. From these results, we concluded that the osmotic pressure due to the heterogeneous distribution of NaI induces the unbinding of lipid bilayers, which results in the formation of ULVs.

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