Effects of cholesterol concentration and osmolarity on fluidity and membrane tension of free-standing black lipid membranes

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Abstract

Although the mechanical properties and compositions of lipid bilayer membranes can change upon deformation, the fundamental relations between composition, membrane tension and fluidity of membranes with little curvature have not been studied yet. In the current study, the membrane tension and the diffusion coefficients of free-standing black lipid membranes (BLMs) based on 1, 2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were observed by systematic control of the cholesterol concentration and the osmotic pressure with the laser induced surface deformation (LISD) and fluorescence recovery after photobleaching (FRAP) techniques. When the osmotic pressure was raised and, therefore, the curvature became larger, both the membrane tension and diffusion coefficients increased as well. On the other hand, when the cholesterol concentration was raised, the membrane tension increased whereas the diffusion coefficient decreased. The importance of the present results goes beyond this quantitative evaluation of the relation between membrane tension and fluidity, as it clarifies the changes in the fundamental properties of lipid bilayers upon natural fluctuations and perturbative deformation that were hitherto unknown.

Keywords: membrane tension, fluidity, osmotic pressure, lipid bilayers, black lipid membranes, fluorescence recovery after photobleaching, laser induced surface deformation.
Introduction

The lipid bilayer that separates the interior of the cell from the outside environment is an important component of the cell membrane. It controls various processes of the cell functions together with other embedded molecules such as steroids, carotenoids and membrane proteins.\textsuperscript{1-7} The properties of the cell membranes can be affected by various parameters of the lipid bilayer membrane, such as shape, composition, embedded molecules and surrounding environment. Membrane tension is also an important parameter which is sensitive to applied forces, deformations and functions of some membrane proteins.\textsuperscript{8,10} Lipid structures and compositions are also related to the deformation and fine structures of membranes.\textsuperscript{4,6,7} For the characterization of the membrane tension and the functions of cell membranes and their models, studies involving a large deformation of the membranes have been conducted, for instance, using the micropipette aspiration method.\textsuperscript{11-14} However, the relations between membrane tension, composition and fluidity upon little deformation and small external force have not been investigated even for the model membranes because of limitations in the measurement methods. This is the case despite the fact that fluctuated structures such as wrinkles are expected to be “ironed out”\textsuperscript{6} when external forces are applied for the measurements.

We have recently developed a non-invasive technique to measure the membrane tension upon natural fluctuations\textsuperscript{15} by applying the laser induced surface deformation (LISD) method to models of cell membranes, i.e. free-standing black lipid membranes (BLMs) fabricated using the folding method,\textsuperscript{16-21} that contain low amounts of residual organic solvents. The LISD method is a method that detects the membrane tension and the interfacial tension by monitoring the optical response to radiation pressure.\textsuperscript{22} It has also been employed for the evaluation of the viscoelastic properties of cells.\textsuperscript{23,24} When the LISD method was applied to BLMs, small deformations of the membranes during the detection of the membrane tension
were observed. In our previous work, we reported that the membrane tensions of free-standing BLMs have values 100 times smaller than those reported by the other methods.\textsuperscript{15} The composition dependence of the cholesterol amount was also discussed and the membrane tension was found to become larger as cholesterol increased in the BLMs. We interpreted such increase as the result of the strengthening of the hydrophobic interaction due to the larger amount of cholesterol.\textsuperscript{15}

When the lipid membrane is deformed, stronger forces such as the Laplace pressure are balanced with the membrane tension that increases upon stretching of the membrane. From a molecular point of view, the origin of the increased membrane tension might be some kind of interaction between molecules. For the evaluation of such interactions, fluidity can be an important measurable parameter because it becomes small when there are strong interactions, while it becomes larger with smaller interactions. However, the relations between membrane tension, membrane deformation and fluidity have not yet been understood because the measurement methods and samples were totally different for the systematic discussions in the previous measurements. In the present work using the LISD method the relations between membrane tension and cholesterol concentration for 1, 2-dioleoyl-sn-glycero-3-phosphocholine (DOPC)-based flat BLMs are discussed through an evaluation of the membrane flatness by controlling the osmotic pressure. For the evaluation of the fluidity, the fluorescence recovery after photobleaching (FRAP) technique\textsuperscript{25} was also applied to the BLMs fabricated in the same chamber and with the same geometry. By measuring both the membrane tension and the diffusion coefficient with the LISD and FRAP methods for similarly prepared BLMs, a systematic discussion of the relations between membrane tension, molecular composition, osmotic condition and diffusion coefficient is presented.
Experimental

Reagents and chemicals

1, 2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) was purchased from Avanti Polar Lipids. Cholesterol was purchased from Kanto Chemical, Japan, and was used after repeated recrystallization with methanol (infinity pure, Wako Chemical, Japan). Glucose, sucrose and KOH were purchased from Kanto Chemical. 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiIC<sub>18(3)</sub>) and KCl were purchased from Wako chemical, Japan. HEPES free acid of molecular biology grade was obtained from CALBIOCHEM. Chloroform (HPLC-grade, Kanto Chemical, Japan) was purified by aluminum oxide column chromatography before use. Water was purified using a Milli-Q Integral 3 system from Millipore (Japan).

Preparation of the BLMs.

The BLMs were prepared using the folding method.<sup>16-21</sup> In this method, a vertical lipid bilayer is formed by simultaneous folding up of two lipid monolayers on two separate aqueous surfaces facing a hole in a PTFE film (20 μm thickness, 200 μm diameter) separating the two solutions. The chamber and the procedure for the BLMs formation are the same as in our previous work.<sup>15</sup> Because a difference in the refractive index is necessary for LISD measurements, two kinds of aqueous solutions of sugar (glucose and sucrose) were used for the extraction of the lipid monolayers. KCl was also dissolved in each solution with a 1 mol/L concentration. The pH of each solution was adjusted to 7.4 by using 10 mmol/L of HEPES/KOH buffer solution. Each chamber was filled with the respective solution (2.0 mL each) so that the surface level of the sugar solutions was below the hole. A 60–80 μL volume of a chloroform solution of DOPC and cholesterol was then dropped onto each surface to form the lipid monolayer. After letting chloroform evaporate from the surface, each surface was raised up
in order to fold the two monolayers into a lipid bilayer. The weight ratios of DOPC and cholesterol were DOPC:cholesterol = 8:2 to 10:0, respectively, with a total concentration of 2 mg/mL in chloroform. For the FRAP measurements, DiIC18(3) was used as the fluorescent probe molecule and was added to obtain a 4 mol% ratio. The formation and the thickness of the BLMs were evaluated with an LCR meter (ZM2353, NF Corporation, Japan) by applying a 50 mV voltage with 1 kHz frequency.

**Membrane tension measurements with the LISD method**

The optical setup for the LISD measurements is, in principle, the same as that of our previous work,\(^\text{15}\) except that additional optics are inserted for the FRAP measurements as shown in Fig. 1(a). Briefly, the light source for the membrane deformation is a 532 nm Nd:YVO\(_4\) green laser (300 mW) and the membrane deformation is optically detected by a 633 nm helium-neon red laser. Both laser beams are expanded and set in a collinear geometry by focusing with an \(f = 20\) mm objective lens (M Plan Apo x10, Mitsutoyo, Japan). Because each sugar solution facing the BLM has a different refractive index, the position-dependent radiation pressure of the green laser induces a membrane deformation. The periodic intensity modulation of the green pump light by an acousto-optic modulator induces propagating waves on the BLM. The maximum frequency of the induced waves is limited by the pump beam diameter \(2w\), because the half-wavelength of the propagating wave cannot become smaller than the beam diameter. According to the dispersion relation, assuming a wave number \(\pi/2w\) at a frequency \(f_c\), the relation between \(f_c\) and the interfacial tension of the BLM membrane, \(\gamma\), can be roughly estimated to be:

\[
2\pi f_c \approx \left( \frac{\gamma}{\rho_1 + \rho_2} \right)^{\frac{1}{2}} \left( \frac{\pi}{2w} \right)^{\frac{3}{2}} \tag{1}
\]

where \(\rho_1\) and \(\rho_2\) are the densities of each aqueous phase. The laser beam diameter was evaluated
by monitoring the scattered image of the laser beam on the PTFE film with a CMOS camera. The characteristic frequency $f_c$ was estimated from the LISD spectra as the frequency where the LISD signal amplitude becomes half of the static value at low frequency. The membrane tension was then obtained from Eq. (1).

**Diffusion coefficient measurements by FRAP**

For the measurements of the diffusion coefficient of the BLMs using the same chamber and the same lipid/cholesterol ratio as in the LISD measurements, we embedded the FRAP measurement system into the LISD measurement system. The additional optics are described in Fig. 1(b). To apply the FRAP technique, DiIC$_{18}(3)$ was mixed in the BLMs and the fluorescence intensity at the position where the photobleaching was induced by the focused pump light was monitored by the illuminated probe light to obtain the fluorescence recovery profile. In this study, the pump light source for the photobleaching was the 532 nm green laser which was used in common with the LISD system. A mechanical shutter was inserted to control the pump irradiation time. Another 532 nm green laser light was separately introduced into the chamber to illuminate the whole BLM region in order to obtain a fluorescence image. The emitted fluorescence was separated by a dichroic mirror, color glass filter and notch filter, and monitored by a cooled CCD camera (Photometrics CoolSNAP HQ2) with a $f = 300$ mm single-lens reflex camera lens. To obtain the FRAP signals, CCD images with a 0.5 s exposure time were saved sequentially using ImageJ software. The diameter of the photobleaching area was determined using the line profile of the image just after the photobleaching, that was also set as the time origin. To obtain the fluorescence recovery profile, the fluorescence intensity at the time origin was subtracted. The bleached fluorescence intensity was also normalized to the illumination intensity which is calculated from the non-bleached area of the fluorescence image to compensate the intensity instability of the illumination. For the profile fitting and calculation
of the diffusion coefficients, the model function by Soumpasis,\textsuperscript{26}

\[ f(t) = e^{-\frac{2\tau_D}{t}} \cdot (I_0 \left( \frac{2\tau_D}{t} \right) + I_1 \left( \frac{2\tau_D}{t} \right)) \]

was used, where \( t, \tau_D, I_0 \) and \( I_1 \) are time, the characteristic diffusion time and the modified Bessel functions of order 0 and order 1, respectively. The diffusion coefficients \( D \), the radius of the photobleaching area \( w \) and \( \tau_D \) are related by \( \tau_D = \frac{w^2}{4D} \).

**Results and Discussion**

*Membrane tension and diffusion coefficients for DOPC BLMs with different cholesterol concentrations*

In the present work, we used DOPC instead of L-\( \alpha \)-phosphatidylcholine (PC) and L-\( \alpha \)-phosphatidylethanolamine (PE), as in our previous paper.\textsuperscript{15} This is because DOPC is a single component lipid with \( \text{L}_\alpha \) phase at room temperature and the system becomes simpler than the PC and PE systems that are the mixture of lipids with different fatty acids. For the evaluation of the effects of cholesterol on the membrane tension and fluidity, the cholesterol concentration in the DOPC-based BLMs was varied and LISD and FRAP were applied. Representative LISD spectra of BLMs based on DOPC are shown in Fig. 2 for five different cholesterol concentrations. At low frequency, the membrane can be deformed following the periodical radiation pressure, while at high frequency, the membrane deformation can no longer follow the periodic radiation pressure and the smaller deformation results in a decrease of the LISD signal intensity. After multiple measurements of the characteristic frequency and laser spot distribution, the membrane tension was calculated and averaged. As observed in Fig. 2, a shift of the spectrum to higher frequency was observed as the cholesterol concentration increased. The resulting membrane tension of five kinds of BLMs with different cholesterol ratios (DOPC : cholesterol = 8:2 (32 mol % cholesterol), 8.5:1.5 (26 mol %), 9:1 (18 mol %),
9.5:0.5 (10 mol %), and 10:0 (0 mol %) (w/w)) was obtained as shown in Fig. 3. The concentration of both sugar solutions for this measurement was 1.5 mol/L. The observed membrane tension became larger when the amount of cholesterol increased. Our previous results for BLMs using PC and PE also showed that the membrane tension increased when the cholesterol ratio was incremented. As described in the paper reporting our PC and PE results, the increase of the membrane tension for the current DOPC BLMs can also be ascribed to the strengthening of the interaction between cholesterol and the lipid molecules due to the higher amount of cholesterol in the BLMs. Nonetheless, the obtained membrane tension value was smaller in the current study than in the previous PC and PE study. This might be because of the difference in the characteristics of the lipid molecules: PC and PE contain saturated alkyl chains that can increase the ordered structures and rigidity, whereas DOPC contains uniform unsaturated alkyl chains.

FRAP measurements of the DOPC BLMs were also conducted using the same chamber with the addition of DiIC<sub>18</sub>(3). A typical FRAP time profile of the BLMs based on DOPC are shown in Fig. 4. From the fits of these profiles the diffusion coefficients for five kinds of BLMs with different cholesterol ratios (DOPC : cholesterol = 8:2, 8.5:1.5, 9:1, 9.5:0.5, and 10:0 (w/w)) were obtained and are shown in Fig. 3. The obtained values of the diffusion coefficient were in the 8-16 μm<sup>2</sup> s<sup>−1</sup> range. This was about 10 times larger than that reported for fluid-phase phosphatidylcholine with saturated dilauroyl and dipalmitoyl alkyl chains. Given that we used DOPC with non-saturated alkyl chains which are expected to possess a higher fluidity than the saturated one, our measured larger fluidity is reasonable. A previous study reported a diffusion coefficient of 7-11 μm<sup>2</sup> s<sup>−1</sup> for DOPC bilayers in spanning bilayers, and 4 μm<sup>2</sup> s<sup>−1</sup> in supported DOPC bilayers. The present results are similar, but somewhat larger than those earlier experimental values. We can ascribe this difference to the condition of our BLMs which are flat and free-standing, unlike in the aforementioned study. The diffusion coefficient
decreased as the cholesterol concentration became larger. As the increase of cholesterol induced a stronger hydrophobic interaction between the membrane components, we can also expect that such strengthening of the molecular interaction, in turn, resulted in a larger membrane tension and a lower fluidity of the compositions. This is because the stronger interaction suppressed the motion of the molecules.

**Effect of the osmotic pressure on the membrane tension and diffusion coefficients**

The obtained membrane tension was much smaller than the values reported by other methods. We considered that this is because the influence of the membrane deformation is very small in our BLMs because it is formed using the folding method in a circular hole on a flat film. Whenever there is a pressure difference between the inside and the outside, the membrane bends and the pressure difference is balanced with the membrane tension as the Laplace pressure. When additional external pressure is applied to the membrane and the curvature becomes larger, the membrane itself is expected to stretch so that the membrane tension increases in order to balance the applied pressure. To minimize the effects of such stretching and deformations, evaluations of the flatness of the BLMs are important. For the characterization of the curvature of BLMs fabricated using the folding method, we controlled the osmotic pressure between the two aqueous phases by varying the concentration differences between the two sugar solutions. We prepared two series of concentration differences to control the osmotic pressure and the direction of the subsequent deformation. In one case, the osmotic pressure was larger in the sucrose solution, as the concentration of glucose solution was fixed to 0.75 mol/L while the concentration of sucrose was set to 0.75, 0.78, 0.80, and 0.82 mol/L. In the other case, the osmotic pressure was larger in the glucose solution, as the concentration of sucrose solution was fixed to 0.75 mol/L while the concentration of glucose was set to 0.75, 0.78, 0.80, and 0.82 mol/L. We expected a convex deformation towards the glucose solution in the case of higher
sucrose concentration, and vice versa in the case of higher glucose concentration at the equilibrium.

The concentration difference dependence of the membrane tension when either the sucrose or glucose concentration was varied is shown in Fig. 5. In both cases, the membrane tension became larger as the concentration difference increased. If a flat membrane is formed when there is no concentration difference, a concentration difference is expected to induce a similar curvature of the membrane but in opposite directions depending on the osmotic pressure. The result that the observed membrane tension increases simultaneously in both cases, confirms the flatness of the fabricated BLM in the absence of a concentration difference. Moreover, we note the similarity of the deformation in the BLMs at each concentration difference although the deformation direction is different. More precisely, there was still a slight difference between the two series of concentration differences. We do not know the precise reason, but one possible explanation is that, when totally flat BLMs are obtained, the concentrations are slightly different from zero $\Delta$concentration because of the instrumental conditions. Another explanation might lie in the different directions of the induced LISD deformations between the two series. The induced LISD curvature is always toward the glucose solution, whereas the deformation induced by the osmotic pressure is dependent on the concentration difference between the sucrose and glucose solutions. Therefore, the actual response to the deformation in the LISD measurement might be different between the case when the radiation pressure has the effect of decreasing the curvature, and the case when the radiation pressure increases the curvature.

Relation between fluidity and deformation

FRAP measurements were also carried out on the BLMs using a 0.75 mol/L glucose solution and a 0.75-0.82 mol/L sucrose solution: the results are shown in Fig. 6. These results indicate that the membrane fluidity increased as the concentration difference became higher and,
consequently, the osmotic pressure was larger. The relations between the fluidity and the membrane tension when changing either the cholesterol concentration or the osmotic pressure are shown in Fig. 7.

Unlike the decrease in the fluidity observed as the membrane tension increased for different cholesterol concentrations, here the fluidity increased as the membrane tension became larger by varying the osmotic pressure. This is due to the different mechanisms leading to the higher membrane tension in these two systems. In the case of the cholesterol concentration dependence, the addition of cholesterol resulted in the internal binding of the lipids. On the other hand, the osmotic pressure itself did not change the membrane composition but it induced a membrane deformation that resulted in the larger membrane tension. Hence the higher fluidity is thought to originate from the deformation and stretching of the membrane accompanying the relocation of molecules and interaction with the surrounding solutions.

In a study on the osmolarity and generalized polarization (GP) value of a laurdan fluorescence probe for a DOPC lipid bilayer membrane of vesicles, a decrease of the GP value of the red-shifted laurdan fluorescence was observed as the osmolality difference increased and the vesicle swelled with larger mechanical strain. The decrease of the GP value indicated a larger polarization of the penetrated water by disordered lipid molecules and larger fluidity. Molecular dynamics simulations of the effect of the membrane tension for the DOPC lipid bilayer were also reported and they indicated the relations between larger membrane tension, deeper penetration of water and larger fluidity. Hence, as to our results, we can also conclude that the larger deformation and stretching of the BLMs by the larger osmotic pressure resulted in movable disordered lipid compositions with larger penetrations from the aqueous phase that resulted in the larger fluidity.

The observed deformation effect of the membrane tension induced by the osmotic pressure is much smaller than the previously reported effect of the osmotic pressure on the
membrane tension of giant vesicles by Yamazaki and coworkers. This is because our BLMs involve quite small deformations and stresses derived from the flat shape. In order to retain the curvature of giant vesicle membrane, a certain membrane tension is required, whereas a flat membrane does not require such tension. In other words, the present result can be interpreted as a perturbative deformation effect of the membrane tension on a flat membrane with natural fluctuations.

Conclusions

In the current study, we have investigated the effects of both compositions and osmotic pressure on the fluidity and the membrane tension of DOPC-based BLMs. In order to minimize the effect of the membrane stretching produced by the deformations, the flatness of BLMs fabricated using the folding method was also evaluated by controlling the osmotic pressure induced by the concentration difference of sugars in both sides of the aqueous phases. The flatness was confirmed for the BLM with no concentration differences. The diffusion coefficients dependence on sugar concentration differences was also evaluated and it was found to increase as the concentration difference became higher and, consequently, the deformation was larger.

By clarifying the flatness of the BLMs without sugar concentration difference, we presented the cholesterol concentration dependences of the membrane tension, and the diffusion coefficient for the flat BLM without deformation effect for the first time. We observed a decrease of the diffusion coefficient when the membrane tension became higher as the cholesterol concentration increased and we attributed this result to the strengthening of the interaction between cholesterol and lipids and the reduced mobility of molecules. On the other hand, as the osmotic pressure rose, both membrane tension and fluidity increased. We concluded that the larger deformation and the stretching of BLMs induced by the larger osmotic pressure
resulted in movable disordered lipid compositions with larger penetration of molecules from aqueous phases that resulted in larger fluidity. These results are not only important because they quantitatively evaluate the relations between membrane tension and fluidity, but also because they provide the membrane tension of flat lipid bilayers under natural fluctuations and perturbative deformation.

Acknowledgements

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References


**Figure Captions**

Figure 1 Optical setups for the LISD and FRAP measurements of the BLMs. (a) For the LISD measurements, the probe laser is focused and detected. (b) For the FRAP measurements, the illumination optics are inserted and the fluorescence image is detected by a cooled CCD camera.

Figure 2 Representative LISD spectra of the BLMs based on DOPC with various cholesterol concentrations. (DOPC : cholesterol = (a) 10:0, (b) 9.5:0.5, (c) 9:1, (d) 8.5:1.5 and (e) 8:2 (w/w)). Circles are the detected modulated light intensity, solid lines are the fitting results, and broken lines indicate characteristic frequencies. The vertical axis corresponds to the modulated light intensity (photocurrents) converted to voltage and detected by a lock-in amplifier.

Figure 3 Membrane tension and diffusion coefficient of the DOPC-based BLMs with various cholesterol concentrations. Error bars represent the ranges of standard deviations for each value.

Figure 4 Typical FRAP time profiles of the BLMs based on DOPC. (DOPC: cholesterol = (a) 10:0, (b) 9.5:0.5, (c) 9:1, (d) 8.5:1.5 and (e) 8:2 (w/w)). Circles are the observed intensity, and solid lines are the fitting results.

Figure 5 Relation between membrane tension and concentration difference when the osmotic pressure was controlled by (cross) the sucrose concentration, or (gray circle) the glucose concentration. Error bars represent the ranges of standard deviations for each value.

Figure 6 Relation between diffusion coefficient and concentration difference when the osmotic pressure was controlled by the sucrose concentration. Error bars represent the ranges of standard deviations for each value.
Figure 7  Relation between diffusion coefficient and membrane tension when (a) the cholesterol concentration was controlled, and (b) the osmotic pressure was controlled. Error bars represent the ranges of standard deviations for each value.
Figure 1

Optical setups for the LISD and FRAP measurements of the BLMs. (a) For the LISD measurements, the probe laser is focused and detected. (b) For the FRAP measurements, the illumination optics are inserted and the fluorescence image is detected by a cooled CCD camera.
Figure 2

Representative LISD spectra of the BLMs based on DOPC with various cholesterol concentrations. (DOPC : cholesterol = (a) 10:0, (b) 9.5:0.5, (c) 9:1, (d) 8.5:1.5 and (e) 8:2 (w/w)). The open circles are the detected modulated light intensity, the solid lines represent the fits to each data set, and the broken lines indicate the characteristic frequencies. The vertical axis is the modulated light intensity (photocurrent) converted to voltage and detected by a lock-in amplifier.
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Relation between diffusion coefficient and concentration difference when the osmotic pressure was controlled by the sucrose concentration. The error bars represent one standard deviations of the average of each value.
Figure 7

Relation between diffusion coefficient and membrane tension when (a) the cholesterol concentration was controlled, and (b) the osmotic pressure was controlled. The error bars represent one standard deviations of the average of each value.
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