Biophysical Journal, Volume *111*

Supplemental Information

Experimental Estimation of Membrane Tension Induced by Osmotic Pressure

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Supporting Material

Experimental Estimation of Membrane Tension Induced by Osmotic Pressure

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S.1. Measurement of osmotic pressure of sucrose and glucose aqueous solution

The osmolality (mOsm/kg) of sucrose solution and glucose solution was measured by analyzing the freezing point depression of these solutions using Osmometer 3250 (Advanced Instrument Inc., Norwood, MA) at Japan Food Research Laboratories, Tama Institute (Tokyo, Japan). By correction of the volume change, we converted these osmolality (mOsm/kg) to the osmolarity (mOsm/L) (Table S1 & S2). Figure S1 shows the linear relationship between osmolarity (y) and molar concentration of sucrose (or glucose), C, when C is at and less than 98 mM, and the fitting equations for sucrose and glucose are $y = (1.03 \pm 0.01)\ C$ and $y = (1.04 \pm 0.02)\ C$, respectively. These results indicate that the ideal equation (van't Hoff's law) for Π can be used for these solutions within experimental errors, which agrees with the previous reports [1,2].

Figure S1

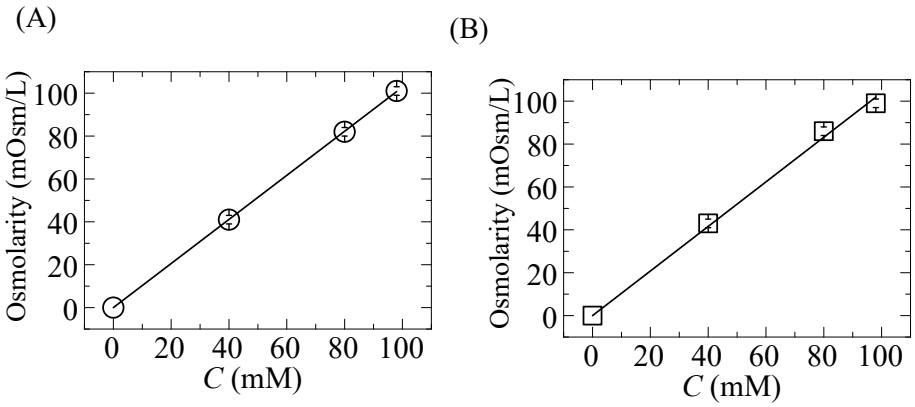

Figure S1**:** Osmotic pressure of sucrose and glucose aqueous solutions. Osmolarity (mOsmo/L) of sucrose solution (A), and glucose solution (B).

Table S1**:** Osmotic pressure of sucrose solution

C (mM)	Osmolality (mOsm/kg)	Osmolarity (mOsm/L)
98.0	104 ± 2	101 ± 2
80.0	84 ± 2	82 ± 2
40.0	42 ± 2	41 ± 2

Table S2**:** Osmotic pressure of glucose solution

C (mM)	Osmolality (mOsm/kg)	Osmolarity (mOsm/L)
98.0	101 ± 2	99 ± 2
80.0	88 ± 2	86 ± 2
40.0	44 ± 2	43 ± 2

Figure S2

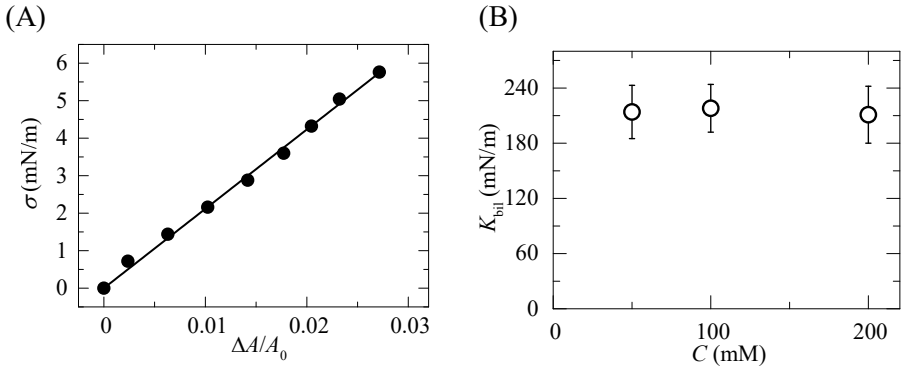

Figure S2**:** The elastic modulus of the bilayer of DOPC-GUV, K_{bil}. (A) Tension versus the fractional change in area of a DOPC-GUV in 100 mM sucrose (inside the GUV) / 100 mM glucose (outside the GUV). The slope of the line provided a K_{bil} value of 212 mN/m. (B) The elastic modulus of the bilayer of DOPC-GUV in various concentrations *C* of sucrose/glucose solutions. GUVs contained sucrose solution in their lumens, and in their outside glucose solution existed, and the concentrations of the sucrose solution and the glucose solution were the same.

Figure S3

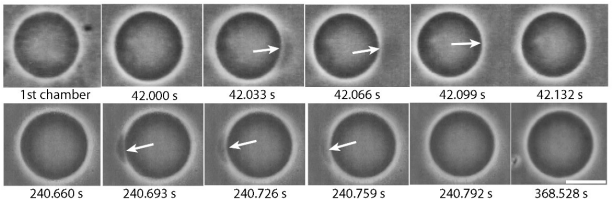

Figure S3**:** Behavior of DOPC-GUV under large Π. Phase-contrast microscopic images of a GUV after it was transferred into chamber B containing 78.0 mM glucose solution (i.e., ΔC^0 = 20.0 mM). The numbers below each image show the time in seconds after the transfer of the GUV into chamber B. The bar corresponds to 25 μm. The white arrows show two rapid, transient leakages of sucrose from DOPC-GUV upon Π.

Table S3: Comparison of $(\sigma_{osm}^{eq})^{ex}$ determined by the Π-induced volume change of GUVs with $(\sigma_{osm}^{eq})^{th}$: the analysis of the data in Figure 2

ΔC^0 (mM)	$\Delta V_{eq}/V_0$	$(\sigma_{osm}^{eq})^{ex}$ (mN/m)	$(\sigma_{osm}^{eq})^{th}$ (mN/m)	$\overline{r_0}$ (μm)
2.0	$(1.7 \pm 0.1) \times 10^{-2}$	2.7 ± 0.1	2.8 ± 0.7	14.5 ± 0.5
3.0	$(2.6 \pm 0.1) \times 10^{-2}$	4.1 ± 0.2	4.3 ± 0.7	16.2 ± 0.6

Table S4: The dependence of P_{Leak} (360 s) on $(\sigma_{osm}^{eq})^{th}$ (mN/m)

ΔC^o (mM)	$\overline{r_0}$ (μm)	$(\sigma_{osm}^{eq})^{th}$ (mN/m)	P_{Leak} (360 s)
1.0	13.0 ± 0.3	1.4	0.0
3.0	13.7 ± 0.4	4.2	0.09 ± 0.02
3.7	14.0 ± 0.3	5.3	0.12 ± 0.01
4.2	14.2 ± 0.3	6.0	0.31 ± 0.08
4.5	14.5 ± 0.5	6.5	0.53 ± 0.04
5.0	15.9 ± 0.5	7.3	0.84 ± 0.06
6.0	14.8 ± 0.5	8.9	0.98 ± 0.02
6.5	15.3 ± 0.5	9.7	1.0
8.0	14.2 ± 0.4	12.1	1.0

Table S5: Comparison of $(\sigma_{osm}^{eq})^{ex}$ determined by the Π-induced volume change of GUVs in the presence of external tension and $(\sigma_{osm}^{eq})^{th}$: the analysis of the data in Fig. 4A

ΔC^0 (mM)	σ_{ex} (mN/m)	$\Delta r_{eq}/r_0$	$(\sigma_{osm}^{eq})^{ex}$ (mN/m)	$(\sigma_{osm}^{eq})^{th}$ (mN/m)	$\overline{r_0}$ (μm)
3.0	4.0	$(8.6 \pm 0.2) \times 10^{-3}$	4.1 ± 0.1	4.3 ± 0.7	14.9 ± 0.6

Table S6: Fraction of ruptured GUVs, P_{pore} (360 s), in the results depicted in Figure 1. $\sigma_t = \sigma_{ex} + \sigma_{osm}^{eq}$
(A) ΔC^0 = 1.9 mM

σ_{ex} (mN/m)	$\overline{r_0}$ (μm)	$(\sigma_{osm}^{eq})^{th}$ (mN/m)	P_{pore} (360 s)	σ_t (mN/m)
4.5	12.5 ± 0.3	2.6	0.58 ± 0.06	7.1
5.0	14.4 ± 0.5	2.6	0.91 ± 0.02	7.6
5.5	13.4 ± 0.3	2.6	0.97 ± 0.02	8.1

Table S7: Fraction of ruptured GUVs, P_{pore} (360 s), in the results depicted in Figure 1. $\sigma_t = \sigma_{ex} + \sigma_{osm}^{eq}$
(B) ΔC^0 = 2.8 mM

σ_{ex} (mN/m)	$\overline{r_0}$ (μm)	$(\sigma_{osm}^{eq})^{th}$ (mN/m)	P_{pore} (360 s)	σ_t (mN/m)
3.3	13.6 ± 0.6	3.9	0.69 ± 0.06	7.2
3.8	13.7 ± 0.3	3.9	0.90 ± 0.02	7.7
4.3	13.8 ± 0.4	3.9	0.99 ± 0.02	8.2

S.2. The sources of the error of the experimental values of $\sigma_{\mathrm{osm}}^{\mathrm{eq}}$

It is necessary to consider the sources of the error of the experimental values of $\sigma_{\mathrm{osm}}^{\mathrm{eq}}$. One possibility is due to permeabilization of sucrose and glucose through DOPC membranes under the experimental conditions. It is reported that the membrane permeability coefficient of glucose, P, through dimiristoylphosphatidylcholine (DMPC) membrane in the liquid-crystalline phase in unilamellar vesicles is 1.5×10^{-12} m/s [3]. In the case of 100 mM and 0 mM glucose in the outside and in the inside of a GUV, respectively (i.e., $C_{\mathrm{out}}^{\mathrm{glu}} = 100$ mM, $C_{\mathrm{in}}^{\mathrm{glu}} = 0$ mM, and the concentration difference of glucose between the inside and the outside, $\Delta C_{\mathrm{glucose}}$, is 100 mM), the flux of glucose from the outside to the inside of the GUV (i.e., influx), J, is $J = P\Delta C_{\mathrm{glucose}} = 1.5 \times 10^{-10}$ mol/s·m^2. Therefore, the maximum rate of the increase in glucose concentration inside the GUV is as follows,

$$\frac{dC_{\mathrm{in}}^{\mathrm{glu}}}{dt} = \frac{1}{V_0} 4\pi r_0^2 J = \frac{3J}{r_0} = 4.5 \times 10^{-5} \quad [\mathrm{mM/s}] \tag{S1}$$

According to eq. S1, the maximum increase in C_{in} during 1800 s (= 30 min) is 0.08 mM, which is less than 5 % of ΔC^0. However, we also consider the permeabilization of sucrose from the inside to the outside of a GUV (i.e., efflux). There is no report to determine the accurate value of P of sucrose of PC membranes in vesicles. If we assume that P of sucrose is the same as that of glucose, we obtain the maximum rate of the decrease in sucrose concentration inside the GUV is 0.08 mM for 30 min, which is cancelled out by the increase in the glucose concentration inside the GUV, and therefore, ΔC^{eq} does not change. However, if the P values are different, ΔC^{eq} changes with time and concomitantly Π changes with time. It is reported that if the P value of substance A outside a GUV is different from that of substance B inside the GUV the diameter of the GUV changes with time [4]. In the control experiment of Fig. 2B (i.e., $\Delta C^0 = 0.0$ mM), we did not observe a significant change in $\Delta V/V_0$ for 20 min after the transfer of the GUV to chamber B. This indicates that the change of ΔC^{eq} due to the difference of the P value of sucrose and glucose is negligible for 20 min.

Another possibility is the increase in the glucose concentration outside the GUV due to the evaporation of water, which would decrease the volume of a GUV and therefore decreases the effects of Π. However, the control experiment of Fig. 2B indicates that the effect of water evaporation is negligible for 30 min (in this control experiment we kept GUVs in chamber A for 10 min and then in the chamber B for 20 min, and hence

we checked the water evaporation during 30 min). As described in the Materials and Methods, we made all the experiments for less than 30 min after we transferred a glucose solution and a GUV suspension to chamber A. Therefore, we consider that the water evaporation effect was negligible in our experiments.

The other possibility is due to the experimental method. In the chamber transfer method, we used a glass capillary with 1 mm diameter containing the same solution of chamber A. After a GUV was transferred into chamber B, the solution inside the capillary can be mixed inside chamber B, which could increase the osmolarity of the solution in chamber B. This would decrease the effective values of Π in the chamber transfer experiments shown in Figs. 2, 3, and 4.

S.3. Effect of Π on the GP values of Laurdan in DOPC-GUVs and DOPC-LUVs

As described in the main text, the transfer of DOPC-GUVs into a hypotonic solution resulted in Π-induced increase in the volume of the GUVs, which increased the area of the GUV membranes (i.e., the stretching of the membrane occurred). This may induce a change in fluidity of the GUV membranes, because it is recently reported that the membrane stretching due to lateral tension increases the fluidity of lipid membranes [5,6] and diffusion coefficient of lipid molecules [5-7]. To monitor such changes, we used a highly environment sensitive amphiphilic fluorophore, Laurdan, whose hydrocarbon chain incorporates into the membrane hydrophobic core while the fluorescing group locates at the region of the glycerols of the phospholipid in the membrane interface. Mechanistically, dipole-dipole interactions and reorientation of water molecules in the membrane interface due to changes in the fluidity and the hydrocarbon packing of lipid membranes cause spectral shifts in the Laurdan fluorescence that can be described by a generalized emission polarization value (GP) [8-10]. It was previously reported that the GP value of Laurdan in LUVs changes when osmotic pressure was applied [11].

S.3.1. Experimental methods

GUVs of DOPC/Laurdan mixture (molar ratio; 40:1) were prepared in water (MilliQ) containing 98.0 mM sucrose by natural swelling method of dry DOPC/Laurdan films described in Section 2.1. To obtain a purified GUV suspension, smaller vesicles and free Laurdan were removed using the membrane filtering method [12]. Firstly, the suspension was centrifuged (13000g, 20 min, 20 °C); the resulting supernatant was filtered through a Nuclepore membrane with 10-μm diameter pores (Whatman, GE Healthcare, UK, Ltd.,

Buckinghamshire, UK) in 98.0 mM sucrose for 1.0 h at a flow rate of 1.0 mL/min at room temperature (20–25 °C); the retained suspension (i.e., that which did not pass through the filter) was collected and used as the purified GUV suspension. Large Unilamellar Vesicles (LUVs) of DOPC/Laurdan mixture (molar ratio; 200:1) were prepared by the extrusion method [13,14]. An appropriate amount of phospholipid was first dissolved in chloroform containing the fluorescence probe Laurdan and the solvent was dried under N_2 gas and kept overnight under vacuum connected with a rotary pump. The lipid film was then hydrated by addition of 100.0 mM sucrose solution and extensively vortexed to form multilamellar vesicles (MLVs) of DOPC. Then the MLV suspension was subjected to freeze–thaw cycles (5 cycles) in liquid N_2 for 1.0 min, followed by warming to room temperature for 25–30 min. The resulting solution was extruded through a 200 nm-pore-size Nuclepore membrane using LF-1 LiposoFast apparatus (Avestin, Ottawa, Canada) until the solution became transparent (almost twenty times pass). To remove free Laurdan molecules in aqueous solution, the LUV suspension was passed through a Sephadex G-75 column equilibrated with 100 mM sucrose solution and the purified LUV suspension was obtained from fractions at the void volume [15].

A Hitachi F7000 spectrofluorometer (Hitachi, Tokyo, Japan) was used for fluorescence measurement. Fluorescence intensities of samples were measured at the excitation wavelength 350 nm, the emission wavelength range was 370-650 nm while both the excitation and emission band-pass were 5 nm. The temperature of the cell was held at 25 °C with a water bath circulator (Cool-Bit circulator, ACE-05AN, KELK Ltd., Tokyo, Japan). In case of LUV experiment, to induce osmotic pressure, we have performed the following method; we prepared five different solutions which have 100 mM sucrose in water as internal solution inside LUV and 90, 80, 70, 60, 50 mM sucrose in water as the external solutions. To induce osmotic pressure, in case of GUV experiments, we have taken certain amount of GUV containing solution (purified) which initially have 98.0 mM sucrose in water both as internal and external solution (osmotic pressure = 0) and then we gradually added water (MilliQ) to make the external solution hypotonic. The fluorescence intensity of the Laurdan in all these solutions were measured after certain incubation times (5 min) to attain the equilibrium. The correction of the fluorescence intensity due to the dilution was done. The GP values of Laurdan in different liposomes were calculated using the following formula: $GP = (I_{439} - I_{483}) / (I_{439} + I_{483})$ where, I_{439} and I_{483} denotes fluorescence intensities at 439 nm and 483 nm, respectively. In this context, it is important to understand that these measurements do not relate to emission polarization but polarization of the

fluorophore itself. In other words, GP does not relate to fluorescence emission polarization but instead to the electric polarization of the fluorophore due to the solvent environment. The lipid concentrations in the GUV suspensions and the LUV suspensions for fluorescence measurements were 14 μM and 180 μM, respectively, which were determined by the Bartlett method [16].

S.3.2. Effect of Π on the GP values of Laurdan in DOPC-GUVs and DOPC-LUVs

Figure S4A shows normalized fluorescence emission spectra of Laurdan in DOPC-GUVs under Π due to ΔC^0. All the spectra show a peak at 483 nm and a pronounced shoulder-like feature around 439 nm. These results revealed that an increase in ΔC^0 caused a decrease in the intensity around the 439 nm shoulder region. In a control experiment in which the GUV suspension was diluted with 98.0 mM sucrose solution, the normalized spectra did not change. Figure S4B shows that the GP values decreased from −0.156 to −0.203 with an increase in ΔC^0 from 0 to 4.0 mM. It is generally considered that with an increase in fluidity of lipid membranes the interaction of water molecules with a Laurdan molecule in the membrane interface increases, which decreases the GP value [8-10]. On the other hand, it is recently reported that the membrane stretching due to lateral tension increases the fluidity of lipid membranes [5,6] and diffusion coefficient of lipid molecules [5-7]. Therefore, the results shown in Fig. S4B suggest that the stretching of the DOPC membrane increases with ΔC^0. For comparison, we investigated the effect of Π on the GP values of Laurdan in DOPC-LUVs with a mean diameter of 200 nm. Figure S4C shows the fluorescence emission spectra of Laurdan in these DOPC-LUVs containing 100.0 mM sucrose solution under Π due to different ΔC^0, and Figure S4D shows that the GP values of Laurdan in these DOPC-LUVs decreased from −0.158 to −0.209 with an increase in ΔC^0 from 0 to 50 mM. Inducing the same decrease in GP values required ~10 times higher ΔC^0 for DOPC-LUVs compared with DOPC-GUVs. By conversion of ΔC^0 into $\sigma_{\mathrm{osm}}^{\mathrm{eq}}$ using the average values of the radius (10 μm for the GUVs and 0.10 μm for the LUVs), we obtained the dependences of the GP values on $\sigma_{\mathrm{osm}}^{\mathrm{eq}}$ for DOPC-GUVs and DOPC-LUVs (Figure S4E). The GP values at the same $\sigma_{\mathrm{osm}}^{\mathrm{eq}}$ were essentially same in DOPC-GUVs and DOPC-LUVs, and the GP values gradually decreased with an increase in $\sigma_{\mathrm{osm}}^{\mathrm{eq}}$.

Figure 3B shows that the probability of leakage, P_{Leak} (360 s), for DOPC-GUVs was almost 0 at $\Delta C^0 \leq$ 3.7 mM and increased with ΔC^0 at $\Delta C^0 \geq 4.2$ mM. This result indicates that $\sigma_{\mathrm{osm}}^{\mathrm{eq}}$ increases with ΔC^0 at $\Delta C^0 \leq 3.7$ mM, because no leakage of sucrose occurs. On the other hand, the GP value for the GUVs decreased with ΔC^0 at $\Delta C^0 \leq 4.0$ mM (Fig. S4B). These results are consistent, because the stretching of the membrane monotonously increases with $\sigma_{\mathrm{osm}}^{\mathrm{eq}}$ at $\Delta C^0 \leq 4.0$ mM, which induces the monotonous decrease in the GP values.

Figure S4

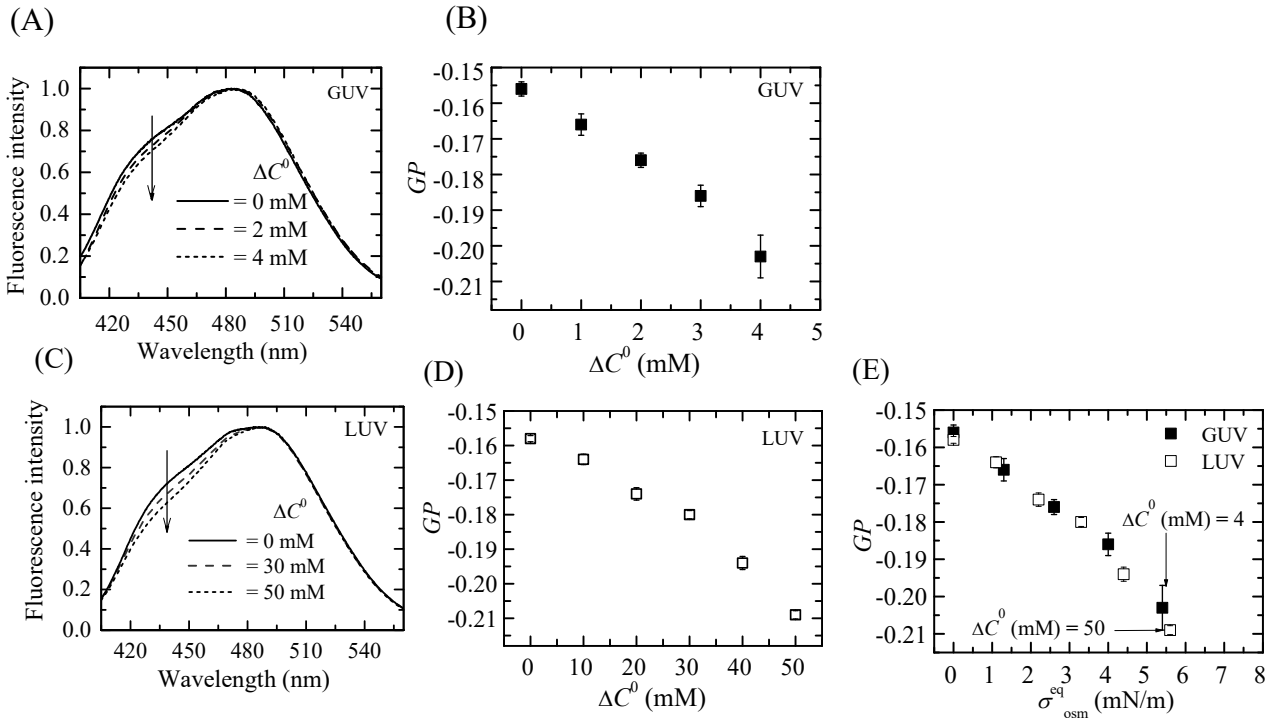

Figure S4**:** Effect of Π on GP values of Laurdan in DOPC-GUVs and DOPC-LUVs. Normalized fluorescence emission spectra of Laurdan in DOPC-GUVs (A) and DOPC-LUVs (C) in sucrose solution with different osmolarities. The GP values of Laurdan in DOPC-GUVs (B) and DOPC-LUVs (D) as a function of ΔC^0 at 25 °C. (E) Effect of $\sigma_{\mathrm{osm}}^{\mathrm{eq}}$ on the GP values of Laurdan in DOPC-LUVs (□) and GUVs (■) at 25 ˚C. (B) (D) (E) Mean GP values of three independent experiments were plotted.

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