Thermally Hydrated DPPC Langmuir Film: A Trial Application to the Analysis of Interaction of Sucrose with DPPC Liposome

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The thermally hydrated dipalmitoyl phosphatidyl choline (DPPC) Langmuir (L) film was investigated to determine whether it could be an appropriate physical model of DPPC liposomes. The interaction of sucrose with the L film and the liposome was investigated for the examination. The conventional DPPC L film without any heating process showed a film shrinkage by bound sucrose on the surface of the L film. The film shrinkage was enhanced with an increase of the concentration of sucrose. This shrinkage was found to affect all of the entire characteristics of the conventional L film. On the other hand, the hydrated L film by a heating process indicated a film expansion by the penetrated sucrose, although the incorporated water in the film avoided the approach of sucrose a little. The expansion increased with the concentration of sucrose, a fact that was contrary to the results of the conventional L film. To compare the hydrated L film with liposome, the fluorescence decay and anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH) in DPPC liposomes were measured. The results indicated that the molecular order of DPPC in the liposome decreased with the concentration of sucrose. The molecular motion of DPPC was reduced with the concentration of sucrose. These phenomena were successfully explained by the results of the hydrated L films. The hydrated L film was consequently proposed to be a much better physical model of liposomes than the conventional L film.

Introduction

Phospholipid liposome has received keen interest as a potential vector of synthesized DNA to be introduced into biological cells for the cancer gene therapy.[1,2] In order to use the DNA enveloped by liposome as a drug, a matter of the DNAs stability against drying or freeze-drying process comes up. Disaccharides, such as sucrose and trehalose, have been noticed as excellent stabilizers for those processes.[3−6] The mechanism of the stabilization has been studied in more than 10 years. The protection mechanism by disaccharide against the drying process has been mainly discussed regarding the retention and the fusion of liposomes. There have been two major hypotheses for the stabilization mechanism: (1) water replacement[3] and (2) vitrification hypotheses.[4] During the drying−rehydration or freezing−thawing processes, the membrane structure of liposome often happens to be destroyed, enabling drug release out of the liposome. If the water molecules on/in surface of the liposome could be replaced by disaccharide molecules that have effective hydroxyl groups, the drying process may not damage the membrane structure so seriously.[5] The vitrification of phospholipids and disaccharides during the freeze-drying process could also contribute to the stabilization. On the other hand, a recent research indicated that the vitrification in itself is not sufficient to preserve the freeze-dried liposomes.[7] In this way, the detail molecular mechanism of the stabilization has been still unclear. The proposed mechanisms are still within the hypotheses. In particular, to the best our knowledge, the molecular interaction has not been reported when disaccharides approach the surface of liposome in wet state (in suspension).

Kato et al. most recently reported[8] that a thermally hydrated Langmuir (spread monolayer) film of DMPA (dimirystoyl−phosphatidic acid) on a water surface was dramatically expanded by the incorporated water molecules in the Langmuir (L) film. The thermal treatment was typically done by raising the temperature once, and the heated L film was cooled down again to the original temperature. Water molecules proved to incorporate in the L film rigidly, during this simple thermal treatment, by the quartz crystal microbalance (QCM) technique.[9] This phenomenon is also the truth of the DPPC L films.

Thus far, the L film without the thermal treatment has been used as a model of liposome to investigate the interaction of liposome with drug[10] or protein,[11] although the problem of the curvature of membrane still remains.[12,13] However, liposome is always prepared by way of heating above the main transition temperature.[3,5,6] The new hydrated L film is then considered to be much more suitable as a physical model of liposome. In this study, the DPPC thermally hydrated L film was investigated to indicate that the hydrated L film is fit for a model of DPPC liposomes. From the results of the interaction of sucrose and the DPPC L film, it was found that there was a big physical difference between the conventional L films and the hydrated L films. Further, the corresponding interaction between DPPC liposomes and sucrose was also studied by fluorescence decay measurements to prove that the liposome has physically similar characteristics to those of the hydrated L film. Our results could eventually indicate that the sucrose stabilized the membrane structure in the liposome even in the wet state.

Experimental Section

(A) Materials. L-α-Dipalmitoylphosphatidylcholine (>99%) was purchased from Sigma Chemical Co. and was used without further purification after drying in a vacuumed ($\sim 10^{-3}$ Torr) desiccater over night. The aqueous solution for both L film and liposome experiments was modified to be pH 7.4 by tris-(hydroxymethyl)aminomethane (Tris), ethylene diaminetetra-acetic acid (EDTA), and KCl. Tris and KCl were guaranteed grade reagents and purchased from Nacalai Tesque, Inc. EDTA was also a guaranteed reagent and obtained from Wako Pure Chemical Industries, Ltd. The pH was checked by a Horiba

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B-112 pH meter. DPH (1,6-diphenyl-1,3,5-hexatriene) as a fluorescence probe was obtained from Nacalai Tesque, Inc. specially prepared for liquid scintillation measurements. The pure water was obtained from a Millipore Co. Model Milli-Q Laboratory water purifier (>18 MΩ/cm) after distillation by a Yamato Scientific Co. Ltd. Model WG-25 autodistiller equipped with ion exchange resin.

(B) Measurements. Surface pressure (π)−molecular area (A) isotherm of DPPC L film was obtained by Kyowa Interface Science Co. Ltd. Model HBM LB film apparatus. The output signals from its preamplifier was further amplified by a Dataforth Corporation Model SCM5B30−03 DC-amplifier modules attached to a Micro Science Co., Ltd. Model SCM-6798BPC board with a Model UBX-01CB-AC DC-source supplier. The analog output signals from the amplifiers were converted to digital signals by a Nippon Filcon Model JJ. JOKER J-1 A/D converter with a resolution at 12 bit. The digital signals were collected by an IBM ThinkPad 220 personal computer. A driver program for collecting, displaying, and saving data was coded by the first author in Quick BASIC language. The compression speed of L films was fixed at 2.4 Å^2 molecule^{-1} min^{-1}. The DPPC L film was prepared by spreading DPPC/chloroform solution at a concentration of 1 mg/mL on aqueous solutions. The temperature of aqueous solution in the trough was controlled by a Neslab Instruments, Inc. Model RTE-110 water circulator.

All of the fluorescence-decay measurements were carried out on a Horiba Model NAES-500 single-photon-counting fluorescence lifetime measurement apparatus at 25 °C. The H_2 arc lamp was used as a pulse-light source. The detail optical system will be mentioned later. The wavelength of incident light was determined as 380.6 nm by the results of a steady-state fluorescence measurement for the corresponding DPPC/DPH liposome suspension measured at 25 °C on a Shimadzu Model RF-5000 spectrofluorophotometer. The two low-pass filters were HOYA L-38 and L-39 glass filters that effectively cut wavelength below 380 and 390 nm, respectively. The depolarizer, Model DEQ-2S, was purchased from Sigma Koki Co., Ltd. For the measurements of the decay function of the incidental light, the scattered light from a diluted colloidal suspension of silica in water (LUDOX solution made by DuPont) was collected. The turbidity of the diluted LUDOX solution was checked in advance by a Beckman DU-600 UV−vis spectrophotometer. The observed turbidity against λ^{-4} (λ: wavelength) showed a fairly good linearity in our wavelength region.

(C) Preparation of DPPC Liposome. DPPC was dissolved by chloroform with DPH. The molecular ratio of DPPC and DPH was 800:1. A very thin homogeneous film without any crystals was obtained in a round-bottom 20 mL glass flask by an evaporator to evaporate the solvent. In order to fully remove the solvent from the thin film, the flask was placed in a vacuumed desiccater over night. The film was then hydrated under nitrogen by a 15 mL of 10 mM Tris-buffer solution by a vortex mixer at 50 °C or higher until the suspension came to seem pale in color. This color indicates that the diameter of the suspended particulates became comparable with the wavelength of the near-ultraviolet light. Then the suspension was filtered through polycarbonate filter, with pores of 0.4, 0.2, and 0.1 μm five times each at about 50 °C. The polycarbonate filters were made by Millipore Co., Ltd. Each filtered suspension was measured by an Otsuka Electronics Model DLS-7000 Super Dynamic light-scattering spectrophotometer with Marquadt method to determine the size distribution of our liposomes by courtesy of Otsuka Electronics Co., Ltd. After the preparation of DPPC/DPH liposome, sucrose was added in the liposome suspension.

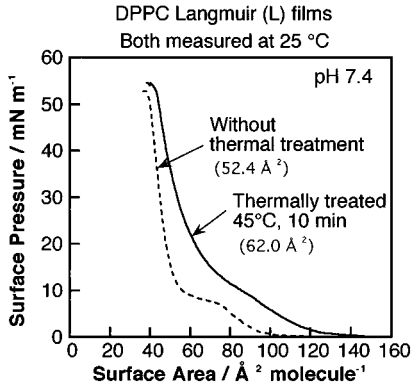

Figure 1. $\pi-A$ isotherms of the conventional DPPC L film without any thermal treatment (broken line) and DPPC L film with thermal treatment (solid line). The figures in the parenthesis indicate the limited surface areas.

Results and Discussion

(A) DPPC Langmuir Films and Sucrose. A π−A isotherm of the thermally hydrated DPPC Langmuir film was measured to check the film-expansion effect.[8] To prepare the hydrated L film, 70 μL of the DPPC solution in chloroform of 1 mg mL^{-1} was dropped on a subphase solution (pH 7.4) at 25 °C, and it was left for 10 min to evaporate the solvent. This temperature is below the main-transition temperature (circa 41 °C) of DPPC.[14] The L film was then heated up to 45 °C by heating the subphase solution. The temperature was maintained for 10 min, and it was cooled down to 25 °C again. The heating and cooling speed was about 1 and 0.5 °C min^{-1}, respectively. The thermally treated film was then compressed from a surface area of 150 Å^2 molecule^{-1} at a speed of 2.4 Å^2 molecule^{-1} min^{-1} to obtain a π−A isotherm. The isotherm is drawn in Figure 1 by a solid line. The limited area at the surface pressure of 0 mN m^{-1} was determined to be 62.0 Å^2 molecule^{-1}. For reference, an isotherm for the conventional DPPC L film without the thermal treatment was also measured (the broken line in Figure 1). The limited area of this L film was obtained to be 52.4 Å^2 molecule. The film expansion effect due to the incorporated water molecules[8] was thus clearly recognized through our experiment, too. The incorporated water in the DPPC L film was estimated to have an architecture similar to that of the boundary water at the interface between the film and the subphase solution. According to Teissié et al.,[15,16] the interfacial water molecules that are in close contact with the lipid polar head groups are oriented and have an enhanced order parameter. This may indicate that the boundary water molecules have a much stable structure compared with that of bulk water. We then considered two experimental schemes as shown in Figure 2 to investigate the boundary water effect on the interaction between the film and sucrose. In the upper scheme, sucrose was added in the subphase after the thermally hydrated DPPC L film had been prepared. The L film without the thermal treatment or sucrose was named as (A), and the sucrose-added L film with the thermal treatment was named as (A*). Since sucrose was added after the stable boundary water had been constructed in the L film, the interaction between the L film and sucrose was expected to be weakened. The bottom scheme was, on the other hand, considered for reference of the upper scheme. In this case, sucrose had been solved in the subphase solution before the DPPC L film was prepared. The

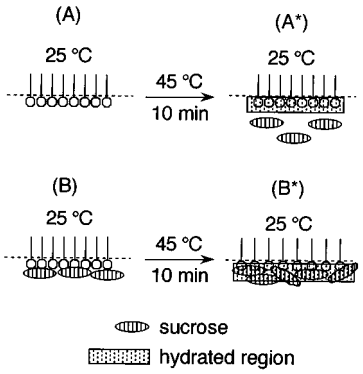

Figure 2. The scheme of our experiments. Open circles and the short solid straight lines indicate hydrophilic and hydrophobic part of DPPC, respectively. The ellipsoids with zebra indicate sucrose molecules. The shaded portion means boundary water region.

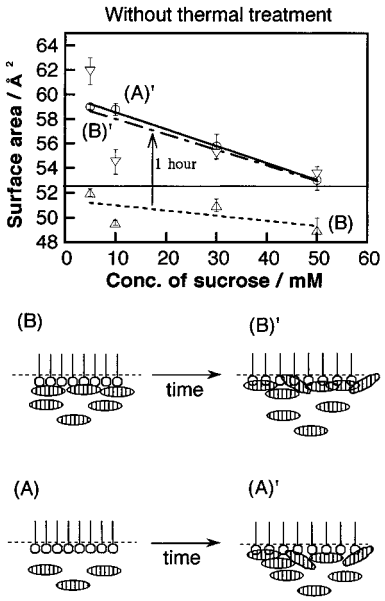

Figure 3. Measured limited surface areas of DPPC L films without the thermal treatment. Detail for the symbols is mentioned in the text. Schematic models are drawn at right hand. The ellipsoids indicate sucrose molecules.

DPPC molecules on the subphase surface would immediately interact with sucrose. The L film after attaining an equilibrium of the interaction was named as (B), and the thermally treated L film of (B) was named as (B*).

To investigate the effect of bound water or sucrose molecules in the L film, the limited-area dependence of type (B) was investigated first on the concentration of sucrose in the subphase solution. For the interaction of the L film and sucrose, no effect was noticed after 10 min at 25 °C, and the limited areas were then obtained by the following compression. The results are summarized and shown by triangle symbols in Figure 3. The straight line at the surface area of 52.4 Å^2 molecule^{-1} in the figure indicates the location of the limited area of the most simple DPPC L film without thermal treatment or sucrose (corresponding to the broken line in Figure 1). All the values of the results are plotted below the straight line. At the same time, the limited area gradually decreases with an increasing of the concentration of sucrose. From these results, we considered that the L film bound with sucrose immediately after the film preparation, and the film shrank because of the strong interaction between the film and sucrose. The calcium salt of DMPA was reported to shrink compared to its interaction with the normal DMPA L film without divalent cation.[17] In this case, the strong interaction of DMPA and Ca^{2+} must have been dominated by the electrostatic interactive force, since the terminal group of DMPA is negatively charged. In our case, on the other hand, the interaction of DPPC L film and sucrose must be much weaker than that of the DMPA and Ca^{2+}, since either sucrose and DPPC have no net charge in their molecules. In place of the electrostatic interactive force, the hydrogen-bonding interactive force could[18] play an important roll for the interaction of DPPC L film and sucrose. The decrease of the limited area with an increase in the concentration of sucrose may be thus explained. However, it is not natural that sucrose binds with only the surface of the L film. The interactive force must have sucrose molecules penetrating more deeply in the L film. To examine this consideration, each L film was left on the subphase containing sucrose for 60 min. The film prepared like this was named (B)′ in Figure 3. Here, prime (′) indicates a state after a long time. Although (B)′ itself is in an expanded state resulting from the penetration of sucrose, it arose from (B) that had been in a condensed state. The results are shown by the reversed triangle symbols in Figure 3. All of the values are above the straight line, indicating that the L film was expanded by the penetrating sucrose. At a low concentration of sucrose, the film expansion is very large, and the limited area goes up to about 59.0 Å^2 molecule^{-1}. On the other hand, at a high concentration of sucrose, the expansion was little. In the previous discussion about the L film of (B), the interaction of the L film and sucrose is strong to make the film rigid at a high concentration of sucrose. Once the L film becomes rigid, the mobility of sucrose molecules into the L film must be reduced. The observed results for (B)′ are quite consistent with this discussion. To examine the results of (B)′ again, another experiment was taken place. This time, the DPPC L film was prepared on subphase solution without sucrose first (identical to (A)). Sucrose was then solved in the subphase solution successively, and 60 min was required to make the L film attain an equilibrium of the interaction of the L film and sucrose. This L film was named as (A)′ (Figure 3). Then, the limited area was measured by compressing these L films. The results are shown by open circles in Figure 3. The fitted straight line for these data is quite similar to that of (B)′. This proved that the measurements for (B)′ were reliable for our discussion, although the deviation is considerably large. From these results and discussion, it was concluded that the conventional DPPC L film without the thermal treatment was once made rigid by the bound sucrose near the surface of the L film, and it was then penetrated by sucrose molecules successively.

Next, the interaction of the new thermally treated DPPC L film and sucrose was investigated like the previous way. In the beginning, DPPC L film was prepared on a subphase solution including sucrose (identical to type (B)). This film was then thermally treated in the same way to have the L film hydrated. This treatment is schematically shown as the process from (B) to (B*) in Figure 4. The limited areas for the hydrated films of (B*) on the concentration of sucrose are shown in Figure 4 by reversed triangles. Before the discussion of the results, we should mention the detail feature of the isotherms. The isotherms of the thermally treated L film showed specific curve around the condensed-state region. The condensed part comprised two steps, while the ordinary L film gives one linear part. The lower-pressure part of the two steps is very short (not shown) with moderate slope. After passing this region, the main condensed part was turned out. Although the lower-pressure part was always seen for the hydrated L films, the length of this part did not have good reproducibility. This two-step phenomenon may indicate the exclusion process of sucrose from the L film during the compression. However, we only

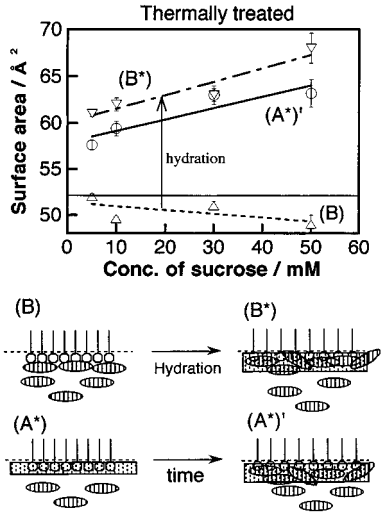

Figure 4. Measured limited surface areas of thermally hydrated DPPC L films. Detail for the symbols is mentioned in the text. Schematic models are drawn at right hand. The ellipsoids indicate sucrose molecules. The shaded portion means boundary water region.

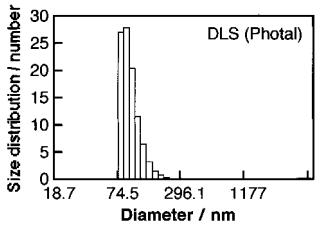

Figure 5. Size distribution of our DPPC liposomes measured by DLS method.

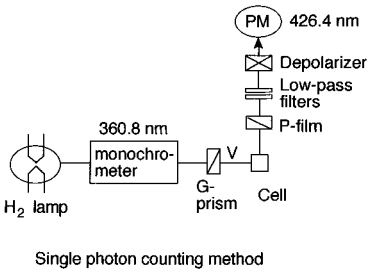

Figure 6. Schematic diagram of the single-photon-counting method for the fluorescence decay measurements. G-prism and P-prism refer to Gran and Polaroid prisms, respectively, that are polarizers.

discuss the limited area obtained from the main condensed part, since the quantitative discussion of the lower part seems difficult. To the contrary of the results for the conventional L film (Figure 3), in Figure 4, the limited surface area gradually increases with an increase of the concentration of sucrose. These results could be explained by the film expansion caused by the penetration of sucrose and water molecules into the L film during the heating process. Even if the L film was once made rigid by the binding sucrose on the surface of the film ((B) in Figure 4), the heating process would make the L film soft[14] and would provide a way to the sucrose and water molecules to be mobile in the L film. With this mechanism, it must be natural that the film expansion is getting larger at a high concentration of sucrose. On the other hand, the next experiment will give more interesting results. This time, the hydrated L film of type (A*) was prepared in advance. This film, at this stage, does not have sucrose molecules in or on the film surface. To this hydrated L film, sucrose was added in the subphase solution. After attaining an equilibrium, the limited surface area was measured. These L films refer to type (A*)′ in Figure 4. The results are summarized in Figure 4 by open circles. The general tendency against the concentration of sucrose is very similar to that for (B*). Their values of (A*)′ are, however, always below those for (B*). In the surface of the L film of (A*), water molecules are incorporated by the thermal treatment, enlarging the room among the DPPC molecules. This should prevent the L films from forming domain structure.[17] In other words, DPPC molecules may disperse in the L film. It is easily understood that sucrose would be incorporated spontaneously in this expanded L film by, for example, the hydrogen-bonding force. The results of (A*)′ show, however, that the interaction between the L films and sucrose is a little avoided, compared with the results of (B*). As a possible reason, it was considered that the stable boundary water layer, at around the polar region in the L film, may have prevented the sucrose molecules from freely approaching the L films.

From the entire view over Figures 3 and 4, it was found that the hydrated DPPC L film had quite different physical characteristics from that for the conventional L film without the thermal treatment. This difference is symbolized by the fact that the slope of the results in Figure 3 is negative, whereas that is positive in Figure 4. In this way, it was proposed that the thermal hydration might be an essential factor to carry out experiments with an L film as a model of a liposome, since liposome is always made through the thermal-hydration process.

(B) DPPC Liposomes and Sucrose. All of the above discussion was made for L film, not for liposome. The correspondence between the L film and liposome should be now investigated to examine whether the L film could be used as an appropriate model for liposome. We then studied the interaction between the DPPC liposome and sucrose by fluorescence decay-time measurements at the same concentrations in the previous discussion.

The preparation of DPPC liposomes in our study was mentioned in detail in the Experimental Section. The prepared liposomes were checked by the dynamic light-scattering (DLS) method to investigate how large they were. A result of the a DLS measurement after a liposome suspension was five times filtered through a polycarbonate filter with 100 nm pores is shown in Figure 5. It shows that our liposome has a single dispersion of number-of-liposome against the diameter, having maximum at about 87 nm. Dust and small fragments of liposome were not detected. The liposome suspensions through the filters with 200 nm or larger pores did not show the single-dispersion. The average diameter and dispersion of our prepared liposomes depended on the diameter of pores of filters. Miyajima et al. once applied the negative stain electron micrograph technique to their egg yolk-L-α-phosphatidylcholine (egg-PC) liposomes of several kinds of diameter. They pointed out that their liposomes with 100 nm or larger diameter had considerable number of oligolamellar or multilamellar liposomes. Since there is a difference in component of phospholipid between their liposomes (egg-PC) and ours (DPPC), a direct comparison between them could not be appropriate. Judging from the results of our DLS measurements, the liposome of DPPC prepared by our extrusion method[5] with a filter with 100 nm pores was recognized to be a fine sample for the fluorescence decay measurements. After the repeated 10 times measurements of fluorescence decay, our DPPC liposomes showed very stable results as 11.4 ns at 25 °C. From this stability, we finally appreciated our liposomes fine enough for our discussion.

In the fluorescence decay measurements, the vertically polarized UV light at 360.8 nm was used for the excitation of DPH in the liposomes (Figure 6). The polarized components of the emission from the liposome suspension were individually

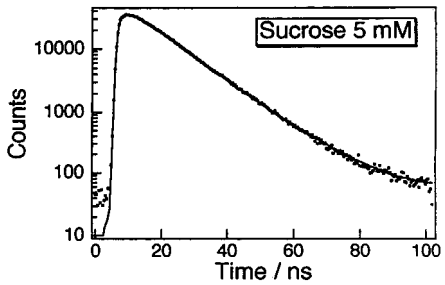

Figure 7. Fluorescence decay curve for the observation (dots) of DPH in the DPPC liposomes with sucrose at the concentration of 5 mM. The solid line is the deconvoluted curve.

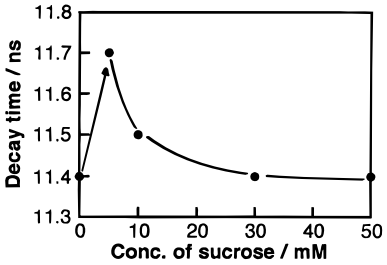

Figure 8. Fluorescence decay time of DPH in DPPC liposomes at various concentrations of sucrose.

measured by a photomultichannel analyzer through a polarizer made of Polaroid film. From now on, each observed vertically and horizontally polarized emission will be written as I_{VV} and I_{VH}, respectively. The total emission, S, defined as

$$S(t) = I_{VV}(t) + 2I_{VH}(t) \quad (1)$$

was analyzed by the deconvolution method[18] to obtain the fluorescence decay time. Since we placed a depolarizer between the polarizer and the detector, the polarization correction was not necessary. For the deconvolution analysis, a single-exponential equation, $s(t)$, was employed for the net decay curve of DPH, and another similar equation, $s_d(t)$, was employed for the scattered light.

$$S(t) = \alpha \exp(-t/\tau) \quad (2)$$

$$S_d(t) = \alpha_d \exp(-t/\tau_d) \quad (3)$$

Here, τ is the decay time. Although two low-pass filters were doubly placed behind the polarizer (Figure 6), the leak light (360.8 nm) emerged from the scattering by the liposome suspension was taken into account by the second equation in case. With the apparatus function $P(t)$, the total observed intensity I_T (eq 4) was compared with $S(t)$ until the difference converged.

$$I_T = \int_0^t P(t-x)[s(x) + s_d(t)]dx \quad (4)$$

An example of the analyzed data is shown in Figure 7. This figure is for DPPC liposome with sucrose at a concentration of 5 mM marked by DPH. The dots are the observed results, and the solid line is the deconvoluted line. They agree fairly well with each other. The same kinds of measurements at the concentrations of 0, 10, 30, and 50 mM were also carried out. The chosen concentrations are corresponding to those in the previous section for the L film. The results are summarized in Figure 8. At the concentration of 5 mM, the decay-time once jumped up to 11.7 ns. Above this concentration, the decay time gradually decreased monotonously with an increase of the concentration of sucrose. The decay time has got to do with the degree of organization of the membrane molecules. For example, the decay time decreases drastically at the phase transition temperature in elevating the temperature.[14] Figure 8 indicates that sucrose once adsorbs on the surface of liposomes to make the liposome a little rigid at a low concentration of sucrose. At a high concentration, however, sucrose deeply binds in the surface of the liposome, leading the molecular order lower. This speculation is schematically drawn in Figure 9. The second step in this figure is supported by an assumption that the number of bound sucrose molecules in the liposome surface increases with the concentration of sucrose. This assumption is consistent with the results of the thermally hydrated L film (Figure 4). Then, Figure 8 indicates that the hydrated L film can be an appropriate model for liposome, while the conventional L film without the thermal treatment is not suitable for the model (Figure 3).

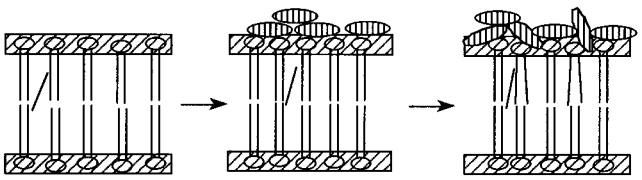

Figure 9. Schematic drawings of DPPC liposome with the concentration of sucrose. The ellipsoids indicate sucrose molecules. The shaded portion means boundary water region.

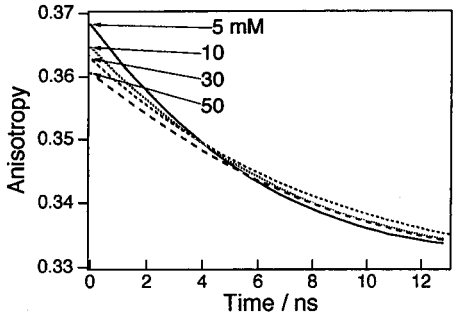

Figure 10. Time dependence of anisotropy of DPH in DPPC liposomes. The figures by the curves indicate the concentration of sucrose.

In order to investigate the change of rigidity of the liposomes on the concentration of sucrose, the time-dependent anisotropy was measured for DPH in the liposomes. To get the anisotropy $r(t)$, the following equations were employed.

$$D(t) = I_{VV}(t) - I_{VH}(t) \quad (5)$$

$$r(t) = D(t)/S(t) \quad (6)$$

$$I_D = \int_0^t P(t-x)[r(x)\, s(x) + s_d(t)]dx \quad (7)$$

With the apparatus function $P(t)$, the differential observed intensity I_D (Eq. 7) was compared with $D(t)$ until the difference converged. The obtained anisotropy is shown in Figure 10. The anisotropy at $t = \infty$ gradually increases with the concentration of sucrose. This may be regarding the viscosity of the liposome suspension or that the liposome is getting rigid due to the adsorbed sucrose.[19,20] At any rate, the change is a little too small to give a further discussion of the rigidity of the liposome. Then, the wobble angle of DPH in the liposome was calculated with the following equation.[21,22]

$$r(\infty)/r(0) = [{}^1/_2\cos\theta_0\,(1 + \cos\theta_0)]^2 \quad (8)$$

Here, $r(0)$ and $r(\infty)$ are the anisotropy at $t = 0$ and a long time later, respectively. θ_0 is the wobble angle of the long axis of

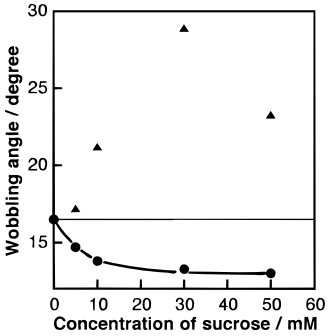

Figure 11. Wobble angle of the long axis of DPH molecule in DPPC liposomes as a function of the concentration of sucrose. The results for the liposomes containing sucrose in only the outer-water phase are shown by solid circles, and the results for the liposomes containing sucrose in both the inner- and outer-water phases are shown by solid triangles.

a DPH molecule. The anisotropy depends on both anisotropies at $t = 0$ and $t = \infty$. The calculated wobble angles against the concentration of sucrose are shown by solid circles in Figure 11. It is found that the wobble angle monotonously decreases with the concentration. This shows that the mobility of the probe molecule (DPH) in the liposomes was reduced. The decrease of the wobble angle on the concentration of sucrose was understood as a result by the overlap of the two effects: the disordering of DPPC molecules (Figure 8) and the kinetic stability of the DPPC molecules in liposome. The outer layer of the bilayer in liposome could not be so expanded compared with the L film, since the expansion of the outer layer is limited by the interaction with the inner layer that is free from sucrose. In this condition, the increase of the disordering (*gauche* conformation is ever increasing in DPPC) due to the penetration of sucrose molecules should make the room for the motion of DPH smaller. The decrease of the corelation time of the time-dependent anisotropy (Figure 10) may be also regarding these overlapped effects. To support the above discussion, the assumption that the outer layer is stabilized by the inner layer should be ascertained. We then prepare another type of liposome containing sucrose in the inner water phase as well as in water outside the liposome. To the dried thin film layer of DPPC/DPH on the inside wall of a round flask, aqueous "sucrose solution" (pH 7.4 by Tris) was added. This flask was vortexed at above 50 °C, and the suspension was subjected to the extrusion method to give the liposome containing sucrose in the inner-water phase. The wobbling angle of DPH in this liposome was also analyzed by the same way for the previous results. The wobbling angles are shown in Figure 11 by solid triangles. All of the results are above the angle for the liposome without sucrose (indicated by a straight line in Figure 11). It was inevitable to have unstable results for this type of liposome, since there were sucrose molecules in the suspension during the vortex or extrusion process at above the transition temperature. The reproducibility of the amount of the penetrated sucrose molecules was then inevitably bad. At least, however, we found that the wobbling motion increases when the inner layer was also expanded by the penetration of sucrose. These results support the outer-layer was stabilized by the rigid inner layer when the sucrose was contained only in outside water of liposome.

Conclusion

From our results and discussion, there are big differences between the conventional L film and the new thermally hydrated L film against the concentration of sucrose. The interaction of sucrose with the hydrated L film could explain the molecular penetration of sucrose in the surface of DPPC liposome, while the conventional L film was not fit for the analysis of liposomes. This result suggested that the thermally hydrated DPPC L film could be a much more appropriate physical model for liposomes than the conventional L film. With the hydrated model, furthermore, we monitored the interaction of sucrose with DPPC liposomes in aqueous suspension. The DPPC liposome was made a little rigid by the adsorption of sucrose at the first stage, and the molecular structure of DPPC was disordered by the penetration of sucrose at the second stage. Since the outer layer of the bilayer in liposome is stabilized by the interaction with the inner layer, the direct comparison of liposome and L film is not appropriate. However, through the comparison of the results by L film and liposome, a detail mechanism of the interaction of sucrose with DPPC liposome in aqueous suspension was clarified.

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References and Notes

(1) Sawa, Y.; Suzuki, K.; Bai, H. Z.; Shirakawa, R.; Morishita, R.; Kaneda, Y.; Matsuda, H. *Circulation* **1995**, *92*, 11479.

(2) Lee, R. J.; Huang, L. *J. Biol. Chem.* **1996**, *271*, 8481.

(3) Crowe, J. H.; Crowe, L. M.; Carpenter, J. F.; Rudolph, A. S. *Biochim. Biophys. Acta* **1988**, *947*, 367.

(4) Green, J. L.; Angell, C. A. *J. Phys. Chem.* **1989**, *93*, 2880.

(5) Tanaka, K.; Takeda, T.; Fujii, K.; Miyajima, K. *Chem. Pharm. Bull.* **1991**, *40*, 1.

(6) Viera, L. I.; Alonso-Romanowski, S.; Borovyagin, M. R.; Feliz, M. R.; Disavo, E. A. *Biochim. Biophys. Acta* **1993**, *1145*, 157.

(7) Crowe, J. H.; Leslie, S. B.; Crowe, L. M. *Cryobiology* **1994**, *31*, 355.

(8) Kato, T.; Shimizu, Y.; Kameyama, M.; Kimura, Y.; Okahata, Y. *Thin Solid Films* **1996**, *284/5*, 32.

(9) Okahata, Y.; Ariga, K. *Thin Solid Films* **1989**, *178*, 465.

(10) Hasegawa, T.; Ushiroda, Y.; Kawaguchi, M.; Kitazawa, Y.; Nishiyama, M.; Hiraoka, A.; Nishijo, J. *Langmuir* **1996**, *12*, 1566.

(11) Nakagaki, M.; Okamura, E.; Kubota, S. *Bull. Chem. Soc. Jpn.* **1983**, *56*, 3730.

(12) Gruner, S. M.; Shyamsunder, E. *Ann. N. Y. Acad. Sci.* **1991**, *625*, 685.

(13) Bramhall, J. *Biochemistry* **1986**, *25*, 3479.

(14) Barrow, D. A.; Lentz, B. R. *Biophys. J.* **1985**, *48*, 221.

(15) Tocanne, J-F.; Teissié, J. *Biochim. Biophys. Acta* **1990**, *1031*, 111.

(16) Gabriel, B.; Prats, M.; Teissié, J. *Biochim. Biophys. Acta* **1994**, *1186*, 172.

(17) Lösche, M.; Möwald, H. *J. Colloid Interface Sci.* **1989**, *131*, 56.

(18) Donnamaria, M. C.; Howardo, E. I.; Grigera, J. R. *J. Chem. Soc., Faraday, Trans.* **1994**, *90*, 2731.

(19) Kinosita Jr., K.; Kataoka, R.; Kimura, Y.; Gotoh, O.; Ikegami, A. *Biochemistry* **1981**, *20*, 4270.

(20) Kawato, S.; Kinosita, K., Jr.; Ikegami, A. *Biochemistry* **1977**, *16*, 289.

(21) Iwamoto, K.; Sunamoto, J. *J. Biochemistry* **1982**, *91*, 975.

(22) Sunamoto, J.; Iwamoto, K.; Kondo, H.; Shinkai, S. *J. Biochemistry* **1980**, *88*, 1291.