Binding Mechanism of Sucrose to Dipalmitoylphosphatidylcholine Langmuir Films by Fourier Transform Infrared Reflection−Absorption Spectroscopy and Quartz-Crystal Microbalance Technique

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Quartz-crystal microbalance (QCM) and Fourier transform infrared (FTIR) reflection−absorption (RA) techniques are employed to investigate the binding mechanism of fully hydrated (thermally treated) DPPC Langmuir (L) films with sucrose in the aqueous solution. Sucrose molecules incorporated in the fully hydrated L film strongly bind to the L film even when the L film is laterally compressed to a high surface pressure, while those in partially hydrated (without thermal treatment) L films are squeezed out by the compression. When the L film is compressed to a low surface pressure corresponding to the liquid-crystalline (soft) state, the incorporated sucrose molecules remain well in both fully and partially hydrated L films. The amount of bound sucrose in the fully hydrated soft L film linearly increases with the concentration of sucrose. This indicates that the fully hydrated soft DPPC L film forms a mingled layer where a hydrated DPPC film and deeply incorporated sucrose molecules are inseparable from each other. In other words, incorporated sucrose molecules in the surface of the fully hydrated soft DPPC L films are very stable.

Introduction

Disaccharide molecules are biologically fundamental compounds that are commonly found in living cells. Disaccharide in the biological system is known to be an effective cryo- and dehydration-protective agent for living cells.[1−15] For the maintenance of biological membranes and phospholipid bilayers, water is essentially indispensable. However, in the last few decades, certain disaccharides including simple sucrose have been recognized as appropriate substitutes of water. In the living creatures, for example, a desert resurrection plant is known to have a large amount of sugar or its derivatives.[4] This is the truth of polysaccharides on cells.[16,17] The surviving mechanism under a dried or frozen state has been described by two major hypotheses: water-replacement and vitrification mechanisms.[1−15] A large amount of physicochemical evidence has been accumulated to support these mechanisms.

Crowe et al. investigated the protein stability in phospholipid vesicles with an aid of sugars by infrared spectroscopy. They indicated that a direct molecular interaction between sugar and vesicles completely blocked a wavenumber shift of the amide I band (1660 cm^{-1}) during a drying process.[4] Miyajima et al. investigated the stability of eggPC (phosphatidylcholine) liposomes protected by trehalose as a function of their diameter during a freeze−thaw stress.[7] They investigated the retaining ability of liposomes in terms of their structural rigidity. These studies are related to the water-replacement and vitrification mechanisms. However, it is not clear which mechanism predominantly protects the liposomes against the drying or freezing stress. Crowe et al. questioned whether the vitrification mechanism is really effective for the cryo-protection.[14]

Viera et al., on the other hand, investigated the osmotic volume change in DPPC-trehalose liposomes by fluorescent-decay measurement technique.[18] They indicated that the incorporation of sugar in liposome membranes was an essential factor for stabilization of dehydrated liposomes. This study is also related to the two hypotheses in principle. However, it should be noted that the incorporation process takes place in an aqueous solution before the dehydration.

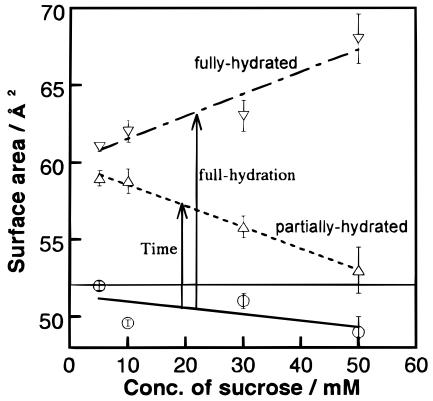

Figure 1. A comparison of film expansion effects by incorporation of sucrose in fully hydrated (▽) and partially hydrated (△) DPPC L films after an adequate equilibrium.[19] The open circles show the results just after the spread of L films, indicating that the L films shrink through a contact with sucrose solution before the equilibrium. The straight line at 52.3 $Å^2$ means the standard molecular cross-section area of DPPC L film spread on pure water at 25 °C.

In the line of these studies, we previously reported[19] the availability of thermally fully hydrated[19,20] Langmuir (L) films (spread monolayers on the aqueous solution) for the investigation of molecular interaction between the phospholipid membrane and sucrose. The fully hydrated DPPC L films were prepared by a heating process via a temperature beyond the transition temperature (41 °C)[7] of DPPC L film. A surface pressure−surface area (π−A) isotherm of a fully hydrated DPPC L film indicated an expansion due to incorporated water molecules in the L film (Figure 1). It was shown that the fully

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hydrated L film was increasingly expanded with the concentration of sucrose, while the partially hydrated L film (see a note below ref 19) was decreasingly expanded even after an adequate equilibrium. The decreasing expansion was considered as a result of the film shrinkage before the equilibrium (shown by open circles in Figure 1).

With the fully hydrated L film, we indicated that the incorporation mechanism of sucrose in the fully hydrated DPPC L film was similar to that in DPPC liposomes by using fluorescent decay analysis. We concluded in the study that the thermally fully hydrated L film was an appropriate physical model for the investigation of liposomes.

In the present study, a finer mechanism of incorporation of sucrose is investigated by a combined technique of quartz-crystal microbalance (QCM)[21,22] and Fourier transform infrared (FTIR) reflection−absorption (RA)[23] spectroscopy. The Langmuir−Blodgett (LB) technique[24] is also employed to perform QCM and RA measurements. QCM is a powerful technique to quantitatively measure the mass of ultrathin films on a nanogram scale. Since QCM and RA techniques have almost the same measurement surface made of a flat gold layer, this combined technique is convenient to study the thin films from different angles.

We investigate in detail an appropriate buffer condition for the main purpose of the present study in advance. Then, a mechanism of molecular interaction between sucrose and thermally fully hydrated DPPC L films is described.

Experimental Section

L-α-Dipalmitoylphosphatidylcholine (DPPC, >99%) was purchased from Sigma Chemical Co., and used without further purification after drying in a vacuumed ($\sim 10^{-3}$ Torr) desiccator overnight. After the drying, DPPC was dissolved in chloroform at a concentration of 1 mg mL^{-1}, and stored in a glass bin under the atmosphere of saturated vapor of chloroform at room temperature to maintain the concentration.

Acyl-deuterated DPPC (DPPC-d_{62}, >99%) was delivered by Avanti Polar Lipids in an ample tube at a concentration of 10 mg mL^{-1} in chloroform. DPPC-d_{62} consists of two deuterated acyl chains with a nondeuterated headgroup. The original solution was diluted by chloroform to 1 mg mL^{-1}. In RA study, DPPC-d_{62} was used in place of DPPC, since DPPC gives C−H stretching vibration bands in the same region of the bands derived from sucrose.

Chloroform, ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid disodium salt (EDTA·2Na), and sucrose were purchased from Wako Chemical Industries Ltd. Chloroform was spectra-grade, and other reagents were guaranteed reagents. Tris(hydroxymethyl)aminomethane (Tris, $NH_2C(CH_2OH)_3$) and potassium chloride were guaranteed reagents provided by Nacalai Tesque, Inc. The buffer solution was prepared by Tris, HCl, EDTA, and KCl with a molar ratio of 100:83.3:1:500. In the present paper, we describe a buffer of 2 mM Tris with the above molar ratio as "2 mM Tris/HCl". With this molar ratio, all buffer solutions gave pH 7.4 ± 0.1. The pH was measured by a Horiba Model B-112 pH meter.

Water used in our experiments was made by a Millipore Milli-Q Laboratory water purifier after distillation by a Yamato Scientific Model WG-25 autodistiller equipped with an ion-exchange resin. The water circulation line in the water purifier was cleaned by 3% H_2O_2 to remove organic contaminants in advance. The electric resistance of the finally obtained water was above 18.2 MΩ cm.

Surface pressure−surface area ($\pi-A$) isotherm measurements and LB film depositions were performed by a Kyowa Interface Science Model HBM LB film apparatus. Data acquisition from this apparatus was carried out with a handmade digital data collector described in the previous paper.[19] Gold-evaporated glass slides as substrates of LB films for FTIR RA measurements were purchased from Sinyo Co. The substrate comprises gold and chromium layers evaporated on a glass plate. The chromium layer was sandwiched between the gold layer and the glass plate to stabilize the preparation of the gold layer by evaporation. The layer thickness of gold and chromium was respectively 3000 and 1500 Å, and their preparations were made on only one side of the glass. Since the thickness of the evaporated gold layer was sufficiently large, the top surface of the gold layer was considered to have the flat morphology that is appropriate for RA measurements. The surface morphology was confirmed by an atomic-force microscopic (AFM) measurement[25] to be sufficiently flat. Surface cleaning of substrates was performed by sonication for 5 min each in pure-water, ethanol, acetone, chloroform, and dichloromethane, successively.

The LB transfer of an L film onto a gold-evaporated glass slide was carried out at a dipping speed of 0.5 cm min^{-1}. The transfer ratio was almost unity through the experiments. The compression speed of the L film was fixed at 14.0 cm^2 min^{-1}.

FTIR RA measurements were performed on a Nicolet Magna 850 FTIR spectrophotometer equipped with a deuterated triglycine sulfate (DTGS) infrared detector. A variable-angle reflection accessory, Harrick Model VRA-1DG, was used for RA measurements. The angle of incidence of the infrared beam was fixed at 80° from the surface normal, and the reflected ray was collected through a Hitachi AgBr wire-grid infrared polarizer. Polarization was set to the *p*-polarized beam condition. The accumulation number of interferograms was 2048 for every RA measurement, and the resolution of the spectra was 4 cm^{-1}.

For QCM measurements, an ULVAC Model CRTM deposition monitor for metal evaporation was used. A temperature-controlled sample chamber was newly designed for isolating the QCM measurement part in a dried and thermally stable condition. The relative humidity outside the sample chamber was controlled to be under 38% by an air conditioner, and nitrogen gas was always flowed in the sample chamber, resulting in an almost ideal dried condition for QCM measurements of LB films. The temperature was controlled by a water circulator at 25 °C throughout the experiments. A QCM plate of 12.35 mm in diameter, Model PKG-5, was purchased from ULVAC Techno Ltd. Both sides of the plate are furnished by evaporated gold layers to be electrodes. The intrinsic vibrational frequency of the plate is 5 MHz. When we transfered an L film onto the QCM plate, the plate was held by Teflon tweezers, and it was dipped into a subphase solution before the transfer. After preparation of an L film on the subphase solution, the L film was transferred by withdrawing the plate with tweezers (LB method) at a speed of 0.5 cm min^{-1}. The transfer ratio of this transfer should be the same as that for the gold-evaporated glass slides (unity), since the QCM plate and the gold-evaporated glass slide have almost the same surface.

Two-dimensional correlation analysis[26] of RA spectra depending on a concentration was calculated by a software written by Mr. D. Adachi, Kwansei Gakuin University, Nishinomiya, Japan. This software, named "2D-Pocha", can calculate the generalized two-dimensional spectra[26] of synchronous and asynchronous correlations, and can display results by contours or a 3-dimensional color image. In this paper, the contour images will be used.

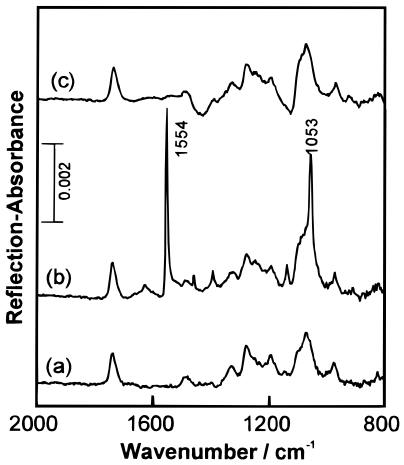

Figure 2. FTIR RA spectra of DPPC-d_{62} monolayer LB films. Each of them was transferred from an L film on (a) pure water, (b) a 10 mM Tris/HCl buffer, and (c) a 2 mM Tris/HCl buffer.

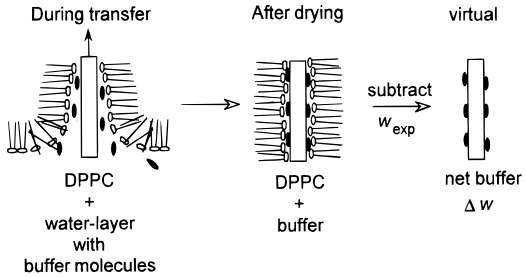

Figure 3. Scheme of the estimation procedure of the net mass of adsorbed buffer molecules on the surface of DPPC L films.

Results and Discussion

(A) Binding Property of Buffer Molecules on DPPC L Films. In our previous study,[19] the L films were prepared on a 10 mM Tris/HCl buffer solution. The concentration of 10 mM, however, may be too high to perform QCM and RA measurements in the present study, since Tris would appear in the results of the measurements. In most QCM studies, thus far, QCM measurements have been performed using solution without buffer (pure water) or solution without organic buffer reagents for feasibility of analysis.[9,10] However, the pH modification by Tris is essential for biophysical study, since it is widely used in vitro experiments. In this section, we investigate an appropriate condition of the Tris buffer for QCM and RA experiments.

Figure 2(a) shows an RA spectrum of a DPPC-d_{62} monolayer LB film prepared on pure water. The same LB film prepared on a 10 mM Tris/HCl buffer solution gives an RA spectrum in Figure 2(b), showing a spectrum similar to spectrum (a), except for accompanying strong sharp peaks at 1554 and 1053 cm^{-1} arising from Tris molecules. These bands indicate that Tris molecules exist near the surface of the DPPC-d_{62} L film.

We found, on the other hand, that the $\pi-A$ isotherm of a DPPC-d_{62} L film measured on a 10 mM Tris/HCl buffer solution gave an isotherm identical to that on pure water (not shown). This means that Tris molecules are not incorporated in the DPPC-d_{62} L film, but adsorb weakly on the film surface.

To investigate the amount of adsorbed Tris molecules on the L film, QCM technique is employed. The analytical procedure is schematically summarized in Figure 3. During or just after an LB transfer, a water layer containing Tris is sandwiched between the monolayer and the QCM plate. After an adequate drying of the LB film under dry nitrogen gas flow, the increase

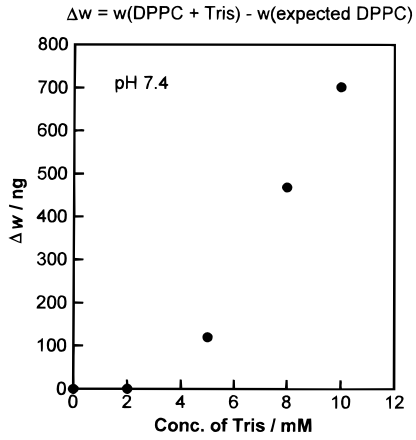

Figure 4. The net mass of adsorbed buffer molecules on the surface of DPPC L films depending on the buffer concentration.

of mass of the QCM plate is measured by the QCM method at each concentration of Tris/HCl buffer. DPPC (and sucrose in the later section) may still be partially hydrated even after the drying process. For our study, however, the equilibrium conditions should be adequate to investigate the qualitative difference between partially and fully hydrated DPPC L films.

The expected increase of mass (w_{exp}) due to a dried DPPC-d_{62} monolayer is calculated from a limited area taken from a $\pi-A$ isotherm in advance with eq 1.

$$w_{exp} = \frac{d^2\pi}{4}\frac{M_W}{A_{\pi=30}N_A} \tag{1}$$

Here, d, M_W, $A_{\pi=30}$, and N_A respectively refer to the diameter of the QCM plate, the molecular weight of DPPC, the molecular cross-section area of DPPC L film at 30 mN m^{-1}, and Avogadro's number. The expected increase of mass is subtracted from the observed increase of mass (w_{obs}) by QCM, to give a net mass (Δw) of the adsorbed buffer molecules by the following equation.

$$\Delta w = w_{obs} - w_{exp} \tag{2}$$

The obtained net mass is shown in Figure 4. In this figure, it is obviously found that the net mass of adsorbates, mainly due to Tris, is not proportional to the concentration of the buffer solution. This means that we can neglect the adsorption of Tris on the L film below 2 mM. We examined whether the 2 mM Tris/HCl buffer had a sufficient ability to control the aqueous solution to pH 7.4 $\pm$ 0.1 for a long period of time. We then concluded that the 2 mM Tris/HCl buffer is an appropriate buffer for QCM study.

This result was also checked by infrared RA spectroscopy. An RA spectrum of DPPC-d_{62} monolayer LB film prepared on a 2 mM Tris/HCl buffer solution is shown in Figure 2(c). The result gives a spectrum very similar to that in Figure 2(a). The most notable thing is that the two strong peaks arising from Tris molecules (found in Figure 2(b)) perfectly disappear in Figure 2(c). This result supports the conclusion by the QCM measurements and proves that 2 mM Tris/HCl buffer is appropriate for RA study, too. These results further indicate that Tris molecules are concentrated near the surface of DPPC-d_{62} L films when the bulk concentration of buffer is higher than 2 mM.

(B) Study of Binding Mechanism of Sucrose in/on DPPC L Films. *(i) Study of Compressed Langmuir Films.* FTIR RA and QCM measurements were performed to analyze the binding

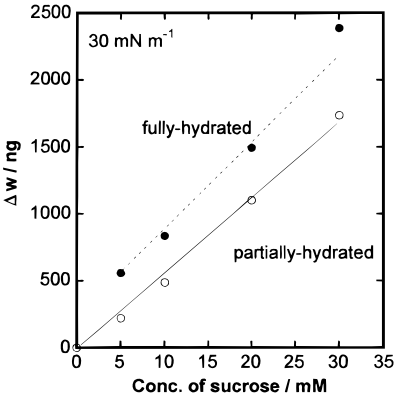

Figure 5. The mass of bound sucrose on DPPC L films at 30 mN m^{-1}. The results for fully hydrated and partially hydrated L films are respectively shown by solid and open circles.

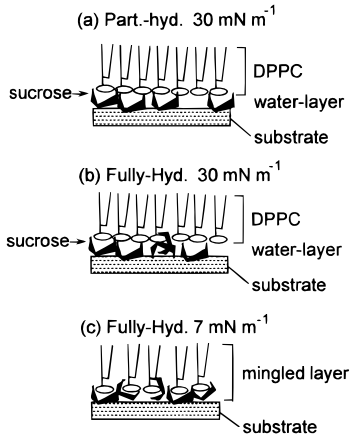

Figure 6. Schematic models of the binding mechanism of sucrose to (a) a compressed partially hydrated DPPC L film, (b) a compressed fully hydrated DPPC L film, and (c) a soft fully hydrated DPPC L film.

mechanism of sucrose molecules on (or in) DPPC L films on a 2 mM Tris/HCl buffer. The QCM technique is first applied to measure the mass of bound sucrose on partially hydrated DPPC L films.

Partially hydrated DPPC L films spread on various concentrations of sucrose (0−30 mM) with 2 mM Tris/HCl were transferred onto a QCM plate by the LB method at 30 mN m^{-1}. This surface pressure corresponds to the gel state of DPPC L film.[27] The mass increase due to the transferred L film with sucrose on the QCM plate was measured in a dried condition as described in the previous section. The mass of adsorbed sucrose on the L film was calculated by using eqs 1 and 2.

The results are summarized in Figure 5 by open circles. It is clearly shown that the increase of mass due to bound sucrose is almost proportional to the concentration of sucrose. This is explained by a simple model (Figure 6(a)): the proportional increase is caused by an increase of sucrose concentration in the water layer between the monolayer film and the substrate. In other words, the proportionality indicates that the concentration of sucrose in the water layer simply reflects the concentration in the bulk sucrose solution, and sucrose molecules are not incorporated in the surface of DPPC L films at 30 mN m^{-1}. This is consistent with the results of $\pi-A$ isotherms of the partially hydrated L films before attaining an equilibrium (Figure 1).

The binding properties of sucrose molecules on thermally fully hydrated DPPC L films are investigated in the same way. In this case, the L film was heated to 45 °C by heating the subphase solution, and the temperature was maintained for 10 min. After the L film was cooled back to 25 °C again, then the L film was laterally compressed to 30 mN m^{-1}. The $\pi-A$ isotherms were almost identical to those in our previous report,[19] indicating the film expansion. The transferred L film on a QCM plate was measured to obtain the film mass after an adequate drying.

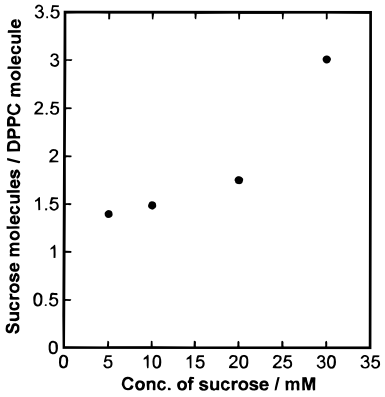

Figure 7. The estimated number of bound sucrose molecules to DPPC L films per one DPPC molecule.

The results are summarized in Figure 5 by solid circles. They also show linear relationship between the sucrose concentration and the measured mass. The fitted line to the plots, however, does not pass through the origin at all. This shift of the line indicates that an additional increase of mass newly appears. This additional increase of mass is explained by incorporated sucrose molecules in the L films (Figure 6(b)). To elucidate the incorporation of sucrose molecules, the number of incorporated molecules is estimated from the additional increase of mass.

The additional increase of mass obtained from the difference between the closed and open circles in Figure 5 is converted to the number of incorporated sucrose molecules per one DPPC molecule in an L film by the following equation. (The equation is easily deduced from a simple ratio of numbers of the two compounds.)

$$\frac{N_{\mathrm{suc}}}{N_{\mathrm{DPPC}}} = \frac{2w_{\mathrm{inc}}N_{\mathrm{A}}A_{\pi=30}}{\pi M_{\mathrm{suc}}d^2} \tag{3}$$

Here, N_{suc}, N_{DPPC}, w_{inc}, and M_{suc} are respectively the number of sucrose molecules on a QCM plate, the number of DPPC molecules on the QCM plate, the mass of incorporated sucrose, and the molecular weight of sucrose. Other variables are the same defined in the previous section.

The calculated number ratios are plotted in Figure 7 by solid circles. They show that the number of incorporated sucrose molecules gradually increases with the concentration of sucrose. The sudden increase at 30 mM may due to an experimental error caused by an excess drop of subphase solution remaining on the QCM plate after the LB deposition, since the 30 mM sucrose solution has relatively high viscosity and the diameter of the QCM plate is small for the size of the excess drop (reproducibility was confirmed). The gradual increase is consistent with the results of $\pi-A$ measurements[19] (refer to Figure 1 in the present paper) of fully hydrated DPPC L films. With the results of QCM experiments, the sucrose molecules prove to be incorporated in the thermally fully hydrated DPPC L films. The calculated ratio (about 1.5) seems to be relatively high. This suggests that the incorporation mechanism is not a simple one, and additional sucrose and water molecules should bind to the incorporated molecules.

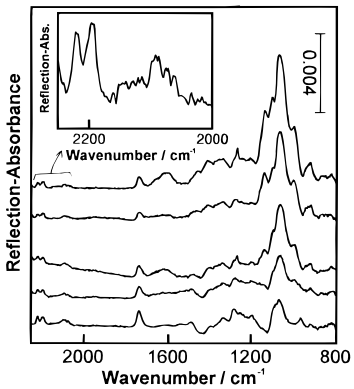

Figure 8. The FTIR RA spectra of DPPC-d_{62}/sucrose monolayer LB films. They were, from the bottom, prepared on 0, 5, 10, 20, and 30 mM sucrose solutions at 30 mN m^{-1}.

To investigate the molecular structure in LB films, FTIR RA technique is next applied to LB films that correspond to those for the QCM measurements. As described in the Experimental Section, DPPC-d_{62} was used for RA experiments. Thermal behavior of DPPC-d_{62} is different from that of a nondeuterated one. The transition temperature of DPPC-d_{62} is about 35 °C,[28] while that of the nondeuterated one is about 41 °C.[7] Then, it is not necessary to heat DPPC-d_{62} L films up to 45 °C for the full-hydration. However, to make the molecular mobility of sucrose consistent with that for the QCM measurements, the L films were also thermally treated via 45 °C.

The RA spectra of fully hydrated DPPC-d_{62} monolayer LB films on a gold-evaporated glass slide are shown in Figure 8. In the spectral range from 800 to 2250 cm^{-1}, there are major vibration bands arising from DPPC-d_{62} and sucrose. All LB films were prepared on a 2 mM Tris/HCl buffer. In any spectrum, we confirmed that bands at 1554 and 1053 cm^{-1} derived from Tris molecules did not appear. This proves that the present RA measurements are not infected by Tris molecules at the concentration. With an increase of sucrose concentration, a few peaks centered at 1069 cm^{-1} increase clearly. In this region, many bands arising from DPPC and sucrose overlap with each other.

To confirm which band is derived from sucrose, two-dimensional (2D) synchronous and asynchronous correlation analyses were applied to two regions in the RA spectra. The two regions are a region of OH stretching vibration of sucrose centered at 3378 cm^{-1} and a region including the bands at around 1069 cm^{-1} mentioned above (900−1200 cm^{-1}). Calculation was carried out with RA spectra in the concentration range of 0−30 mM. The result is shown in Figure 9. There is a strong correlation peak between the two regions in the synchronous spectrum. In particular, a cross-peak at 1069−3378 cm^{-1} is most intense. On the other hand, there is almost no correlation at 1069−3378 cm^{-1} in the asynchronous spectrum. These results strongly indicate that the band at 1069 cm^{-1} is arising from sucrose.

Therefore, the intensity change of the band at 1069 cm^{-1} in Figure 8 is plotted against the sucrose concentration in Figure 10 by open circles. From the increase in intensity of the band, the number of sucrose molecules bound in the fully hydrated DPPC-d_{62} L films is expected to increase with the concentration of sucrose.

We investigated, on the other hand, the molecular conformational change of the alkyl chains of DPPC-d_{62} due to the incorporation of sucrose. We tried to investigate the molecular conformational change through the location of a CD stretching vibration band, but the band location was unfortunately insensitive to the conformational change. The antisymmetric CD_2 stretching vibration band stayed at 2195 cm^{-1} with no shift within an experimental error, for example. This may be because the surface pressure is fixed at a high surface pressure (30 mN m^{-1}).

Besides, it is noted that the relative band intensity of CD stretching vibration modes in the spectra above 5 mM of sucrose is a little different from that in the spectrum at 0 mM (Figure 8). Therefore, the intensity of the antisymmetric CD_2 stretching vibration band is plotted against the sucrose concentration in Figure 11. If the molecular organization in L films is disordered by incorporated sucrose molecules, the intensity of the band would increase more, considering the surface selection rule of RA spectra.[23] Contrary to the expectation, the intensity decreases in a low-concentration region, while the shapes of spectra seem to change little (Figure 8). This intensity change is explained as follows. The film organization itself is maintained well, since the surface pressure of the L films is fixed at 30 mN m^{-1}. With an increase of sucrose concentration, however, the sucrose molecules spontaneously incorporate in

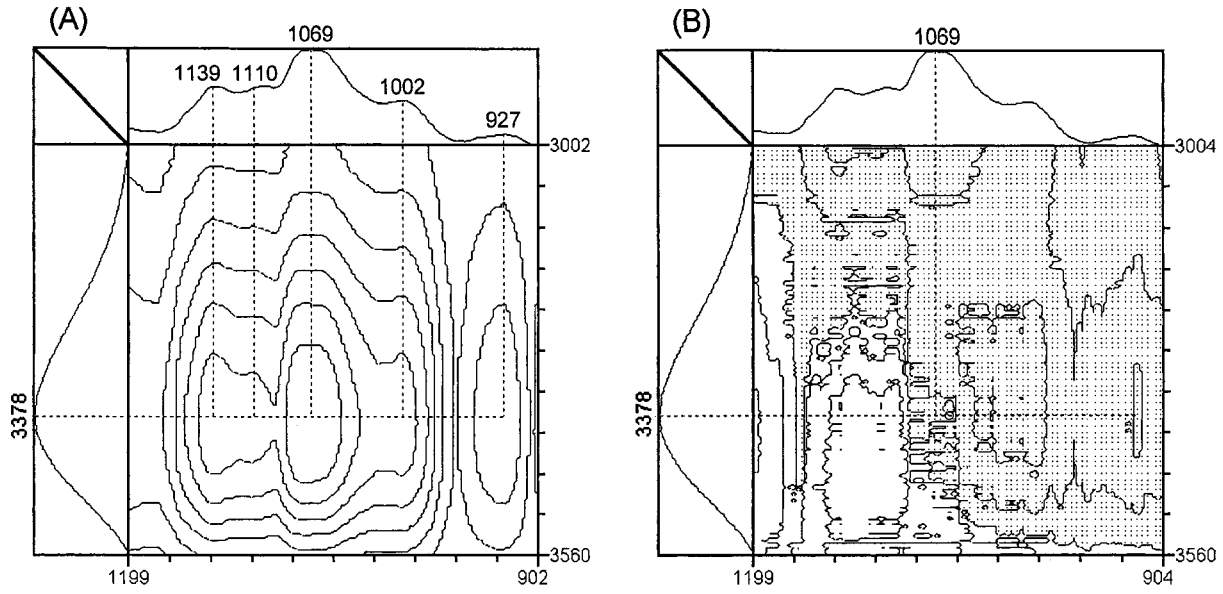

Figure 9. (A) Synchronous and (B) asynchronous two-dimensional correlation spectra calculated from RA spectra in Figure 8.

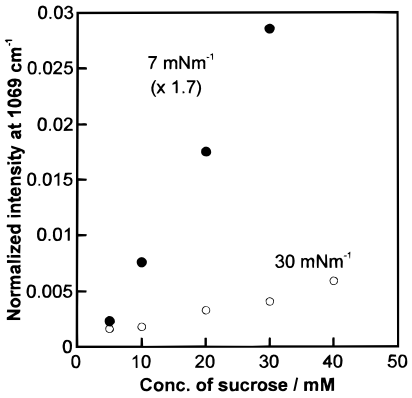

Figure 10. The intensity profile of the C−O stretching vibration band at 1069 cm^{-1} in RA spectra of DPPC-d_{62}/sucrose LB films against sucrose concentration. The results of LB films prepared at 7 and 30 mN m^{-1} are respectively indicated by solid and open circles.

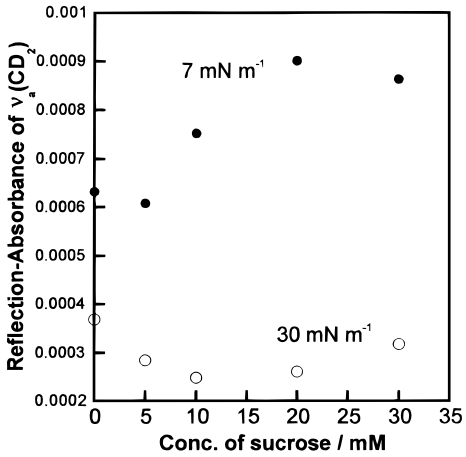

Figure 11. The intensity profile of the antisymmetric CD_2 stretching vibration band in RA spectra of DPPC-d_{62}/sucrose LB films against sucrose concentration. The results of LB films prepared at 7 and 30 mN m^{-1} were respectively indicated by solid and open circles.

the L films, resulting in the film expansion. Due to the expansion of the film, the surface density of the film decreases, resulting in a decrease of intensity of the band. At a high concentration of sucrose, on the other hand, the band intensity increases again. This is because the molecular tilting angle from the surface normal becomes larger due to a strong incorporation of sucrose, and the increase in intensity depending on the surface selection rule exceeds over the decrease in surface density. The increase in band intensity is very small indicating that the molecular disorder (or tilting) is not so apparent. This is why we could not monitor the disorder through the band shift.

The RA results support the incorporation of sucrose in fully hydrated L films, being consistent with the results of QCM.

(ii) Study of Soft Langmuir Films. The surface pressure of L films (30 mN m^{-1}) seems to be too high to study a biological model membrane. Biological cells and liposomes are known to have fluidity of membrane molecules.[29] It is necessary to investigate the molecular interaction at a lower surface pressure. We then prepared fully hydrated L films at 7 mN m^{-1} where they are in a liquid-crystalline state.[19] This L film is softer than a compressed film at 30 mN m^{-1}.

After the transfer of L films at 7 mN m^{-1} onto a QCM plate or a substrate for RA spectroscopy, the same experiments in the previous sections were performed for the soft L films. The results of QCM are summarized in Figure 12. It is noted that the increase of mass due to bound sucrose (solid circles) in fully hydrated L films is proportional to the concentration of sucrose.

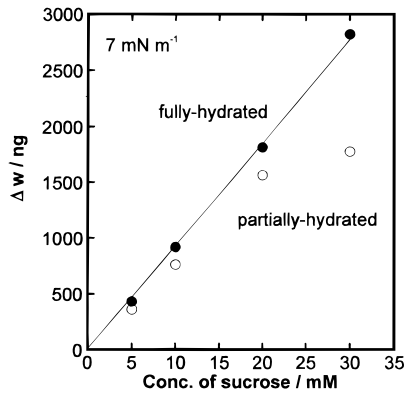

Figure 12. The mass of bound sucrose in DPPC L films at 7 mN m^{-1}. The results for fully hydrated and partially hydrated L films are respectively shown by solid and open circles.

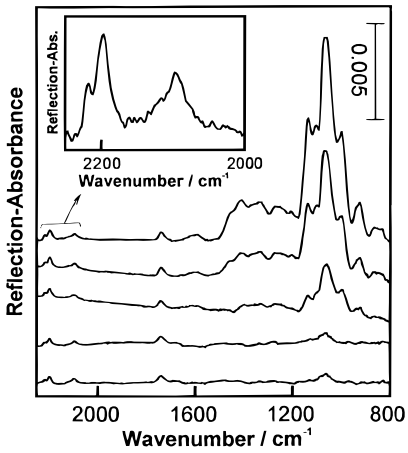

Figure 13. The FTIR RA spectra of DPPC-d_{62}/sucrose monolayer LB films. They were, from the bottom, prepared on 0, 5, 10, 20, and 30 mM sucrose solutions. The LB deposition was carried out at 7 mN m^{-1}.

This result is largely different from that of L films prepared at 30 mN m^{-1} (Figure 5). The proportionality is explained as follows with a schematic model (Figure 6(c)). Since the LB films are prepared at a low surface pressure, the originally prepared L films are soft and they may consist of sucrose and water as well as DPPC-d_{62} that make a whole. The whole layer forms a mingled layer on the gold substrate where a water layer is inseparable from a DPPC monolayer. With this mingled layer model, it is understandable that the number of sucrose molecules increases proportionally with the concentration of the sucrose solution. To further investigate this model, FTIR RA spectra will be referred.

The RA spectra of monolayer LB films transferred at 7 mN m^{-1} from fully hydrated DPPC-d_{62} L films are shown in Figure 13. Although an entire feature is very similar to that in Figure 8, the relative intensity of CD stretching bands is different (see inset). This may reflect the fact that the DPPC-d_{62} L films at 7 mN m^{-1} are soft with a lower molecular order than condensed L films at 30 mN m^{-1}. The difference in molecular order depending on surface pressure was previously investigated by Okamura et al.[27] However, in our case, the results are more complicated.

The band intensity of the antisymmetric CD_2 stretching vibration mode in Figure 13 is plotted against sucrose concentration in Figure 11 by solid circles. The values were multiplied by 1.7 considering the surface density to be normalized to the results at 30 mN m^{-1}. On the contrary to the results of the condensed films (open circles in Figure 11), the band intensity

increases with concentration after a small drop at 5 mM. Judging from the surface selection rule of RA spectra, this increase with an increase of sucrose concentration strongly indicates the lowering of molecular organization in DPPC-d_{62} L film. This means that the soft films retain well the incorporated sucrose and water molecules in the films, and do not squeeze them out. This is supported by the fact that the spontaneous film shrinkage at a constant surface pressure of 7 mN m^{-1} is almost negligible (not shown) for a long period of time. Then, we can conclude that the effect of molecular disordering exceeds that of the lowering of surface density.

We tried to investigate this molecular disorder by way of the band location of the antisymmetric CD_2 stretching vibration mode. The band is, however, always found at 2199 cm^{-1} for all LB films irrespective of the sucrose concentration. This is because the films are not ordered even at 0 mM sucrose when the surface pressure is low. This means that the band location is also insensitive to the conformational change in the case of soft films. It is notable, however, that the band location is higher than that of the highly compressed LB films (2195 cm^{-1}).

The intensity change of the band at 1069 cm^{-1} derived from sucrose in the soft films is, on the other hand, plotted in Figure 10 by solid circles. The values are multiplied by 1.7 considering the surface density as described above. It is impressive that the change is linear to the concentration. This result supports the above discussion that the incorporated sucrose molecules are not squeezed out from the soft L films. In other words, the mingled layer is very stable.

The mingled layer model could be a base model accounting for the water replacement and vitrification hypotheses. When the mingled layer is dried, sucrose molecules are deeply entangled with the DPPC layer, and the sucrose molecules act as substitutes of water molecules. In a freezing process, the strongly incorporated sucrose molecules can be vitrificated with DPPC molecules.

Conclusion

Our present study gives an important fact that incorporation of sucrose molecules in fully hydrated L films is very strong, while that in partially hydrated L films is weak. The incorporated sucrose molecules in the partially hydrated L films are easily squeezed out by a lateral pressure in L films. For the fully hydrated films, sucrose binds in the films strongly, so that it remains even in a condensed state film. It is also found that the film molecules are largely disordered by incorporated sucrose molecules when an L film is soft and fully hydrated. In this case, the L film is considered to form a mingled layer consisting of L film and sucrose solution as a whole. This mingled layer could be a base model for both water replacement and vitrification hypotheses. In this aspect, the mingled layer model is important to elucidate the protective function of disaccharide on cells.

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