

Interactions between Sex Hormones and a 1,2-Di-*O*-myristoyl-*sn*-glycero-3-phosphocholine Molecular Layer: Characteristics of the Liposome, Surface Area versus Surface Pressure of the Monolayer, and Microscopic Observation

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The interactions between sex hormones (estradiol and testosterone) and a 1,2-di-*O*-myristoyl-*sn*-glycero-3-phosphocholine (DMPC) molecular layer were investigated by observing the liposome and the surface area (A) vs. surface pressure (π) of the DMPC monolayer at an air–water interface. The shape and size of the liposome changed with the addition of estradiol and testosterone. The π–A isotherm, the time variation of the surface pressure, and the microscopic observation of monolayer were examined to clarify the different effects of the steroid hormones. These experimental results are discussed in relation to the formation of a rigid membrane due to hydrogen bonding between estradiol and DMPC.

The "nongenomic effects" of steroid hormones, which refers to a short-term response in the cell membrane, have been investigated in contrast to typical "genomic effects," which refers to a long-term response in the cell membrane.[1] For example, estradiol is generally considered to act via its nuclear receptor with a lag period of several hours.[2] On the other hand, Tsutsumi and Denda reported the effect of estradiol within 1 h on epidermal permeability barrier homeostasis as a "non-genomic effect."[3] Dicko et al. investigated the nongenomic effect of estradiol and tamoxifen on brain membranes by infrared and fluorescence spectroscopy.[4] However, "nongenomic effects" have not yet been clarified, e.g., interaction between steroid and cell membrane, penetration of steroids through the cell membrane, and function of individual steroid receptors. An artificial membrane system is useful for not only reducing the complexity of biomembranes but also for understanding the intrinsic dynamics of a target molecule.[5] There have been several experimental[6–9] and theoretical[10] studies on the effects of the interaction of cholesterol or steroid on lipid molecular membranes.

In this study, we examined the shape and size of a 1,2-di-*O*-myristoyl-*sn*-glycero-3-phosphocholine (DMPC) (Figure 1) liposome under the addition of steroid sex hormones (estradiol and testosterone) as an artificial model membrane. The surface pressure (π)–surface area (A) isotherm gives us abundant information on the stability or fluidity of the monolayer under the compression of the membrane. The π–A isotherm, the time variation of π, and the microscopic observation of monolayer were examined to clarify the interaction between steroid and DMPC molecules at the air–water interface. In particular, we examined the effect of hydroxy groups in steroid molecules by using several steroids with a different number of hydroxy group. Our experimental results suggest that the interaction of steroid with the DMPC monolayer plays an important role in the nongenomic effect of sex hormones, even in the absence of steroid receptors.

Experimental

1,2-Di-*O*-myristoyl-*sn*-glycero-3-phosphocholine (DMPC), estradiol, and testosterone were purchased from Sigma-Aldrich (St. Louis, MO). Cholesterol, cholesteryl benzoate, and androstendione were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Progesterone and estrone were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Estriol was obtained from Wako Pure Chemicals (Kyoto, Japan). 1,2-Di-*O*-hexanoyl-*sn*-glycero-3-phosphocholine (DHPC) was purchased as lyophilized powders from Avanti polar lipids. 3-Trimethylsilyl-2,2,3,3-tetradeuteropropionic acid, sodium salt (TSP-d4) was purchased from ISOTEC.

Formation of Liposome and Analysis. Liposomes were prepared as described previously,[11] as follows. DMPC–steroid mixtures with different mass ratios (DMPC/steroid: 1/0, 100/1, 10/1, 2/1, and 1/1) were dissolved in a chloroform/methanol solution (volume ratio: 2/1). The solvents were removed by evaporation in a rotary evaporator for 1 h. The residual lipid film, after being allowed to dry under vacuum for 3 h, was hydrated with 10 mM HEPES buffer solution (pH 7.3, volume: 1 mL), and Nile red (final concentration: 4 μM) was

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added to the liposome solution at room temperature. Two mL of the liposome solution on a slide glass was covered with a preparation, and the sample was observed with a fluorescence microscope (IX70, TS Olympus, Tokyo, Japan) equipped with a 75 W xenon lamp and band-pass filters of 340 and 380 nm. Imaging data were recorded with a high sensitivity silicon intensifier target camera (C4742, Hamamatsu Photonics, Hamamatsu, Japan). The shape and size of the liposomes were analyzed on a PC using ImageJ 1.41 software.

π–A Isotherm and Relaxation of π. The used reagents (DMPC and steroids) were dissolved in chloroform, and the chloroform solution was dropped on an aqueous phase with a micro syringe (volume: several tens of μL). The amount in the single sample dropped on water was 1.62×10^{-8} mol. The molar ratio of the DMPC–steroid mixture was 1:1, and the total amount was 3.24×10^{-8} mol. As the aqueous phase, water was first distilled and then purified with a Millipore Milli-Q filtering system (pH of the obtained water 6.3, resistance >20 MΩ).

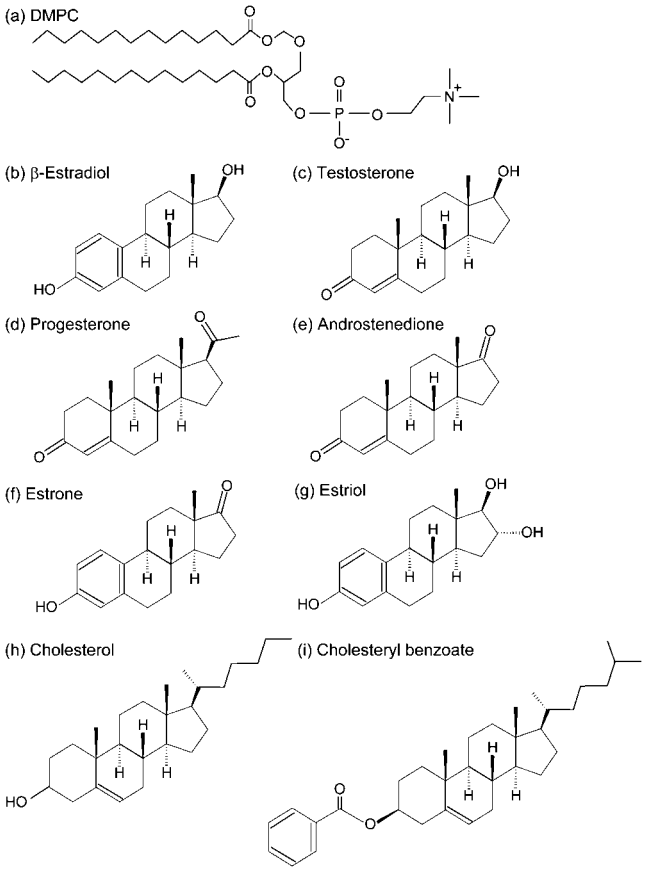

Figure 1. Chemical structures of DMPC (a) and steroids (b–i).

The π–A isotherm was measured with a surface pressure meter (Kyowa Interface Science Co., Ltd., HMB, Saitama, Japan) at 293 ± 1 K. The surface area (A) was decreased from 210 to 40 cm^2 at a rate of 17.6 $cm^2\,min^{-1}$ (e.g., 18 $Å^2\,molecule^{-1}\,min^{-1}$ for a DMPC monolayer without additives). Compression of the monolayer was started 3 min after application of the chloroform solution to eliminate chloroform from water by evaporation. At least four examinations were performed to confirm reproducibility.

AFM Observation. The monolayer was transferred to a mica plate by the horizontal lifting method[12,13] at a surface pressure of 15 $mN\,m^{-1}$. A micrograph of the transferred monolayer was obtained with an atomic force microscope (AFM; Seiko Instruments, SPI3800N and SPA-400). An aluminum-coated silicon detective cantilever (DF-20(Al), spring constant: 11 $N\,m^{-1}$, resonance frequency: 120 kHz) in noncontact mode was scanned over the sample in pure air at room temperature.

Spectrophotometric Analysis. As for ATR FT-IR, spectra were obtained by a FT-IR spectrophotometer (Perkin-Elmer Spectrum One) equipped with an ATR diamond cell (Universal ATR Sample Accessory) at room temperature. The measurements were performed for solid samples with DMPC and/or steroid after drying the chloroform solutions at room temperature (resolution of wavelength: 1 cm^{-1}). As for NMR, ^{13}C NMR spectra were measured with a Lambda 500 spectrometer (JEOL, Japan) at room temperature, and chemical shifts (δ) were with reference to TSP-d4 as an internal standard material in aqueous solutions. To dissolve DMPC in the aqueous solutions, we used a biceller system composed of DMPC and DHPC.[14] The final concentrations of DMPC and DHPC in a phosphate buffer solution (10 mM, pH 6.6) were 200 and 100 mM, respectively.

Results

Characteristic Change in the DMPC Liposome with the Addition of Steroid Compounds. Figure 2 shows typical snapshots of a DMPC liposome with or without the addition of steroid (testosterone or estradiol). The size and shape of the liposome changed with the addition of steroids. To characterize the nature of the liposome depending on the kind of steroid, the

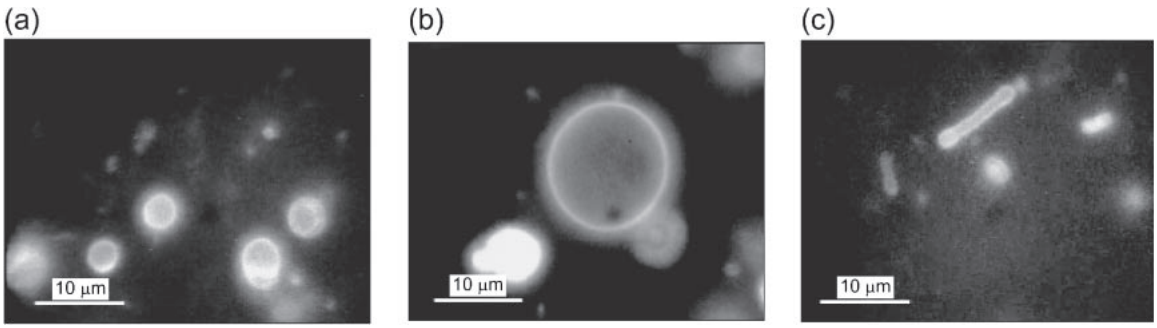

Figure 2. Snapshots of a liposome composed of (a) DMPC without steroid, (b) a mixture of DMPC and testosterone, and (c) a mixture of DMPC and estradiol. The mass ratio (steroid/DMPC) was 1. Bars: 10 μm.

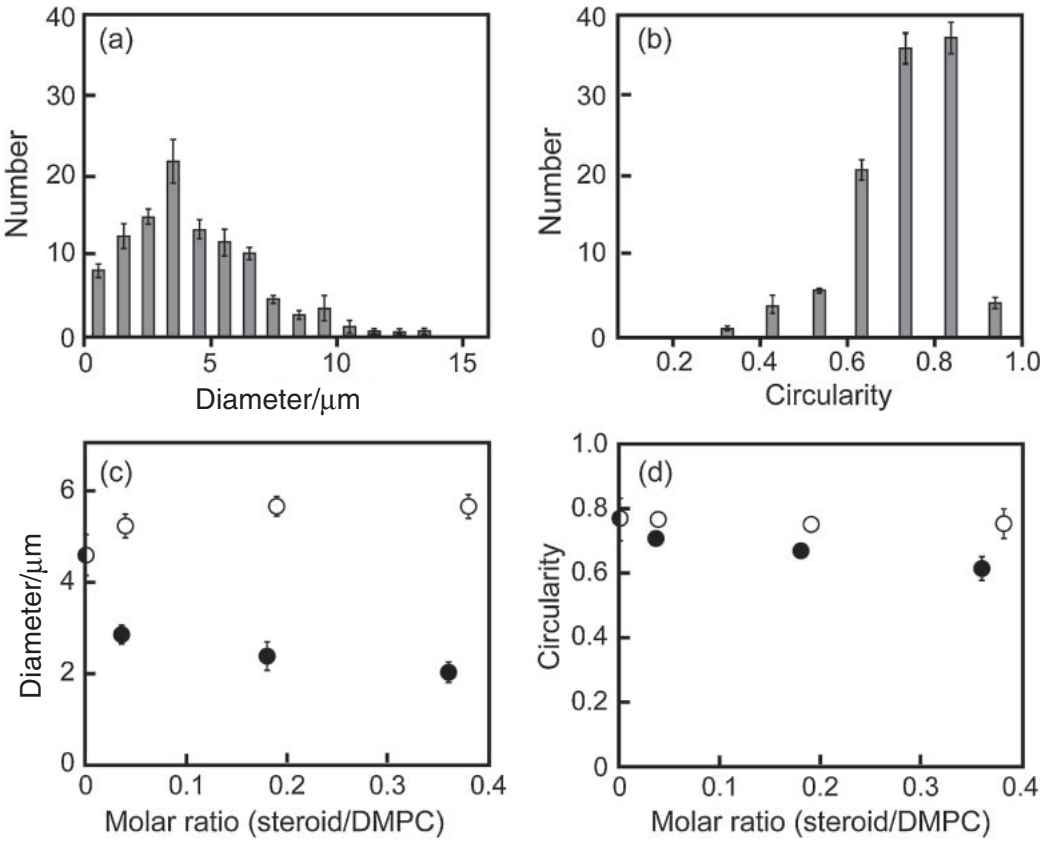

Figure 3. Histogram of (a) diameter (d) and (b) circularity ($4\pi S/L^2$, S: area, L: peripheral length ($=\pi d$)) for a DMPC liposome without steroid. (c) Diameter (d) and (d) circularity ($4\pi S/L^2$) of a DMPC liposome with (empty circle) testosterone and (filled circle) estradiol depending on the mass ratio (steroid/DMPC).

size and shape were analyzed. Histograms of diameter (d) and circularity ($4\pi S/L^2$) for a DMPC liposome without steroid, where S is the cross-section area and L is the peripheral length of the liposome, are shown in Figures 3a and 3b. If the shape of a liposome is circular, $4\pi S/L^2 = 1$. For a DMPC liposome without steroid, both the diameter and circularity show a distribution rather than a specific value, i.e., $d = 4.6 \pm 2.7\,\mu m$ and $4\pi S/L^2 = 0.76 \pm 0.11$. The diameter and circularity of a DMPC liposome with testosterone or estradiol, depending on the mass ratio (steroid/DMPC), are shown in Figures 3c and 3d. The diameter increased but the circularity did not change with an increase in the mass ratio of testosterone. In contrast, both the diameter and the circularity decreased with an increase in the mass ratio of estradiol. Similar features of images were observed in the other samples which were obtained in the same conditions.

***π–A* Isotherm and Relaxation of a DMPC Monolayer with the Addition of Steroid Compounds.** Figure 4 shows the π (surface pressure)–A (surface area per molecule) isotherm of a DMPC, a steroid (testosterone, estradiol, estrone, or cholesterol), and their mixed monolayer. In this π–A isotherm of the mixed monolayer, the number of molecules was expressed not as the total number of DMPC and steroid molecules, but rather only as the number of DMPC molecules, since the surface pressures of steroids other than cholesterol were not very sensitive to compression of the surface area due to the short hydrocarbon chain and were apparently lower than that of DMPC.[6,15] For the π–A isotherm, if steroid molecules do not influence the DMPC monolayer, the surface pressure of the DMPC monolayer (π_{DMPC}) is equal to π_{mix}, where π_{mix} is the surface pressure of the mixture of DMPC and steroid. On the other hand, if π_{mix} is given by the sum of the surface pressures of DMPC and steroid ($\pi_{sum} = \pi_{DMPC} + \pi_s$ (surface pressure of a steroid)), π_{sum} is equal to π_{mix}. π_{mix} for testosterone was nearly equal to π_{sum} at $60 < A < 100$ Å^2 molecule^{-1}, e.g., $\Delta A \approx 0$ Å^2 molecule^{-1} at 3 mN m^{-1} and $\Delta\pi = 0.9 \pm 0.9$ mN m^{-1} at 100 Å^2 molecule^{-1}, where ΔA is the result of subtracting A for the mixture from that for DMPC at the same surface pressure, and $\Delta\pi$ is equal to $\pi_{mix} - \pi_{DMPC}$ at the same A. In comparison with testosterone, π_{mix} for estradiol was clearly larger than π_{sum} at $60 < A < 120$ Å^2 molecule^{-1}, e.g., $\Delta A \approx 10$ Å^2 molecule^{-1} at 3 mN m^{-1} and $\Delta\pi = 4.1 \pm 0.5$ mN m^{-1} at 100 Å^2 molecule^{-1}. π_{mix} for estrone was larger than π_{sum} at $60 < A < 120$ Å^2 molecule^{-1}, e.g., $\Delta A \approx 10$ Å^2 molecule^{-1} at 3 mN m^{-1} and $\Delta\pi = 2.6 \pm 0.3$ mN m^{-1} at 100 Å^2 molecule^{-1}. π_{mix} for cholesterol was significantly larger than π_{sum} at $90 < A < 150$ Å^2, e.g., $\Delta A \approx 30$ Å^2 and $\Delta\pi = 23.0 \pm 4.6$ mN m^{-1}, but the increase in π_{mix} with the decrease in A was suppressed at $A < 90$ Å^2. For the other steroids, $\Delta\pi$ at 100 Å^2 molecule^{-1} for cholesteryl benzoate, estriol, progesterone, and androstendione were 5.6 ± 2.5, 10.6 ± 0.6, 0.9 ± 0.8, and 0.5 ± 0.4 mN m^{-1} (data not shown). Thus, $\Delta\pi$ values were in the order cholesterol $\gg$ estriol $>$ estradiol $>$ estrone $>$ progesterone $\approx$ testosterone $\approx$ androstenedione.

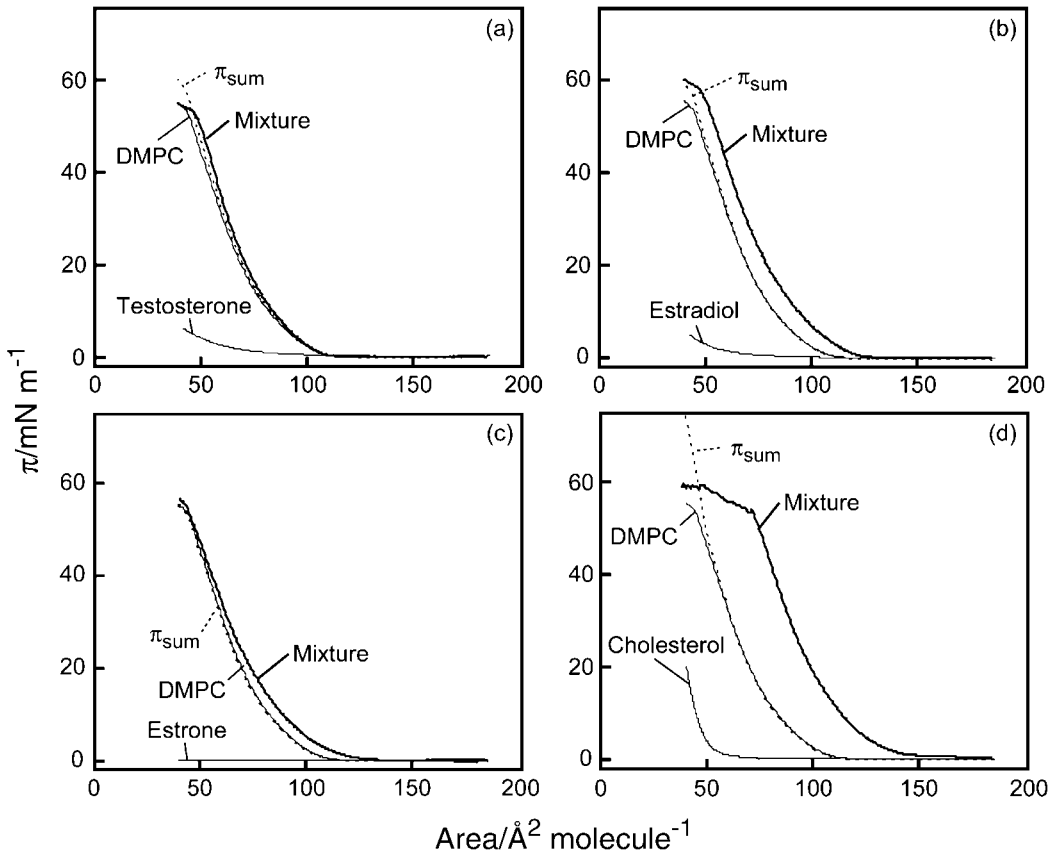

Figure 4. π–A isotherm of a DMPC/steroid mixed monolayer ((a) testosterone, (b) estradiol, (c) estrone, and (d) cholesterol). The surface pressures are for DMPC (solid line), steroid (testosterone, estradiol, estrone, or cholesterol; solid line), a mixture of DMPC and steroid (thick line), and π_{sum} (sum of the surface pressures of DMPC and steroid; dotted line). The horizontal axis, A, is either the area per DMPC molecule (surface pressure of DMPC or a mixture, or π_{sum}) or that per steroid molecule (surface pressure of steroid).

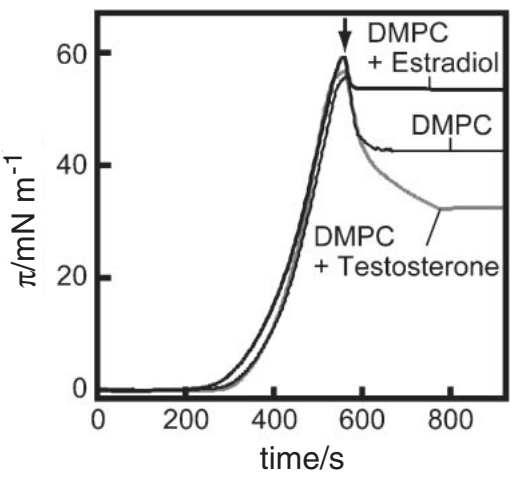

Figure 5. Time variation of π for a DMPC monolayer (thin line), DMPC/estradiol mixed monolayer (thick black line), and DMPC/testosterone mixed monolayer (thick gray line). The surface area was compressed to 50 Å^2 per DMPC molecule, and then held at that value at the time indicated by the downward arrow. The amounts of DMPC and steroids were the same as those in Figure 3.

Figure 5 shows the time variation of π for a DMPC monolayer, DMPC/testosterone mixed monolayer, and DMPC/estradiol mixed monolayer. After compression of the surface area was stopped, the surface pressure decreased with time and finally converged to a constant value. The convergent surface pressure (π_{con}) values for DMPC, a DMPC–estradiol mixture, and a DMPC–testosterone mixture were 39.1 ± 3.2, 49.3 ± 5.0, and 34.6 ± 4.5 mN m^{-1}, respectively. The relaxation times (τ_r) were 40 ± 15, 20 ± 3, and 60 ± 15 s, respectively. Here, τ_r is obtained by an approximation of the equation, $\Delta\Pi = (\pi_0 - \pi_{con}) \times \exp(-t/\tau_r)$, where π_0 is the surface pressure when compression is stopped.

Microscopic Observation. Figure 6 shows AFM images of the DMPC monolayer with or without steroid (testosterone or estradiol) on a mica surface. Concavities (number density: ca. 8 in 10 μm^2, surface area: 1.96 ± 2.78 μm^2, depth: ca. 2 nm) were observed in the DMPC monolayer without steroid. The size and shape of the concavities differed between testosterone and estradiol, i.e., the surface area of the concavities for testosterone and estradiol were 0.43 ± 0.53 and 0.017 ± 0.013 μm^2, respectively. The number densities for DMPC–testosterone and DMPC–estradiol were ca. 20 and 100 per 10 μm^2, respectively. Similar features of images were observed in the other samples which were obtained in the same conditions.

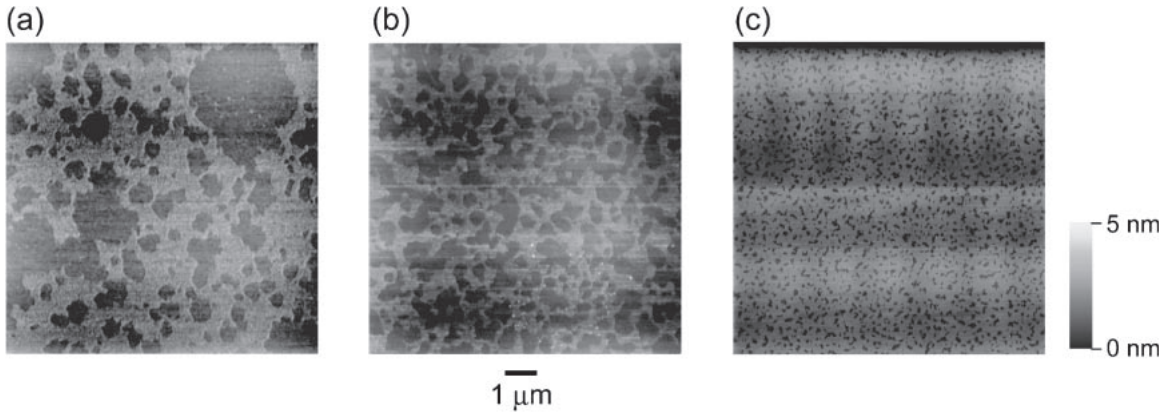

Figure 6. AFM images for a DMPC/steroid mixed monolayer on mica surfaces that were transferred using a horizontal-lifting method. (a) DMPC without steroid, (b) DMPC/testosterone, and (c) DMPC/estradiol. The amounts of DMPC and steroids were the same as those in Figures 4 and 5.

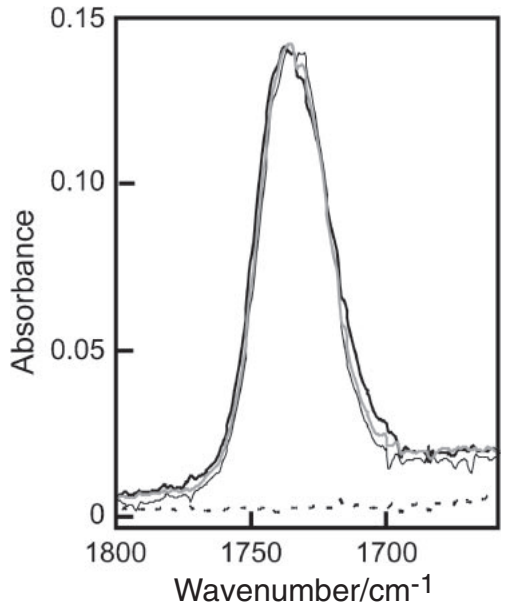

Figure 7. C=O stretching region of the IR spectra for DMPC–estradiol system in the solid state. The compositions of the system were estradiol (dotted line), DMPC (thin line), and a mixture of DMPC and estradiol with different molar ratios (gray thick line (10:1), thick line (1:1)).

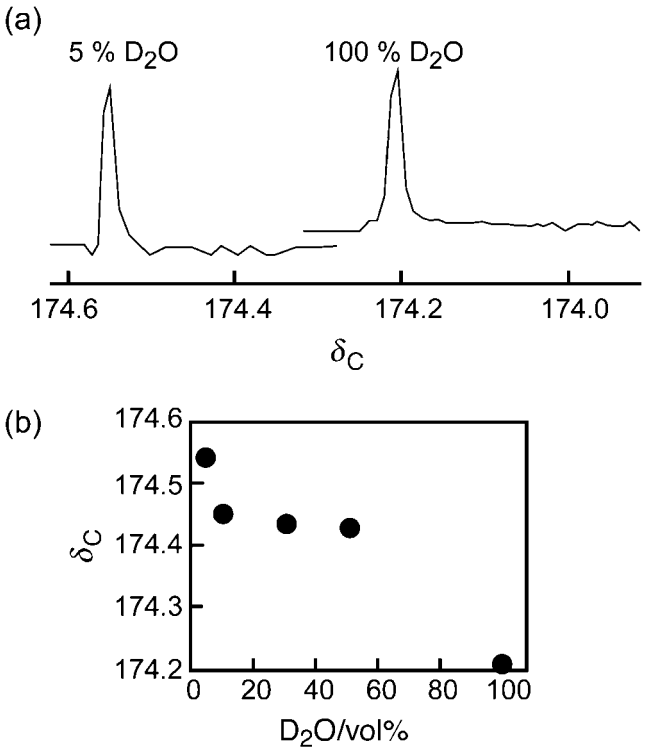

Figure 8. ^{13}C NMR spectra of the mixture (DMPC, DHPC, and estradiol) for different concentrations of D_2O (5 and 100 vol %), and the chemical shift of C=O in DMPC depending on the concentration of D_2O.

Spectrophotometric Analysis. Attenuated total reflection Fourier transform spectroscopy (ATR FT-IR) and nuclear magnetic resonance (NMR) were used to clarify the interaction between DMPC and steroid molecules referring to the related work.[8,9,16] Figure 7 shows the C=O stretching region of IR spectra for DMPC, estradiol, and mixtures of DMPC and estradiol with different molar ratios (DMPC:estratiol = 10:1, 1:1). Here, the added amount of DMPC on the ATR cell was 5.12×10^{-8} mol. The absorbance at 1700–1720 cm^{-1} slightly increased with the addition of estradiol to DMPC. Such a change in the absorbance was not observed with the addition of testosterone.

Figure 8 shows the carbonyl ^{13}C NMR chemical shift of the mixture (DMPC, DHPC, and estradiol) for different concentrations of D_2O (5 and 100 vol %), and the chemical shift depending on the concentration of D_2O in the aqueous solution. The carbonyl chemical shift changed to higher magnetic field with the increase in the concentration of D_2O in the presence of estradiol. On the other hand, the change in the chemical shift was not observed in the absence of estradiol.

Discussion

These characteristic changes in the π–A isotherm may depend on the interaction between DMPC and steroid molecules at the air–water interface. In Figure 4, the large $\Delta\pi$ and ΔA suggest that steroid molecules form a monolayer together with a DMPC monolayer because of hydrophobic and

hydrophilic interactions between them. If $\Delta\pi$ and ΔA are very small, steroid molecules may be randomly folded in hydrophobic sites of the DMPC monolayer rather than the mixed monolayer because of the weak interactions between them. In Figure 5, the higher π_{con} and shorter τ_r suggest that the mixed monolayer is stable even under further compression. In contrast, the lower π_{con} and longer τ_r values suggest that the mixed monolayer is fluid. The largest ΔA and $\Delta\pi$ values for cholesterol are due to hydrogen bonding between the hydroxy group in the A-ring of the cholesterol and the C=O group of the lipid, and hydrophobic interaction between cholesterol and DMPC hydrocarbon chains.[6,8,10] ΔA and $\Delta\pi$ for estrone, estradiol, and estriol are smaller than those for cholesterol due to the lack of the hydrocarbon chain which induces the hydrophobic interaction, but are larger than those for testosterone, progesterone, and androstenedione due to the existence of the hydroxy group in the A-ring.[6,8,10] In contrast, the hydroxy group in the D-ring of testosterone is not very effective because of the higher pK_a of the hydroxy group in the D-ring ($pK_a > 12$) than that in the A-ring ($pK_a = 10.3$)[17] and the methyl group in the D-ring may weaken the adsorption on the air–water interface as the hydrophilic group. Scheidt et al. reported a more extended hydrophilic area around the hydroxy group in the A-ring in comparison with that in the D-ring based on a quantum chemical calculation.[8] $\Delta A \approx 30$ Å^2 for cholesterol corresponds to the cross-section area for the long axis of cholesterol or the maximum surface area at the saturated surface pressure ($\approx$35 Å^2),[7] i.e., cholesterol molecules may completely pack as a monolayer mixed with DMPC molecules. $\Delta A \approx 10$ Å^2 for estradiol is smaller than the cross-section area for the long axis of estradiol ($\approx$25 Å^2), i.e., the steroid body in estradiol may be embedded in hydrocarbon chains in DMPC as a mixed monolayer. A few Å^2 of ΔA for testosterone, progesterone, and androstenedione suggest that the existence of the hydroxy group in the A-ring plays an important role in the packing of the steroid/DMPC mixed monolayer. Thus, it is difficult for these steroids to form a mixed monolayer with DMPC because of the absence of the hydroxy group in the A-ring. The experimental results for $\Delta\pi$ in Figure 4 agree with those for surface tension in a previous study.[6] The time variation of $\Delta\pi$ in Figure 5 also suggests that the fluidity of the estradiol–DMPC monolayer is lower than that of the testosterone–DMPC monolayer due to hydrogen bonding between DMPC and steroid. The experimental results using AFM suggest that the homogeneity of the estradiol–DMPC monolayer is greater than that of the testosterone–DMPC monolayer due to the interaction between DMPC and estradiol molecules. Since the concavities may be generated by the process of desiccation on the mixture monolayer transferred from the air–water interface to the mica surface, the fluid monolayer composed of testosterone and DMPC may be inhomogeneous. Thus, the characteristic nature of the π–A isotherm for a DMPC–steroid mixed monolayer can be discussed in terms of the effect of the different chemical structures, and especially hydrophobic and hydrophilic interactions between them.

With regard to the size and shape of the liposome in Figures 2 and 3, the large number of concavities in the AFM image of estradiol–DMPC may induce a smaller liposome in comparison to the testosterone–DMPC liposome. In addition, the solid estradiol–DMPC monolayer can form a deformed liposome due to the existence of a hydrogen bond.[8,16] In contrast, the fluid testosterone–DMPC monolayer can easily form a large spherical liposome rather than an unstable deformed liposome.

The spectrophotometric analysis suggests the interaction between DMPC and estradiol. That is, the increase in the absorbance at the lower wavenumber (1700–1720 cm^{-1}) on the C=O band (maximum peak: $\approx$1730 cm^{-1}) suggests the interaction between C=O in DMPC and estratiol. The ^{13}C NMR suggests the interaction between C=O in DMPC and OH in estradiol, i.e., the electron density of C in C=O is increased by the H/D exchange of OH in estradiol,[18] the carbonyl ^{13}C NMR chemical shift may change to higher magnetic field due to the hydrogen bond between C=O in DMPC and OH in estradiol.

Conclusion

The π–A isotherm of a DMPC monolayer changed characteristically depending on the kind of steroid used. These characteristic π–A isotherms can be discussed with regard to the chemical structure of the steroid, and especially the hydrophobic and hydrophilic interactions between DMPC and steroid molecules. The time variation of π and AFM observation agree with the experimental results for the π–A isotherm. The spectrophotometric analysis suggests the interaction between DMPC and estradiol. Although the present interpretation is still rather unrefined (for example, the relationship between the liposome and monolayer has not yet been clarified and we should consider the interaction between steroid and DMPC except for the hydrogen bond between hydroxy and carbonyl groups),[8] it is clear that estradiol induces characteristic features of the liposome and a monolayer composed only of a lipid without a receptor protein. In addition, the π–A isotherms for estrone, estradiol, and estriol suggest that the number of hydroxy groups may also enhance the surface pressure in the mixed monolayer in addition to the effect of a hydroxy group in the A-ring.[8]

Estradiol is generally considered to act via its nuclear receptor. However, if this is the case here, a lag period of several hours would be expected before the appearance of physiological changes. Kanda and Watanabe suggested the existence of estradiol receptor on the keratinocyte cell membrane.[2] In this paper, we do not contradict the effect of the steroid receptor but would like to propose that the direct interaction of steroid molecules with a lipid molecular layer should be considered to better understand the nongenomic effects that support the role of the receptor.[19]

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References

1 B. S. McEwen, *Trends Pharmacol. Sci.* **1991**, *12*, 141.

2 N. Kanda, S. Watanabe, *J. Invest. Dermatol.* **2004**, *123*, 319.

3 M. Tsutsumi, M. Denda, *Br. J. Dermatol.* **2007**, *157*, 776.

4 A. Dicko, M. Morissette, S. B. Ameur, M. Pézolet, T. D. Paolo, *Brain Res. Bull.* **1999**, *49*, 401.

5 J. H. Fendler, *Biomimetic Membranes*, Wiley, New York, **1982**.

6 O. A. Dimitrov, Z. I. Lalchev, *J. Steroid Biochem. Mol. Biol.* **1998**, *66*, 55.

7 K. Sabatini, J.-P. Mattila, P. K. J. Kinnunen, *Biophys. J.* **2008**, *95*, 2340.

8 H. A. Scheidt, R. M. Badeau, D. Huster, *Chem. Phys. Lipids* **2010**, *163*, 356.

9 B. Biruss, R. Dietl, C. Valenta, *Chem. Phys. Lipids* **2007**, *148*, 84.

10 S. A. Pandit, D. Bostick, M. L. Berkowitz, *Biophys. J.* **2004**, *86*, 1345.

11 S. M. Nomura, S. Kondoh, W. Asayama, A. Asada, S. Nishikawa, K. Akiyoshi, *J. Biotechnol.* **2008**, *133*, 190.

12 K. Fukuda, H. Nakahara, T. Kato, *J. Colloid Interface Sci.* **1976**, *54*, 430.

13 Y. Tagami, T. Matsufuji, H. Ikigai, T. Narita, Y. Oishi, *Bull. Chem. Soc. Jpn.* **2009**, *82*, 536.

14 J. Dittmer, L. Thøgersen, J. Underhaug, K. Bertelsen, T. Vosegaard, J. M. Pedersen, B. Schiøtt, E. Tajkhorshid, T. Skrydstrup, N. C. Nielsen, *J. Phys. Chem. B* **2009**, *113*, 6928.

15 R. S. Cantor, K. A. Dill, *Langmuir* **1986**, *2*, 331.

16 L. Cegelski, C. V. Rice, R. D. O'Connor, A. L. Caruano, G. P. Tochtrop, Z. Y. Cai, D. F. Covey, J. Schaefer, *Drug Dev. Res.* **2005**, *66*, 93.

17 J.-H. Kwon, H. M. Liljestrand, L. E. Kats, *Environ. Toxicol. Chem.* **2006**, *25*, 1984.

18 P. M. Tolstoy, P. Schah-Mohammedi, S. N. Smirnov, N. S. Golubev, G. S. Denisov, H.-H. Limbach, *J. Am. Chem. Soc.* **2004**, *126*, 5621.

19 C. Rauch, A. Flint, *Vet. J.* **2008**, *176*, 265.