Interactions of a Tetrazine Derivative with Biomembrane Constituents: A Langmuir Monolayer Study

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Supporting Information

ABSTRACT: Tetrazine (Tz) is expected to be used for bioimaging and as an analytical reagent. It is known to react very fast with trans-cyclooctene under water in organic chemistry. Here, to understand the interaction between Tz and biomembrane constituents, we first investigated the interfacial behavior of a newly synthesized Tz derivative comprising a C18-saturated hydrocarbon chain (rTz-C18) using a Langmuir monolayer spread at the air—water interface. Surface pressure (π)—molecular area (A) and surface potential (ΔV)—A isotherms were measured for monolayers of rTz-C18 and biomembrane constituents such as dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylglycerol (DPPG), dipalmitoyl phosphatidylethanolamine (DPPE), palmitoyl sphingomyelin (PSM), and cholesterol (Ch). The lateral interaction between rTz-C18 and the lipids was thermodynamically elucidated from the excess Gibbs free energy of mixing and two-dimensional phase diagram. The binary monolayers except for the Ch system indicated high miscibility or afinity. In particular, rTz-C18 was found to interact more strongly with DPPE, which is a major constituent of the inner surface of cell membranes. The phase behavior and morphology upon monolayer compression were investigated by using Brewster angle microscopy (BAM), fluorescence microscopy (FM), and atomic force microscopy (AFM). The BAM and FM images of the DPPC/rTz-C18, DPPG/rTz-C18, and PSM/rTz-C18 systems exhibited a coexistence state of two different liquid-condensed domains derived mainly from monolayers of phospholipids and phospholipids—rTz-C18. From these morphological observations, it is worthy to note that rTz-C18 is possible to interact with a limited amount of the lipids except for DPPE.

INTRODUCTION

Inverse-electron-demand Diels–Alder (IEDDA) reactions of tetrazine (Tz) and trans-cyclooctene derivatives give stable adducts in high yields and N2 as the only byproduct in organic solvents, water, and cell media. The reaction can achieve fast reaction kinetics and high selectivity without catalysis and is thus well-known as a method of rapid bioconjugation. Currently, this reaction has been applied to amine sensing and protein modification toward tumor imaging. Molecular imaging techniques will play an important role in the clinic and in drug discovery and development. However, it has been largely unknown if the obtained product conjugated to the tumor cell via the IEDDA reaction and unreacting Tz can interact with the surrounding biomembrane constituents.

The structure of cell membranes, which is well established by Singer and Nicolson, is constructed by two asymmetric leaflets of biological membranes. Among lipids, phosphatidylcholine (PC), sphingomyelin (SM), and cholesterol (Ch) are enriched on the outer surface of the bilayers. In particular, Ch is an essential component for the maintenance of SM-rich microdomains or lipid rafts, which are thought to have important biological functions such as membrane signaling and protein trafficking. On the contrary, the inner surface of the bilayers mainly consists of phosphatidylethanolamine (PE) and phosphatidylserine. Phosphatidylglycerol (PG) is a minor component in plasma membranes but exists specifically in pulmonary surfactants. It is noticed that pulmonary surfactants mostly comprise PC (in particular, dipalmitoyl PC or DPPC). The Langmuir monolayer technique at the air—water interface is one of the simple and powerful methods to understand interactions among different molecules. The

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monolayer, which is adopted as an experimental paradigm of biomembranes, is an optimal model to examine the lateral interaction among them as a function of intermolecular distance. Furthermore, the physical behavior of biomembranes (or bilayers) is directly linked to that of monolayers at the surface pressure (π) of 30–35 mN m⁻¹.¹⁷,¹⁸ Thus, many researchers have used the technique to elucidate the interfacial behavior of surfactants and the interaction (or mechanism) between compounds such as natural lipids, nineteen to twenty-one synthesized lipids,²²,²³ and proteins.²⁴,²⁵ Much useful information on the phase variation in monolayers on the micro- and nanoscale can be derived from the microscopic observations using Brewster angle microscopy (BAM),²⁶,²⁷ fluorescence microscopy (FM),²⁸–³⁰ and atomic force microscopy (AFM).³¹ In the present study, we have investigated a reduced Tz comprising a single stearoyl (C18) chain (rTz-C18) because of the rapid reaction kinetics (or the instability) and the aqueous solubility of Tz molecules. The objective of the present study is to clarify the lateral interaction between biomembrane constituents and rTz-C18, which is assumed to be conjugated to tumour cells. The interfacial behavior of two-component monolayers of rTz-C18 and five different lipids such as DPPC, dipalmitoyl PE (DPPE), dipalmitoyl PG (DPGG), palmitoyl SM (PSM), and Ch has been examined using the Langmuir monolayer technique. The lipids except for Ch have the same hydrophobic chain, which is easy to understand the interaction between the Tz moiety and the head groups of the lipids. The surface pressure (π)—molecular area (A) and surface potential (ΔV)—A isotherms of the binary mixtures were measured on a 0.02 M Tris buffer solution with 0.13 M NaCl (pH 7.4) at 298.2 K. The phase change and morphology of the monolayer upon compression were visualized using BAM, FM, and AFM.

### EXPERIMENTAL SECTION

#### Materials.

**N-(6-(6-(Pyridin-2-yl)-1,2-dihydro-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)stearamide**, abbreviated as rTz-C18 (2; see Figure 1), was newly synthesized by the procedure mentioned in the next section. The obtained rTz-C18 was purified by repeated recrystallizations from methanol, and its identification was checked by ¹H NMR, ¹³C NMR (JNM-AL400, Jeol, Tokyo, Japan), FT-IR (JASCO FT/IR-4200, Tokyo, Japan), and FAB-MS (SX102A, Jeol; m/z 559.4042 [M + Na + 2H]⁺). For the synthesis of rTz-C18, stearic acid, DMF, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC-HCl), and 1-hydroxybenzotriazole (HOBt) were purchased from nacalai tesque (Kyoto, Japan). N-Ethyl-N,N-diisopropylamine was obtained from Wako Pure Chemical Industries (Osaka, Japan). These were of analytic grade and were used as received. For the measurements of physicochemical properties, the reagents, 1,2-dipalmitoyl-sn-glycerol-3-phosphocholine (DPPC; purity >99%), 1,2-dipalmitoyl-sn-glycerol-3-phospho-(1′-rac-glycerol) (sodium salt) (DPGG; purity >99%), N-palmitoyl-o-erythro-sphingosylphosphorylcholine (PSM; purity >99%), and the fluorescent probe, 1-palmitoyl-2-[6-[7-nitro-2,1,3-benzoxadizol-4-yl]amino]-hexanoyl]-sn-glycerol-3-phosphocholine (NBD-PC), were purchased from Avanti Polar Lipids (Alabaster, AL). 1,2-Dipalmitoyl-sn-glycerol-3-phosphoethanolamine (DPPE; purity >99%) was obtained from NOF Corporation (Tokyo, Japan). Ch (purity >99%) was obtained from Sigma-Aldrich, Inc. (St. Louis, MO). These lipids were used without further purification. Chloroform (99.7%) and methanol (99.8%) were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan) and nacalai tesque, respectively. The chloroform/methanol (1:1, v/v) mixture was used as a spreading solvent. Tris-(hydroxymethyl) aminomethane (Tris) and acetic acid (HAc) of guaranteed reagent grade for the preparation of the subphase were obtained from nacalai tesque. Sodium chloride (nacalai tesque) was roasted at 1023 K for 24 h to remove all the surface-active organic impurities. The substrate solution was prepared using threis- distilled water (surface tension = 72.0 mN m⁻¹ at 298.2 K; electrical resistivity = 18 MHz cm). The pH of the subphase (0.02 M Tris buffer and 0.13 M NaCl) was adjusted to pH 7.4 with an adequate amount of HAc.

#### Methods. Synthesis of rTz-C18 (2). To a solution of 6-(6-(pyridin-2-yl)-1,2-dihydro-1,2,4,5-tetrazin-3-yl)pyridin-3-amine (1) (50 mg, 0.20 mmol) in DMF were added stearic acid (129 mg, 0.45 mmol), EDC HCl (33 mg, 0.45 mmol), HOBt (70 mg, 0.45 mmol), and N-ethylisopropylamine (59 mg, 0.45 mmol), and the mixture was stirred for 24 h at room temperature. Water (100 mL) was added to the mixture, and the precipitate formed was collected by filtration. Purification by column chromatography on silica gel (hexane/EtOAc 7:3) gave 2 (41 mg, 39% yield) as a white pellet, mp 55–56 °C.

#### Surface Pressure–Area Isotherms.
The surface pressure (π) of monolayers was measured using a commercially available film balance system (KSV Miniflou, KSV Instruments Ltd., Finland).²² The surface pressure sensor had a resolution of 0.004 mN m⁻¹. The pressure-measuring system was equipped with a filter paper (Whatman 541, periphery = 2.0 cm). The trough was made of Teflon (area = 273 cm²), and the Teflon barriers were used in this study. The π–molecular area (A) isotherms were recorded on a 0.02 M Tris buffer solution with 0.13 M NaCl (pH 7.4) at 298.2 ± 0.1 K. Stock solutions of DPPC (1.0 mM), DPPE (1.0 mM), DPPG (0.5 mM), PSM (1.0 mM), and rTz-C18 (1.0 mM) were prepared using chloroform/methanol mixture (2:1, v/v). The spreading solvents were allowed to evaporate for 15 min before compression. The monolayer was compressed at a barrier speed of 10 nm min⁻¹ or a compressing speed of ~0.08 nm² molecule⁻¹ min⁻¹.

#### Surface Potential–Area Isotherms.
The surface potential (ΔV) was recorded simultaneously with surface pressure, when the monolayer was compressed at the air–water interface. It was monitored with a Kelvin probe system (KSV SPOT1, KSV Instruments Ltd.) at 1–2 mm above the interface, whereas a counter electrode was dipped in the subphase.²³ The Kelvin probe had a resolution of 1 mV.

#### Brewster Angle Microscopy.
The monolayers were imaged directly at the air–water interface using a Brewster angle microscope (KSV Optrel BAM 300, KSV Instruments Ltd.) coupled with the KSV Miniflou.²²,²⁴ Using a 20 mW He–Ne laser, which emits p-polarized light with a wavelength of 632.8 nm, in combination with a 10x objective lens allowed for a lateral resolution of ~2 nm. The angle of the incident beam to the interface was fixed to the Brewster angle (53.1°) at 298.2 K. The reflected beam was recorded with a high-grade charge-coupled device (CCD) camera (EHDKamProt02, EHD Imaging GmbH, Germany).

#### Fluorescence Microscopy.
The KSV Miniflou was mounted on the stage of an Olympus microscope BX51WI (Tokyo, Japan) that was equipped with a 100 W mercury lamp (USHI-1030L), an objective lens (SLMPlan; 50x, working distance = 15 mm), and a CCD camera with a camera control unit (IKTU51CU, Toshiba, Japan). Before taking the images, the stock solution of the samples was doped with 1 mol % of the fluorescence probe (NBD-PC). Image processing and analysis were performed using Adobe Photoshop Elements version 7.0 (Adobe Systems Inc., CA). The total amount of ordered domains (dark-gray regions or those not containing NBD-PC) was determined.
and expressed as a percentage per frame by dividing the respective frame into dark and bright regions.

Atomic Force Microscopy. Langmuir−Blodgett (LB) films were prepared using the KSV Minitrough. Freshly cleaved mica (Okenshoji Co., Tokyo, Japan) was used as a supporting solid substrate for film deposition, which was performed through the vertical dipping method. The transfer velocity during the single-layer deposition process, which was performed at selected surface pressures, was 5 mm min\(^{-1}\). During the transfer process, the hydrophilic part of the monolayer was in contact with the mica substrate, whereas the hydrophobic part was exposed to air. LB films deposited at a rate of \(\sim 1\) were used in the subsequent experiments. The images were obtained in the air at room temperature using an SPA 400 instrument (Seiko Instruments Co., Chiba, Japan) in the tapping mode.\(^{36,37}\)

■ RESULTS AND DISCUSSION

\(\pi−A\) and \(\Delta V−A\) Isotherms. The \(\pi−A\) and \(\Delta V−A\) isotherms of the binary monolayers (DPPC/rTz-C18, DPPE/rTz-C18, DPPG/rTz-C18, PSM/rTz-C18, and Ch/rTz-C18) are shown in Figure 2. rTz-C18 (curve 7) forms a typical liquid-condensed (LC) monolayer under the present condition. The \(\pi−A\) isotherm of rTz-C18 monolayers rises up at \(\sim 0.45\) nm\(^2\) upon lateral compression and then reaches a collapse pressure \((\pi^c)\) of \(\sim 50\) mN m\(^{-1}\), where the monolayer state converts to the three-dimensional (3D) bulk state at the air−water surface. The limiting molecular area of rTz-C18 monolayers is \(\sim 0.38\) nm\(^2\), which is larger than the cross-sectional area, \(\sim 0.20\) nm\(^2\), of one saturated aliphatic chain. Considering the area, \(\sim 0.30\) nm\(^2\), of a six-membered ring, the orientation of the rTz moiety is likely to be turned toward the bulk at the closely packed monolayer state. Under the monolayer compression, the \(\Delta V\) value increases monotonously up to \(\sim 350\) mV. The \(\Delta V\) value of monolayers is interpreted as a combination of the vertical dipole moments of molecules in the subphase (layer 1), polar head group of monolayer-forming compounds (layer 2), and terminal group of their hydrophobic chain (layer 3).\(^{38}\) Independent dipole moments and effective local dielectric constants are attributed to each of the three layers. Thus, the \(\Delta V−A\) isotherms indicate changes in the molecular orientation upon compression. The monotonous increment in \(\Delta V\) indicates an improved orientation of C18 chains toward the
vertical direction. The $\pi$–$A$ isotherm of DPPC (curve 1 in Figure 2A) exhibits a first-order transition from a liquid-expanded (LE) phase to an LC phase at $\pi_{eq} \approx 11$ mN m$^{-1}$ (a dashed arrow). Similarly, DPPG (curve 1 in Figure 2C) and PSM (curve 1 in Figure 2D) monolayers undergo the transition at $\pi_{eq} \approx 17$ and $\sim 21$ mN m$^{-1}$, respectively. DPPE (curve 1 in Figure 2B) and Ch (curve 1 in Figure 2E) form typical LC monolayers. As seen in the steepness of $\pi$–$A$ isotherms, Ch monolayers are found to be more rigid than those of DPPE. The $\Delta V$ value of DPPE monolayers at the close-packed state is $\sim 600$ mV, and this value is the largest among the phospholipid monolayers here. This is attributed to the difference in head group species and in orientations of the hydrophobic chains, which are constricted by the spatial size of head groups. Detailed analyses and discussions on monolayer properties of pure lipids are mentioned elsewhere.$^{33,34}$

Analyses of the isotherm for the two-component systems provide fruitful information on mutual interactions from a thermodynamic aspect. The $\pi$–$A$ isotherms for the binary lipids/rTz-C18 monolayer (except for the Ch system) regularly shift within those for pure components (curves 1 and 7). Apparently, all of the systems indicate the $\pi$ variation with a mole fraction of rTz-C18 ($X_{rTz-C18}$). In addition, the incorporation of rTz-C18 induces a solidification of the lipid monolayer for the DPPC, DPPG, and PSM systems, which is characterized by a decrease in $\pi_{eq}$ with increasing $X_{rTz-C18}$. These variations imply the miscibility between the two components in the monolayer state. On the other hand, the complicated behavior is observed in the Ch/rTz-C18 system (Figure 2E). The $\pi$–$A$ isotherms at $X_{rTz-C18} = 0.1$ and 0.3 overlap completely with that of single Ch monolayers (data not shown). The further addition of rTz-C18 slightly shifts the isotherm to larger molecular areas (see curve 2). The difference in $A$ at 40 mN m$^{-1}$ between the isotherms at $X_{rTz-C18} = 0$ (curves 1) and 0.5 (curve 2) is no more than 0.006 nm$^2$, which is not a significant value in terms of the resolution of molecular

Figure 3. Two-dimensional phase diagrams based on the variation in the transition pressure ($\pi^{tr}$, open circle) and collapse pressure ($\pi^c$, solid circle) on a 0.02 M Tris buffer solution with 0.13 M NaCl (pH 7.4) at 298.2 K as a function of $X_{rTz-C18}$. In the $\pi^c$ region, the dashed lines are calculated according to eq 2 for $\xi = 0$. The solid lines are obtained by curve fitting of experimental $\pi^c$ values to eq 2. “M” indicates a mixed monolayer formed by each lipid and rTz-C18 species, whereas oblique-line areas show a bulk phase of the two components (“bulk phase” may be called the “solid phase” and not the monolayer state): (A) DPPC/rTz-C18, (B) DPPE/rTz-C18, (C) DPPG/rTz-C18, (D) PSM/rTz-C18, and (E) Ch/rTz-C18.
areas. At $X_{\text{rTz-C18}} = 0.7$, the isotherm lies between those of the pure components. Judging from the appearance of the $\pi$–$A$ isotherms, it is found that Ch dominates the surface activity of the binary Ch/rTz-C18 monolayer with respect to A or its surface density.

**Excess Gibbs Free Energy of Mixing.** A lateral interaction between lipids and rTz-C18 monolayers can be analyzed thermodynamically by the excess Gibbs free energy of mixing ($\Delta G_{\text{mix}}^{\text{exc}}$), which is estimated from the $\pi$–$A$ isotherms shown in Figure 2. The $\Delta G_{\text{mix}}^{\text{exc}}$ value is calculated from the following equation (eq 1):

$$\Delta G_{\text{mix}}^{\text{exc}} = \int_{0}^{\pi} (\pi_{1} - X_{A} \pi_{1} - X_{A} \pi_{2}) \, d\pi$$

(1)

where $A$, and $X_{A}$ are the molecular area and mole fraction of component i, respectively, and $A_{i,j}$ is the mean molecular area of the binary monolayer. When the intermolecular interactions between components in the mixed monolayer are the same as those in the respective one-component films (2D ideal solution) or when the film components are completely immiscible, the value of $\Delta G_{\text{mix}}^{\text{exc}}$ is zero.\(^{20,40}\) On the contrary, the negative value of $\Delta G_{\text{mix}}^{\text{exc}}$ indicates that the interactions between components of the mixed film are more attractive or less repulsive as those in the respective one-component monolayers.

This provides evidence of ideal mixing or immiscibility between Ch and rTz-C18 within a monolayer state. As for the phospholipids/rTz-C18 systems, it is found that the miscibility and affinity of rTz-C18 for the phospholipids become stronger in the following order: DPPE > DPPG > DPPC \(
\approx\) PSM. The $\Delta G_{\text{mix}}^{\text{exc}}$ analysis has been theoretically simulated using the Joos equation\(^{41,42}\) and assuming a regular surface mixture

$$1 = X_{1} \exp[(\pi_{1} - \pi_{2})/kT] \exp(\xi X_{2}^{2})$$

$$+ X_{2} \exp[(\pi_{1} - \pi_{2})/kT] \exp(\xi X_{1}^{2})$$

(2)

where $X_{1}$ and $X_{2}$ represent the mole fractions of components 1 and 2, respectively, in the two-component monolayer; $\pi_{1}$ and $\pi_{2}$ are the collapse pressures of components 1 and 2, respectively; $A_{1}$ and $A_{2}$ are the molecular areas of components 1 and 2 at $\pi_{1}$ and $\pi_{2}$, respectively; $\xi$ is the interaction parameter; and $kT$ is the product of the Boltzmann constant and the temperature in Kelvin. A solid curve could be obtained at higher surface pressures by adjusting $\xi$ in eq 2 so as to achieve the best fit for the experimentally determined $\pi$ values. Accordingly, the interaction energy ($\Delta \varepsilon$) is given as follows

$$\Delta \varepsilon = \xi \varepsilon \Omega / z$$

(3)

where $z$ is the number of nearest neighbors per molecule (equal to 6 in this case) in a closely packed monolayer. The expression for the interaction energy can be rewritten as $\Delta \varepsilon = \varepsilon^{12} - (\varepsilon^{12} + \varepsilon^{6})/2$, where $\varepsilon^{12}$ denotes the potential interaction energy between components 1 and 2. Note, here, that the Ch/rTz-C18 monolayer is treated as a miscible system to elucidate the $\xi$ value between the two components and to compare it with those for the other systems. When the amount of rTz-C18 in the DPPC (Figure 3A) and PSM (Figure 3D) systems increases, the $\pi$ values increase up to $X_{\text{rTz-C18}} = 0.4$–0.5 and then decrease to that of rTz-C18 monolayers so that both systems have values of $\xi = -4.40$ ($\Delta \varepsilon = -1.82$ kJ mol\(^{-1}\)) and $\xi = -3.50$ ($\Delta \varepsilon = -1.45$ kJ mol\(^{-1}\)) respectively. The DPPC system shows a similar variation; however, the $\pi = X_{\text{rTz-C18}}$ plot can be divided into two regions at the boundary of $X_{\text{rTz-C18}} = 0.5$: $\xi = -1.96$ ($\Delta \varepsilon = -0.81$ kJ mol\(^{-1}\)) for $0 \leq x_{\text{rTz-C18}} \leq 0.5$ and $\xi = -1.20$ ($\Delta \varepsilon = -0.50$ kJ mol\(^{-1}\)) for $0.5 \leq X_{\text{rTz-C18}} \leq 1$. As for the DPPE system (Figure 3B), the monolayer collapse behavior against $X_{\text{rTz-C18}}$ is complicated; $\xi = -1.32$ ($\Delta \varepsilon = -0.55$ kJ mol\(^{-1}\)) for $0 \leq X_{\text{rTz-C18}} \leq 0.25$, $\xi = -2.53$ ($\Delta \varepsilon = -1.05$ kJ mol\(^{-1}\)) for $0.25 < X_{\text{rTz-C18}} \leq 0.5$, and $\xi = -3.13$ ($\Delta \varepsilon = -1.29$ kJ mol\(^{-1}\)) for $0.5 \leq X_{\text{rTz-C18}} \leq 1$. It is noteworthy that the $\pi$ profile has a minimum value of $\sim 36$ mN m\(^{-1}\) at $X_{\text{rTz-C18}} = 0.25$. This value is smaller than those of pure DPPE and rTz-C18.
monolayers, which means that a small amount of rTz-C18 makes DPPE monolayers easy to transfer from the monolayer to the 3D bulk state. In other words, this is interpreted as an attenuation of monolayer stability against lateral pressure. Considering that the minimum value is almost the same pressure as the $\pi$ value of biological membranes, the rTz moiety may induce the instability of biomembranes from the inside by the attractive interaction of the rTz and PE groups. The Ch/rTz-C18 monolayer (Figure 3E) has a positive $\xi$ value of 0.66 ($\Delta \varepsilon = 0.27 \text{ kJ mol}^{-1}$) different from the other systems. This indicates that the interaction between the same molecules (Ch=Ch or rTz-C18=rTz-C18) occurs more favorably than that of Ch/rTz-C18. All of the $\Delta \varepsilon$ values here are smaller than the mean thermal energy ($2RT \approx 5.0 \text{ kJ mol}^{-1}$ at 298.2 K), which suggests that the molecular interaction related to the binary miscibility at high surface pressures is based on nonbonding intermolecular forces, not the functional chemical bond such as a covalent bond.

**Morphological Observations.** Figures 4 and S2 show FM images of the DPPC/rTz-C18 monolayers containing different amounts of rTz-C18 in situ at the air–water interface. In the FM observation, the monolayer contains a small amount of a fluorescent probe (1 mol % NBD-PC). The scale bar in the lower right represents 100 $\mu$m.

Figure 4. FM images of the DPPC/rTz-C18 monolayers for $X_{Tz-C18} = 0.3$ and 0.5 at 10 and 15 mN m$^{-1}$ on a 0.02 M Tris buffer solution with 0.13 M NaCl (pH 7.4) at 298.2 K. The monolayers contained a fluorescent probe (1 mol % NBD-PC). The scale bar in the lower right represents 100 $\mu$m.

Figure 5. FM images of the DPPG/rTz-C18 monolayers for $X_{Tz-C18} = 0.3$ and 0.5 at 15 and 20 mN m$^{-1}$ on a 0.02 M Tris buffer solution with 0.13 M NaCl (pH 7.4) at 298.2 K. The monolayers contained a fluorescent probe (1 mol % NBD-PC). The scale bar in the lower right represents 100 $\mu$m.

to the DPPC system, the LC domain (white arrows) of the mixed DPPG/rTz-C18 monolayers is visualized in the image beyond $X_{Tz-C18} = 0.3$ (Figure 5). However, as opposed to the DPPC system, the LC domain is hard to form the domain network because of the repulsive force derived from a positive charge of DPPG head groups. On the other hand, the size of LC domains at $X_{Tz-C18} = 0.1$ in the PSM system (Figures 6 and S4) becomes larger compared with that of pure PSM monolayers. In addition, the appearance of the domains transforms to a noncircular form. This means the enhancement of the dipole–dipole repulsive interaction among the LC domains by the rTz-C18 incorporation. At $X_{Tz-C18} = 0.3$, the dipole–dipole interaction is improved further so that some LC domains adhere to each other (white arrows). The images at $X_{Tz-C18} = 0.5$ show the two different LC phases: the domain forming the network (PSM–rTz-C18) and that with the diameter of less than 10 $\mu$m (mainly, PSM). The results suggest the limited quantitative ratio of interactions of rTz-C18 with DPPC, DPPG, and PSM.

**Figure 7** shows BAM images of the above-mentioned three systems. These indicate the three distinct phases of dark, white, and ordered domains.47–53 The apparent heterogeneity is resulted from the existence of two kinds of LC domains. One is the LC domain rich in DPPC (less in rTz-C18) and the other is the domain formed by the miscible monolayer of DPPC and rTz-C18, which is indicated by white arrows. This phenomenon is found not to result from the interaction with the probe because the same morphological behavior is observed in the in situ BAM images, which will be discussed in a later section. Both of the LC domains grow in size with an increase in the surface pressure. In other words, the domain growth (e.g. size and shape) evidences the miscible interaction between them, not their immiscibility. At $X_{Tz-C18} = 0.5$, the coexistence of the LC domains is also observed and the domain miscible with each other tends to form a network between the same LC domains. However, it can be said that the LC domain of the mixed monolayers predominates compared with that of the DPPC-rich LC domain. This implies the limited ratio of possible interactions of rTz-C18 with DPPC at the surface.

In the DPPG/rTz-C18 system (Figures 5 and S3), rTz-C18 interacts less strongly with DPPG below $X_{Tz-C18} = 0.1$. Similarly
and bright contrasts (white arrows) in each image. As opposed to the FM image, the dark contrast corresponds to LE phases of monolayers in the BAM image.26,27 The domains with white contrasts in Figure 7a–c are almost the same as those for single DPPC, DPPG, and PSM monolayers in terms of the size and appearance, respectively.33,35 Therefore, the bright domains with interference fringes are the LC domains of monolayers of phospholipids–rTz-C18, which are also seen in the corresponding FM images (Figures 4–6). A comparison of FM photographs with BAM images provides evidence of the following information: (1) the coexistence of two different LC domains is not induced by the interaction with the fluorescent probe, and (2) the LC domains derived from the mixed monolayers of phospholipids–rTz-C18 are more rigid and have higher density than those for single phospholipid monolayers.

In the binary DPPE/rTz-C18 and Ch/rTz-C18 systems, the FM and BAM images exhibit no phase variations upon compression because of the same LC phase for DPPE, Ch, and rTz-C18 monolayers. Thus, to clarify the mutual interaction of the systems, we have performed AFM observations, which provide powerful information on micro-scale and nano-scale molecular distributions based on the height difference in the monolayer state. Figure 8 shows the AFM images of the DPPE/rTz-C18 system at 35 mN m$^{-1}$. The image at $X_{rTz-C18} = 0.5$ indicates dark and bright contrasts. The dark domain reflects DPPE monolayers because the occupied area of the domain decreases when $X_{rTz-C18}$ increases to 0.7 and DPPE is lower in the molecular size or height than rTz-C18. In addition, the increment in $X_{rTz-C18}$ induces a dispersion of the DPPE domain. Considering the length of an ethylene group ($\sim 0.3$ nm), the larger difference between the two monolayers (1.3 and 1.6 nm) means the vertical orientation of the rTz moiety on the mica substrate. These results reveal that rTz-C18 exerts the most highly dispersing effect on DPPE monolayers among the present systems, not the solidifying effect. Furthermore, rTz-C18 is possible to interplay with DPPE over the whole $X_{rTz-C18}$ without the extra amount of the components. Here, we turn our attention to the structural difference between DPPC and DPPE. Both lipids are zwitterions under the present experimental conditions. The difference is only the end of their head groups: a choline group (DPPC) and amino group (DPPE). In the present study, however, the additional effect of rTz-C18 on DPPC and DPPE monolayers is directly opposite. Expectedly, the rTz moiety is easier to approach the nitrogen atom of DPPE head groups upon lateral compression. Therefore, a clue to exert the dispersing effect of the rTz group on biomembranes is an existence of interaction sites (here, amino group) in lipid structures.

On the other hand, the Ch/rTz-C18 system at $X_{rTz-C18} = 0.5$ (Figure 9) is highly dispersed, where the dark domain represents Ch monolayers because of the molecular size. However, as $X_{rTz-C18}$ increases to 0.7, the extra amount of rTz-C18 (indicated by a straight line arrow) is squeezed out of the
mixed Ch/rTz-C18 monolayer. The exclusion does not mean the bilayer formation of rTz-C18 because the mean height difference (1.6 nm) between the two components is quite low in comparison with the bilayer thickness of roughly 5 nm. Considering the difference between 0.9 and 1.6 nm, it is speculated that the rTz moiety lifts up from the surface to the air location because of the π–π interaction between the Ch steroid backbone and the rTz moiety. In this regard, it is suggested that the lateral interaction between Ch and rTz-C18 is quantitatively limited but they are almost ideally miscible with each other rather than the immiscibility.

CONCLUSIONS

rTz-C18 forms a stable monolayer characterized of typical LC phases and takes on the miscibility with all of the lipids treated in this study in the monolayer state. The two-component miscibility of rTz-C18 with the lipids becomes higher in the following order: DPPE > DPPG > DPPC ≈ PSM > Ch. From the thermodynamic analyses, it is suggested that rTz-C18 interacts less strongly with the constituents (DPPC, PSM, and Ch) of the outer surface of biological membranes. The solidification of monolayer LC phases occurs for the DPPC, DPPG, and PSM systems. Furthermore, in these systems, two different LC phases of the phospholipid-rich domains and the domains consisting of the binary components coexist at middle surface pressures. The LC domains (phospholipids–rTz-C18) are more rigid and have higher density. Nevertheless, the coexistence of the two different LC phases suggests the quantitative limited ratio of the interaction between the two components. On the contrary, rTz-C18 interacts slightly with Ch, which is based on the π–π interaction between the Ch steroid backbone and the rTz moiety. In the DPPE system, the addition of a small amount of rTz-C18 induces the instability in DPPE monolayers near the surface pressure (∼35 mN m⁻¹) of biological membranes, whereas the rTz moiety exerts a highly dispersing effect on DPPE monolayers in the large X_rTz-C18 region. This effect is likely to be exerted by the interaction of the rTz group with the amino group of DPPE head groups. In summary, the rTz derivative more strongly interacts with DPPE monolayers compared with other components of biological membranes (DPPC, DPPG, PSM, and Ch). The outstanding properties of this composite rTz will offer useful aspects on the Langmuir monolayer recognition and interaction mechanism against biomembrane constituents.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.langmuir.6b00997.

Plots of the excess Gibbs free energy of mixing with XTz-C18 and FM images of the two-component systems (PDF)

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Notes

The authors declare no competing financial interest.

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