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# Monolayers of a tetrazine-containing gemini amphiphile: Interplays with biomembrane lipids

Hiromichi Nakahara[a], Masayori Hagimori[b], Takahiro Mukai[c], Osamu Shibata[d,*]

[a] Department of Industrial Pharmacy, Daiichi University of Pharmacy, 22-1 Tamagawa-cho, Minami-ku, Fukuoka, 815-8511, Japan
[b] Department of Pharmaceutical Informatics, Graduate School of Biomedical Sciences, Nagasaki University, 1-7-1 Sakamoto, Nagasaki, 852-8501, Japan
[c] Department of Biophysical Chemistry, Kobe Pharmaceutical University, 4-19-1 Motoyama Kitamachi, Higashinada-ku, Kobe, 658-8558, Japan
[d] Department of Biophysical Chemistry, Graduate School of Pharmaceutical Sciences, Nagasaki International University, 2825-7 Huis Ten Bosch, Sasebo, Nagasaki, 859-3298, Japan[1]

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ABSTRACT

The property of a newly synthesized tetrazine derivative comprised of double C18-saturated hydrocarbon chain (C18-rTz-C18) has been studied in situ at the air–water interface. C18-rTz-C18 or a gemini amphiphile contributes to restriction of its tetrazine moiety on the interface, which is expected to be used for bioimaging and analytical reagents. Herein, to understand lateral interactions between Tz and biomembrane constituents, we investigated the interfacial behavior of Langmuir monolayers composed of C18-rTz-C18 and biomembrane lipids such as DPPC, DPPG, DPPE, PSM, and Cholesterol (Ch). The lateral interaction of the binary monolayers was analyzed with the surface pressure ($\pi$)–molecular area ($A$) and surface potential ($\Delta V$)–$A$ isotherms. These thermodynamic data indicate that all of the two-components are miscible with each other. In particular, as opposed to the others, the monolayer stability of DPPE, which is a major constituent of the inner surface of cell membranes, is attenuated by the small-amount addition of C18-rTz-C18. This specific interaction implies the membrane destruction from the inside. The phase behavior during monolayer compression was visualized with Brewster angle microscopy (BAM), fluorescence microscopy (FM), and atomic force microscopy (AFM). The obtained morphologies exhibit a coexistence state of two different liquid-condensed domains derived from extra phospholipids and phospholipids–C18-rTz-C18 monolayers.



## 1. Introduction

Bioorthogonal covalent reactions have been extensively used in chemical biology. Recently, among the reactions, tetrazine (Tz) emerged as a valuable biorthogonal coupling tool, which is based upon the inverse electron-demand Diels–Alder (IEDDA) reactions with dienophiles such as cyclooctyne and *trans*-cyclooctene. The reaction is characterized at fast kinetics, reactivity with no catalysts, high yield even in serum [1,2]. Therefore, the Tz ligation has been investigated towards applications in amine sensing [3], cellular microscopy [2], and tumor imaging [4–6]. In vivo imaging techniques, which is based on the IEDDA cycloaddition onto a labeled cell surface, are highly effective in clinical diagnosis as well as drug discovery and development [7,8]. Thus, it must be clarified how the free Tz and reacted Tz interplay with the surrounding membrane.

Gemini surfactants or amphiphiles contain two hydrophobic chains and two hydrophilic headgroups in a molecule. The hydrophobic chains are covalently connected with a rigid or flexible spacer [9,10]. Gemini surfactants possess extraordinary solution properties such as much lower critical micelle concentration, superior surface activity, and better wetting properties compared to the conventional monomeric surfactant [11–14]. However, in the present study, we adopt the dimerization as an immobilization of Tz derivatives at the air–water interface. Although the above-mentioned unique properties are not obtained in this dimerization, the molecular motion of Tz moieties is regulated in bilateral symmetry. The gemini amphiphile acquiring insolubility to the aqueous medium can form a Langmuir monolayer at the air–water interface. The monolayer is a simple and powerful method to understand interactions among different molecules and therefore, is widely used as a model of biomembranes [15]. Furthermore, combinations with in situ microscopies such as Brewster angle microscopy (BAM) [16,17] and fluorescence microscopy (FM) [18–20] can provide useful information on the phase variation of monolayers at the micro

* Corresponding author at: Department of Biophysical Chemistry, Faculty of Pharmaceutical Sciences, Nagasaki International University, 2825-7 Huis Ten Bosch, Sasebo, Nagasaki, 859-3298, Japan.
*E-mail address:* wosamu@niu.ac.jp (O. Shibata).
[1] http://www.niu.ac.jp/~pharm1/lab/physchem/indexenglish.html.

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scale. The Langmuir-Blodgett (LB) film, which can be fabricated by a transfer of surface monolayers onto a substrate at fixed surface pressures, is possible to be visualized with atomic force microscopy (AFM) [21].

In this study, the gemini amphiphile containing a reduced Tz as a spacer and two stearoyl (C18) chain (abbrev. C18-rTz-C18) has been used to overcome the rapid reaction kinetics and the aqueous solubility of Tz itself, respectively. To our knowledge, there exist few reports on surface immobilization of the functional group as a spacer in gemini amphiphiles although some researchers have studied the monolayer comprised of gemini amphiphiles and lipids or DNA [22–24]. We focus on the lateral interaction of C18-rTz-C18 with biomembrane constituents such as DPPC, DPPE, DPPG, palmitoyl-SM (PSM), and cholesterol (Ch). It is commonly accepted that PC, SM, and Ch are enriched on the outer surface of the biological membranes. On the other hand, PE is selected as a model lipid of the bilayer inner surface. PG, which is a minor component in mammalian plasma membranes, exists specifically in pulmonary surfactants [25–27] and in some bacterial cell membranes [28]. The interfacial properties of the two-component lipids/C18-rTz-C18 monolayers have been in situ investigated from thermodynamic and morphological aspects by the surface pressure ($\pi$)–molecular area ($A$) and surface potential ($\Delta V$)–$A$ isotherms, BAM, and FM. Furthermore, the LB film transferred onto a mica substrate is characterized with AFM.

## 2. Materials and methods

### 2.1. Materials

*N,N'-((1,2-dihydro-1,2,4,5-tetrazine-3,6-diyl)bis(pyridine-6,3-diyl))distearamide* or C18-rTz-C18 (Fig. S1), was obtained according to the synthetic procedure in the following section. Purification of the crude C18-rTz-C18 was made by repeated recrystallizations from methanol. The purified C18-rTz-C18 was identified with $^1$H NMR, $^{13}$C NMR (JNM-AL400, Jeol, Tokyo, Japan), FT-IR (JASCO FT/IR-4200, Tokyo, Japan), and FAB-MS (SX102A, Jeol); $m/z$ 803.6669 $[M+3H]^+$. DFM, 1-hydroxybenzotriazole (HOBT), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC HCl), and stearic acid were bought from Nacalai Tesque (Kyoto, Japan). *N*-Ethyldiisopropylamine of analytic grade was purchased from Wako Chemical (Osaka, Japan). 1,2-dipalmitoyl-*sn*-*glycero*-3-phosphocholine (DPPC) with the purity >99%, 1,2-dipalmitoyl-*sn*-*glycero*-3-phospho-(1′-*rac*-glycerol) (sodium salt) (DPPG), *N*-palmitoyl-D-*erythro*-sphingosylphosphorylcholine (PSM), and 1-palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-*sn*-*glycero*-3-phosphocholine (NBD-PC) with the purity >99% were obtained from Avanti Polar Lipids (Alabaster, AL). 1,2-dipalmitoyl-*sn*-*glycero*-3-phosphoethanolamine (DPPE) and Cholesterol (Ch) with the purity of >99% were purchased from NOF Corporation (Tokyo, Japan) and Sigma (Sigma-Aldrich, Inc, St Louis, MO), respectively. These lipids were used as received. The other reagents including water were the same as those used in the previous study [29]. A spreading solvent was the mixed solvent of chloroform/methanol (2/1, v/v). The monolayer was prepared on 0.02 M Tris buffer solution with 0.13 M NaCl (pH = 7.4).

### 2.2. Methods

#### 2.2.1. Synthesis of C18-rTz-C18

Stearic acid (123 mg, 0.43 mmol), EDC HCl (32 mg, 0.44 mmol), HOBT (66 mg, 0.43 mmol), and N-ethyldiisopropylamine (56 mg, 0.43 mmol) were added to the DMF solution containing 6,6′-(1,2-dihydro-1,2,4,5-tetrazine-3,6-diyl)bis(pyridin-3-amine) (50 mg, 0.19 mmol). Then, the solution was stirred for 24 h at rt. The precipitate formed after the addition of proper amount of water was collected by filtration. C18-rTz-C18 (70 mg, 45% yield, mp 77–78 °C) was obtained as a white pellet by purification with column chromatography on silica gel (hexane/EtOAC 7/3, 4/6).

#### 2.2.2. Isotherm measurements

Monolayers were characterized simultaneously by measuring surface pressure ($\pi$)–molecular area ($A$) (KSV Minitrough, KSV Instruments Ltd., Finland) and surface potential ($\Delta V$)–$A$ isotherms (KSV SPOT1) on 0.02 M Tris buffer solution with 0.13 M NaCl (pH 7.4) at 298.2 ± 0.1 K [30]. The $\pi$ value was detected using filter paper with periphery = 2.0 cm. The other information on the apparatus and experimental setup was described elsewhere [29]. The concentration of stock solutions except for DPPG (0.5 mM) was kept at 1.0 mM. A phase transition pressure ($\pi^{eq}$) from liquid-expanded (LE) to liquid-condensed (LC) states and collapse pressure ($\pi^c$) of monolayers were determined by a kink on the $\pi$–$A$ and $\Delta V$–$A$ isotherms. In all the measurements, the compression rate of monolayers was kept constant to ~0.08 nm$^2$ molecule$^{-1}$ min$^{-1}$ to compare with the previous study [29]. The compressibility modulus ($Cs^{-1}$) and excess Gibbs free energy of mixing ($\Delta G^{exc}_{mix}$) were calculated from $\pi$–$A$ isotherms with the following Eqs. (Eq. (1) and (2)), respectively:

$$Cs^{-1} = -A\left(\frac{\partial \pi}{\partial A}\right)_T \tag{1}$$

$$\Delta G^{exc}_{mix} = \int_0^{\pi} (A_{12} - X_1 A_1 - X_2 A_2)\mathrm{d}\pi \tag{2}$$

where $A$, $X$, and $T$ are respectively the molecular area, mole fraction, and temperature. The subscript represents each component in the binary monolayer. The interaction parameter ($\xi$) and energy ($\Delta\varepsilon$) were calculated from the following Eqs. (3) and (4) under the assumption of a regular surface mixture, respectively [31,32]:

$$1 = X_1 \exp\left\{\left(\pi^c - \pi_1^c\right) A_1/kT\right\} \exp\left(\xi \cdot {X_2}^2\right) + X_2 \exp\left\{\left(\pi^c - \pi_2^c\right) A_2/kT\right\} \exp\left(\xi \cdot {X_1}^2\right) \tag{3}$$

$$\Delta\varepsilon = \frac{\xi RT}{6} \tag{4}$$

where $k$ is the Boltzmann constant.

#### 2.2.3. Brewster angle microscopy (BAM)

The phase behavior of monolayers during compression was visualized in situ at the air–water interface with Brewster angle microscope (KSV Optrel BAM 300). The emission of *p*-polarized light (632.8 nm) was done with a He–Ne laser (20 mW). During the observation, the incident angle of the laser beam against the surface was kept at the Brewster angle of 53°. More details for the BAM observation were mentioned in the previous papers [33,34].

#### 2.2.4. Fluorescence microscopy (FM)

The in situ phase variation at the interface was imaged using an Olympus microscope B×51WI (Tokyo, Japan). 1 mol% NBD-PC as a fluorescence probe was embeded in the surface monolayer during observations [35].

#### 2.2.5. Atomic force microscopy (AFM)

Single layer deposition of monolayers onto mica substrates (Okenshoji Co., Tokyo, Japan) was performed with the KSV Minitrough in the vertical dipping method. The lift-up speed of mica was 5 mm min$^{-1}$ and the surface pressure was kept constant during the deposition process. The Langmuir-Blodgett (LB) films with a transfer ratio of ~1 were selected for AFM observations. The LB