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Partitioning of Hydrophobic Molecules to Liposome Membranes Can Induce Variations in their Micro-Polarity and Micro-Viscosity

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The micro-polarity and micro-viscosity of liposome membranes were evaluated to develop a platform for the localization of hydrophobic substrates in aqueous solution. The distribution ratios of benzaldoxime (BO) onto the zwitterionic 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) liposome and onto the cationic 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) liposome were almost the same, while those of benzonitrile oxide (BNO) onto DOTAP liposomes were higher than those of BO. Through the analysis of a polarity-sensitive 6-lauroyl-2-dimethylaminonaphthalene, the membrane surface of the DOTAP liposome was found to be dehydrated in the presence of substrates. Using a fluorescent probe 1,6-diphenyl-1,3,5-hexatriene, we found that the micro-viscosity of the DOTAP liposome membrane increased with BNO. These results indicate that the interaction of hydrophobic substrates induce variations in the microscopic membrane environment.

1. Introduction

Click chemistry in water solution has been developed as a simple and secure process for asymmetric syntheses [1]. Diels-Alder reactions have been studied as powerful chemical reactions to be used in high-yield and high-stereoselective methods [2-3]. The 1,3-dipolarcycloaddition reaction, one of the well-known Dields-Alder reactions, requires each reactant to have a high-diffusion coefficient within one phase in multiple phase systems, although most of the substrates are hydrophobic and are not readily dissolved in the water phase. In order to achieve such an organic synthesis in the water phase, the self-assembly systems, such as micelles, oil-in-water emulsions, and vesicles, have been studied to improve the yield and stereoselectivity [4-6]. Liposomes are self-assembled phospholipid bilayers in water, where the basic structure of a lipid membrane is the lipid bilayer with a 5 nm-thickness consisting of a polar membrane surface and a non-polar inner membrane [7]. Due to the hydrophobic interior of these systems, it becomes possible for hydrophobic molecules to homogeneously diffuse in aqueous solution [8]. By utilizing the liposome membrane systems, a novel platform for organic synthesis, e.g. 1,3-dipolarcycloaddition reaction of nitrile oxide and maleimide [6], can be established. The solution properties, such as the polarity of the solution and its viscosity, are key factors that control the Diels-Alder reactions [9]. It is therefore important to characterize the microscopic properties of the liposome membrane as a "platform" for the accumulation of substrates and for the improvement of chemical reactions in water.

In our previous study, the liposome, which is composed of a variety of phospholipids, was developed as a platform for molecular recognition and biological reaction [10]. It has also been reported that liposomes can recognize $_{L}$-tryptophan selectively, but not $_{D}$-tryptophan, while chiral selectivity was not much higher in a liquid-liquid solvent extraction system [11]. The physicochemical properties of the liposome membrane can provide a clue to improving the recognition of target molecules [10, 12-15]. The polarity and fluidity (viscosity) of the liposome membranes can be regulated by cholesterol modification or temperature control [16]. It is therefore expected that the liposome membrane can be utilized as a "tunable" environment for the localization and reaction of substrates.

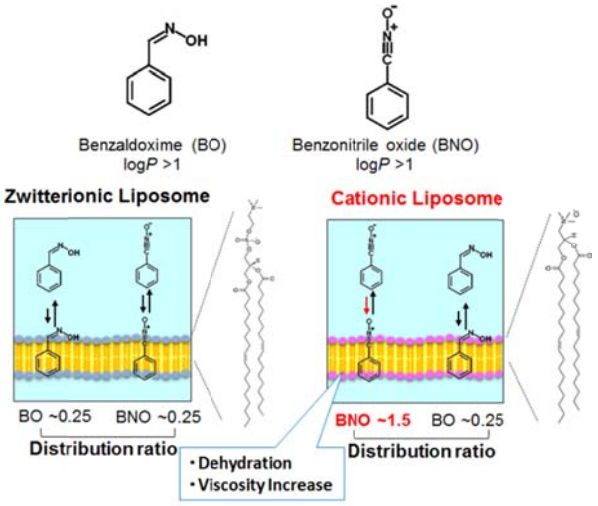

Figure 1. Schematic illustration of this study.

The aim of this study is to investigate the microscopic properties of liposome membranes with the localization of hydrophobic molecules (Figure 1). Benzaldoxime (BO) and benzonitrile oxide (BNO) diffuse with difficulty in aqueous solution due to their hydrophobicity. BNO is known as the substrate for the 1,3-dipolarcycloaddition reaction [4]. By using the zwitterionic 1,2-dioleoyl-*sn*-glycero-3-phospho-choline (DOPC) and the cationic 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) liposomes, the partitioning behaviors of BO and BNO were determined. Since the partitioning of substrates to the self-assembly system is one of the key factors to improve the 1,3-dipolarcycloaddition reaction [4-5], the partitioning of BNO, but not BO, onto liposome would improve the reaction in aqueous solution. The microscopic environment of the liposome membrane was evaluated by using a polarity-sensitive probe, 6-lauroyl-2-dimethylamino naphthalene (Laurdan), together with micro-viscosity analysis evaluated by using 1,6-diphenyl-1,3,5-hexatriene (DPH).

2. Experimental

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Benzaldoxime (BO), 1,6-diphenyl-1,3,5-hexatrien (DPH) and 6-lauroyl-2-dimethylaminonaphthalene (Laurdan) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other chemicals were purchased from Wako Pure Chemical (Osaka, Japan) and were used without further purification. The benzonitrile oxide (BNO) was prepared according to the literature [4]. The formation of BNO was determined by UV-vis spectroscopy (UV-1800; Shimadzu, Kyoto, Japan), where the conversion of BO was >85 %. The obtained solution was used as the BNO solution. The prepared BNO solution was stable for at least 3h, and the experiments were performed soon after the BNO was prepared.

The liposome suspension was prepared as previously reported [16]. In brief, a solution of lipids in chloroform was dried in a round-bottom flask by rotary evaporation under vacuum. The obtained lipid thin

film was kept under high vacuum for at least 3 h, and then hydrated with distilled water at room temperature. The obtained liposome suspension was frozen at -80°C and then thawed at 50°C; this freeze-thaw cycle was repeated 5 times. A large unilamellar vesicle was obtained by extruding the vesicle suspension 11 times through 2 layers of a polycarbonate membrane with a mean pore diameter of 100 nm using an extruding device (Liposofast; Avestin Inc., Ottawa, Canada).

The distribution ratios (D) of the substrates in the liposome membranes were evaluated by using the ultrafiltration method. Each substrate was incubated with the liposome solution for 30 min at room temperature, and the liposome and substrates distributed in the membrane were removed with the ultrafiltration unit USY-5 (molecular weight cutoff: 50,000, Advantec Toyo, Ltd., Tokyo, Japan). The distribution ratios of substrates on the filtration membranes were lower than 0.05, and the fraction of each substrate was calculated with the corrected concentration of the substrates. The D values of the substrates were calculated as follows:

$$D = C_m / C_w \quad (1)$$

where C_m and C_w are the concentrations of the substrate in the membrane and in the water solution, respectively. The concentration of the reactant in the eluted solution was measured by UV-vis spectroscopy.

The fluorescence spectra of Laurdan and DPH were measured by using a fluorescence spectrophotometer (FP-8500; Jasco, Tokyo, Japan). The excitation wavelength of Laurdan was 340 nm, measured with a 10 mm path length quartz cell. The fluorescence intensity of Laurdan was normalized with the spectrum of each liposome without substrates. The fluorescence anisotropy (r) of DPH was calculated based on the previous report [17]. A fluorescent probe DPH was added to the liposome suspension with a molar ratio of lipid/DPH of 250/1; the final concentrations of lipid and DPH were 100 and 0.4 μM, respectively. The fluorescence polarization of DPH (Ex = 360 nm, Em = 430 nm) was measured after incubation at room temperature for 30 min. The sample was excited with vertically polarized light (360 nm), and emission intensities both perpendicular ($I_\perp$) and parallel ($I_\parallel$) to the excited light were recorded at 430 nm. The anisotropy (r) of DPH was then calculated using the following equations:

$$r = (I_\parallel - GI_\perp) / (I_\parallel + 2GI_\perp) \quad (2)$$

$$G = i_\perp / i_\parallel \quad (3)$$

where $i_\perp$ and $i_\parallel$ are the emission intensities, perpendicular and parallel to the horizontally polarized light, respectively, and G is the correction factor. Based on the previous report [17], the micro-viscosity (η_{DPH}) was calculated using the following equation:

$$\eta_{DPH} = 2.4r / (0.362 - r) \quad (4)$$

The η_{DPH} values of the oleic acid vesicles in the literature [17] and in our study were 59 cP and 39 cP, respectively, indicating that this method could estimate the micro-viscosity of lipid membranes.

3. Results and Discussion

3.1 Partitioning behaviors of BO and BNO onto liposome membranes

The log$P$ values of BO and BNO were calculated via both computational [18,19] and experimental methods (Table 1). The results indicated that BO and BNO were hydrophobic, implying that they can be accumulated in the hydrophobic regions of liposome membranes. Figure 2 shows the distribution ratios (D) of BO and BNO onto DOPC and DOTAP liposomes. In the case of the DOPC liposome, the D values of

Table 1. Log*P* values of BO and BNO.

	Mw	logP_{cal} [*1]	logP_{cal} [*2]	logP_{ex} [*3]
BO	121.14	1.9	1.91	1.29
BNO	119.12	1.1	1.82	>1

[*1] Referred by PubChem database [18]: http://pubchem.ncbi.nlm.nih.gov/ [*2] Referred by ChemSpider database [19]: http://www.chemspider.com/ [*3] Determined by partitioning coefficient in water/1-octanol two-phase system.

BO and BNO were almost the same (Figure 2(a)), suggesting that the partitioning of hydrophobic molecules onto the zwitterionic liposome could be driven by hydrophobic interaction. In contrast, the *D* values of BNO onto the cationic DOTAP liposome were higher than those of BO (Figure 2(b)). Despite the difference in the partitioning behaviors in liposome membrane systems, the partitioning behaviors of BO and BNO in liquid-liquid two phase systems (i.e. water/1-octanol system) were not significantly different (Table 1). It was therefore found that the DOTAP liposome could accumulate BNO, suggesting that there exists multiple interactive forces, i.e. hydrophobic and electrostatic interaction. Because BNO is one of the substrates in 1,3-dipolarcycloaddition reactions [4,5], it is expected that the DOTAP liposome membrane surface can be utilized as a platform for the 1,3-dipolar-cycloaddition reaction. It has been reported that the interaction between liposomes and target molecules can induce the dehydration of the membrane surfaces [14, 20]. The solvent polarity and viscosity are key factors in regulating the yield and stereoselectivity in the Diels-Alder reaction [9]. It is therefore important to investigate the microscopic polarity and viscosity of the liposome membranes with the localization of hydrophobic molecules.

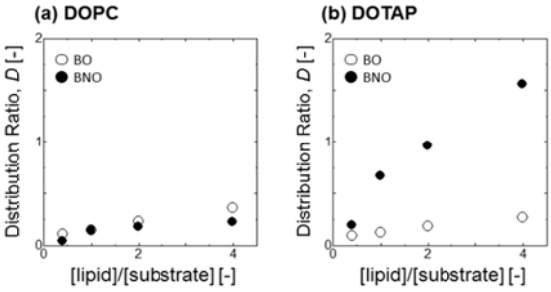

Figure 2. Distribution ratios of BO (open circle) and BNO (closed circle) in liposomes. BO or BNO (250 μM) was incubated with the liposome solution at room temperature, and the distributed substrates were separated by ultra-filtration.

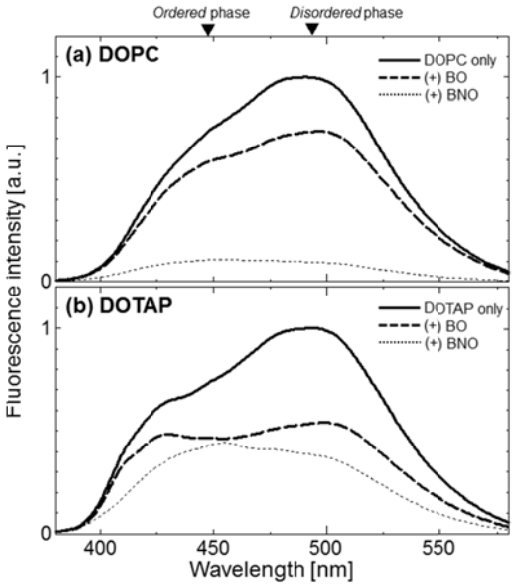

Figure 3. Fluorescence spectra of Laurdan in liposomes. (a) DOPC, and (b) DOTAP. Lines indicate 1) in the absence of substrates (*bold line*), 2) in the presence of BO (*dashed line*), and 3) in the presence of BNO (*dotted line*). The total concentrations of the lipid, Laurdan, and the substrate were 0.5 mM, 20 μM, and 5 mM, respectively.

3.2 Variation of the polar environment of liposome membranes in the presence of substrates

It has been previously reported that the micro-polarity of the liposome membrane surface can be monitored by Laurdan [16,21]. The fluorescent probe Laurdan, embedded in the hydrophilic- hydrophobic interface region of liposome membranes, is sensitive to the polarity around itself, which reflects the surface polarity of lipid membranes to be determined [22]. In

liposome membranes, Laurdan shows a peak at around 440 nm in the gel phase (ordered phase), where the lipid molecules are tightly-packed in membranes. On the other hand, Laurdan shows a peak at around 490 nm in the fluid phase (disordered phase), where the lipid molecules laterally diffuse in the membranes. The fluorescence spectra of Laurdan in the presence of BO and BNO were investigated (Figure 3). By adding BNO, a decrease of fluorescence intensity and a blue-shifted peak around 440 nm were observed. In our previous report, the blue-shifted peak was evidence of the "ordered" phase, and such ordered phases are dehydrated, as compared with disordered phases [16]. The blue-shifted peak was also observed in the presence of the anionic polynucleotides [14]. This indicates that the liposome membrane surface is dehydrated in the presence of BNO. The fluidity (viscosity) of liposome membranes also relates to the variations in the Laurdan spectra [16,22]. In addition, the viscosity of the solvent is one of the factors that affect the behavior of fluorescent probes [23-25]. Our results suggest that the micro-viscosity of the liposome membrane can vary with the localization of BNO.

3.3 Variations in the micro-viscosity of liposome membranes in the presence of substrates

The micro-viscosity of the self-assembly systems has been studied by using fluorescent probes: the C_{12}TAB micelle, 11.9 cP [26]; the polymer assembly 30-70 cP [24]; the oleic acid vesicle, 59 cP [17]. Based on the previous report [17], the micro-viscosities (η_{DPH}) of the liposome membranes were evaluated by using DPH. Figure 4 shows the η_{DPH} values of the liposomes in the presence of BO and BNO. The η_{DPH} values of DOPC and DOTAP liposomes were 64.7 cP and 48.4 cP, respectively. It was found that the DOPC and DOTAP liposomes, in disordered phases, showed the lower micro-viscosities, while the liposomes in ordered phases showed much higher micro-viscosities: DPPC, 783 cP (26.1°C); DOPC/cholesterol =1:1, 177 cP (26.1°C); DODAB vesicles, 805 cP [17]. It is therefore suggested that the micro-viscosity of the liposome is relating to its phase state. In the presence of BNO, the η_{DPH} values of the

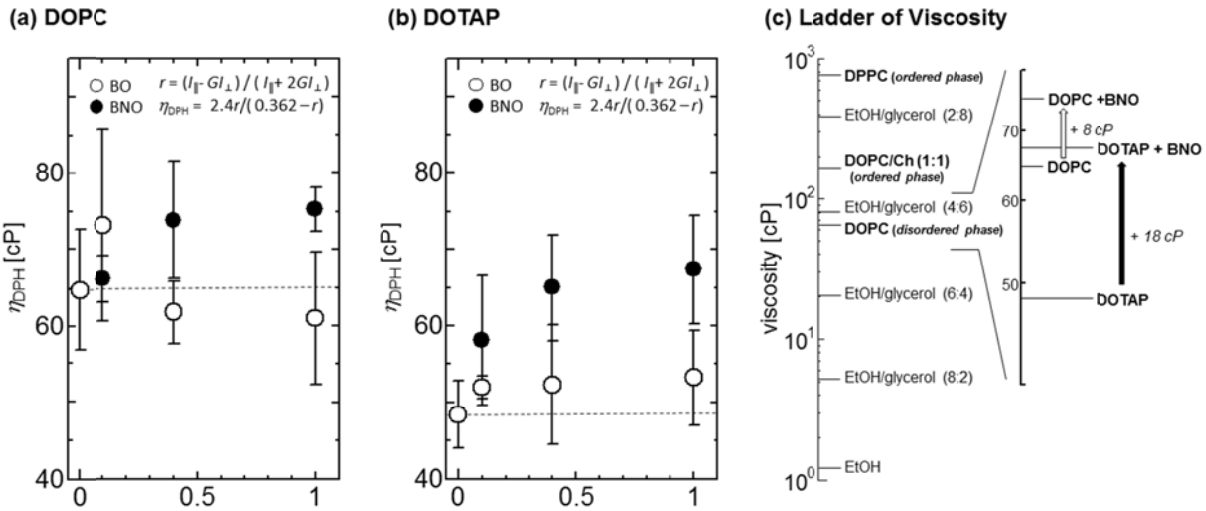

Figure 4. Analysis of the micro-viscosity of liposome membranes by using DPH. (a) DOPC and (b) DOTAP. Symbols indicate BO (*open circle*) and BNO (*closed circle*), respectively. Each dotted line indicates the η_{DPH} value in the absence of substrates. The values of the DPH anisotropy (r) and the micro-viscosity (η_{DPH}) were calculated based on previous the report [17]. The total concentrations of lipid and DPH were 0.1 mM and 0.4 μM, respectively. (c) Ladder chart of viscosity. The viscosity of ethanol/glycerol systems was reported previously [27]. Open and closed arrows indicate the increased η_{DPH} value of DOPC and DOTAP in the presence of BNO, respectively.

DOTAP liposomes increased, on the other hand, those of the DOPC did not increase much, indicating that the interaction between BNO and DOTAP liposomes could affect both the micro-polarity (Figure 3) and the micro-viscosity (Figure 4). It has been reported that the solvent relaxation time of the DOTAP liposome was increased in the presence of zwitterionic DOPC [28], which suggests that the dipolar interaction between lipid molecules can stabilize the lipid membranes. It is therefore found that the localization of BNO in the DOTAP liposome could induce the stabilization of the membrane with the variation of the membrane surface state (disordered phase to ordered phase). These results suggest that the BNO can be localized on the DOTAP liposome and, furthermore, BNO and lipid molecules are tightly packed within the dehydrated membrane surface.

4. Conclusion

The micro-polarity and micro-viscosity of the liposome membranes were evaluated by using Laurdan and DPH, respectively. The distribution ratio of BNO in the DOTAP liposome was higher than that of DOPC, where increased dehydration and micro-viscosity of the DOTAP liposome membrane were induced by BNO. We found that the membrane surface of the DOTAP liposome varied with the partitioning of substrates, indicating the "dehydrated" and "viscous" membrane surface could affect the chemical reactions of the substrates. Because the environmental properties around the substrates are one of the key factors to regulate the yield and stereoselectivity, it is therefore expected that the liposome membrane surface can be utilized as a platform for chemical reactions in water.

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