Fluorescent Anisotropy Evaluation of Bicelle Formation Employing Carboxyl BODIPY and Pyrromethene

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Abstract: Bicelles are extensively used as the parent assemblies of functional membrane materials. This study characterizes membrane fluidity in fatty acid/detergent bicelles containing carboxyl boron-dipyrromethene (BODIPY C12) and pyrromethene as fluorescent probe molecules. The anisotropy value of BODIPY C12 and pyrromethene in the phospholipid vesicles depended on the phase state of the vesicles. The anisotropy of the fluorescent probe molecules in bicelles of oleic acid/3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxypropane sulfonate (OA/CHAPSO) was then evaluated. The OA/CHAPSO bicelles were prepared by mixing CHAPSO detergent solution with OA vesicles at different molar ratios, \( X_{\text{OA}} = \left( \text{[OA]}/(\text{[OA]}+\text{[CHAPSO]}) \right) \). The anisotropies of the probes in the OA/CHAPSO bicelles increased with decreasing \( X_{\text{OA}} \). BODIPY C12 in the range 0.30 ≤ \( X_{\text{OA}} \) ≤ 0.70 exhibited a distinctly larger anisotropy than pyrromethene. This result agreed with the increase in packing density associated with the adsorption of CHAPSO molecules on the OA bilayer membrane in the OA/CHAPSO bicelle, revealing that the anisotropy of BODIPY C12 molecule enables membrane-fluidity evaluation in OA/CHAPSO bicelles.

Key words: bicelle, anisotropy, BODIPY, pyrromethene

1 Introduction

A bicelle is a molecular assembly of two parts: a bilayer membrane structure formed from long-chain lipid molecules and a micellar assembly of detergent molecules\(^1\). A non-spherical bicelle can form via interaction between the bilayer membrane and the detergent molecule\(^2\). The structural change of the bicelle caused by this interaction depends on the concentration and composition of the bicelle components\(^3\). By controlling the interaction, a continuum of functional membrane materials such as vesicles and supported lipid bilayers can be formed from the parent bicelle\(^4,5\). We have constructed a phospholipid vesicle from an oblate bicelle containing a bilayer membrane of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) with 1,2-dihexanoyl-sn-glycero-3-phosphocholine (DHPC) detergent by diluting the DMPC/DHPC bicelle in a simple flow system\(^6\). This technique is used in continuous vesicle preparation for drug encapsulation. Hydrophilic drugs can be enclosed in an inner aqueous solution of vesicles, whereas hydrophobic drugs can be held in the hydrocarbon chain region of the bilayer membrane\(^5\). Prior to dilution, the DMPC/DHPC bicelle is dispersed in DHPC solution through interactions between the DMPC and DHPC molecules\(^6\), but after dilution, the hydrated DHPC molecules leak from the DMPC/DHPC bicelles into the DHPC solution\(^6\). Finally, a phospholipid vesicle is formed by fusion of the DMPC/DHPC bicelles\(^4\). The DMPC bilayer membrane in the DMPC/DHPC bicelles maintains its initial membrane fluidity after dilution\(^6\). The result showed that the bicelles’ initial membrane properties, such as their membrane fluidity and morphology, are important factors in the design strategy of functional membrane materials with bicelle parents. Bicelle morphology is usually monitored by transmission electron microscopy (TEM) or cryo-TEM\(^6,7\). However, morphology must be analyzed from different characterization techniques to avoid microscope image artifacts. The authors of combined static light scattering and dynamic light scattering (DLS) to evaluate the persistence of warm micelles in aqueous solution\(^8\). This technique detects the scattering vectors of various concentrations of micelles, but is not practical for morphological analysis because the morphology of micelles depends on their con-
Fluorescent probe analysis, which is independent of lipid concentration, can reproducibly characterize the membrane properties such as membrane fluidity. 1,6-Diphenyl-1,3,5-hexatriene (DPH) is a fluorescent probe of polarization that detects local mobilities in a biomembrane or protein. When excited under polarized light, a DPH molecule in a biomembrane develops a dipole moment and the magnitude of its emission light versus rotation profile depends on the viscosity in biomembranes. Fluorescent polarization using DPH has estimated the membrane fluidities of vesicles, bicelles, and micelles. The membrane fluidity is calculated as the inverse polarization 1/P, which determines whether the membrane phase is ordered or disordered. 1/P = 6 is the threshold value between the disordered and ordered phases (where 1/P > 6 suggests an ordered phase). We previously confirmed the bilayer membrane structure of DMPC/DHPC bicelles using the probes DPH and 6-dodecanoyl-membrane structure of DMPC/DHPC bicelles using the CHAPSO solutions with decreasing a mixed molar ratio bicelles and those sizes were reduced upon the addition of CHAPSO bicelles with broad peaks showed multi-dispersed 354

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human digestive process. Here we evaluate the anisotropies of OA/CHAPSO bicelles with different composition ratios ($X_{OA} = \frac{[OA]}{[OA] + [CHAPSO]}$) at a concentration of 20 mM.

2 Materials and Methods

2.1 Materials

DMPC, DHPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). OA (95%), CHAPSO, pyromethene, and other chemicals were purchased from KANTO Chem. Co., Inc. (Osaka, Japan). BODIPY was purchased from Thermo Fisher Scientific K.K. (Tokyo, Japan).
2.2 Phospholipid vesicle and bicelle preparation

Phospholipid vesicles and bicelles were prepared as references of the phase states and morphologies (Fig. 2). A chloroform solution of phospholipids was dried in a round-bottomed flask by rotary evaporation under vacuum conditions. The obtained lipid film was re-dissolved in chloroform and the solvent was evaporated. The obtained lipid thin film was hydrated with phosphate buffer (50 mM, pH 7.0). The obtained vesicle solution was frozen at 213 K and then thawed at 337 K. This freeze–thaw cycle was performed five times. LiposoFast extruder (Avestin Inc., Ottawa, Canada) was used to prepare the vesicles’ sizes to 100 nm or 50 nm. The vesicles were characterized at 298 K, where the bilayer membrane is ordered in the DPPC vesicle but disordered in the DOPC vesicle. Near or above the phase-transition temperature of DMPC vesicles (~295 K), the morphology of DMPC/DHPC bicelles may not be oblate, and the DMPC membranes of the DMPC vesicles and DMPC/DHPC bicelles are ordered at 289 K. At \(X_{\text{DMPC}} = 0.33, 0.60\), and 0.67, DMPC/DHPC bicelles were reported to be oblate.

2.3 OA/CHAPSO bicelle preparation

First, OA vesicles (pH 8.51, [NaCl] = 50 mM) were prepared as the source of the OA bilayers. The OA vesicles were then mixed with BODIPY C12 or pyrromethene molecules at 1.0 mol%. OA/CHAPSO bicelles with different \(X_{\text{OA}}\) values (0.3, 0.5, and 0.7) were prepared by mixing 20 mM CHAPSO stock solution with 20 mM OA vesicle solution (Fig. 2). The total concentration (20 mM) exceeded the critical micelle concentration of CHAPSO (CMC(CHAPSO) = 8 mM). The TEM images (HP-2000, Hitachi Ltd., Osaka, Japan) were obtained after staining the bicelles with uranium acetate.

2.4 Anisotropy evaluation of the OA/CHAPSO bicelles

Anisotropy of the OA/CHAPSO bicelles was evaluated by the fluorescent probes BODIPY C12, pyrromethene, and DPH. The fluorescent intensity was measured using a fluorescence spectrophotometer (RF-2019b, The MathWorks Inc., Natick MA, USA). The emission intensity of the scattered laser was transiently measured using a charge-coupled device detector to obtain its auto-correlation functions, \(G(\tau)\). The size distribution of the OA/CHAPSO bicelles was calculated by an approximate solution of the \(G(\tau)\) based on the CONTIN algorithm. The calculation was performed in MATLAB (R2019b, The MathWorks Inc., Natick MA, USA).

3 Results and Discussion

3.1 Effect of probes on anisotropy of phospholipid vesicles

We first evaluated the anisotropy of BODIPY C12 in phospholipid vesicles, for which the packing densities of the bilayer membranes are known. The anisotropy value of BODIPY C12 was compared with those of pyrromethene and DPH. Figure 3 displays the spectra of these probes in a DPPC vesicle at different directions of light propagation. Changing the direction of the polarizing plate changed the intensities of the fluorescence but not the overall spectral shapes. The \(r\) value was calculated from the peak intensity in each spectrum.

Figure 4 compares the \(r\) values of the probes in the DOPC and DPPC vesicles. The different anisotropies of DPH (\(r_{\text{DPPC}}\)) in the DOPC and DPPC vesicles can be explained by the different phase states of the bilayer membranes. Whereas a DOPC molecule has an unsaturated bond in its hydrophobic chain and a disordered phase in the bilayer membrane, DPPC is a saturated phospholipid that builds an ordered bilayer membrane. Consequently, the rotation of the DPH molecule was more suppressed in the
DPPC vesicle than in the DOPC vesicle. Similar results were obtained for the anisotropy of pyrromethene ($r_{\text{pyrromethene}}$), indicating that a pyrromethene molecule is also suitable for membrane-fluidity evaluation. However, the threshold value may be newly set for the type of probe molecule. On the other hand, the rotation of BODIPY C12 was thought to be more suppressed than those of pyrromethene and DPH, and an ordered phase (as in the DPPC vesicle) was assumed in the $r_{\text{BODIPY}}$ calculation. However, the results suggested a more fluidic membrane in the DPPC vesicle. The difference between the anisotropies of BODIPY C12 ($r_{\text{BODIPY}}$) in the DOPC and DPPC vesicles was also smaller than that of other probes. As the BODIPY C12 molecule has a fluorescent site and a hydrophobic chain terminated with a carboxyl group, it was probably inserted into the vesicles like phospholipids (Fig. 2). In general, the center of a bilayer membrane is disordered and mobile. It was suggested that the BODIPY core located at the most hydrophobic center of the bilayer membranes of the DPPC and DOPC vesicles; thus, the difference between the $r_{\text{BODIPY}}$ values of DOPC and DPPC represented a slight difference in fluidity near the center of the membrane. Figure 5 shows the $r_{\text{BODIPY}}$ values in a DMPC vesicle, in DMPC/DHPC bicelles at $X_{\text{DMPC}} = 0.67, 0.60, and 0.33$. The DMPC vesicles of 100 nm and 50 nm showed similar $r_{\text{BODIPY}}$ values; it was considered that $r_{\text{BODIPY}}$ doesn’t depend on the vesicle size. DMPC-rich bicelles with $X_{\text{DMPC}} = 0.67$ and $0.60$ also showed similar $r_{\text{BODIPY}}$ values to the vesicles. Below the phase-transition temperature, the DMPC bilayer membranes in both the DMPC vesicle and DMPC/DHPC bicelle at $X_{\text{DMPC}} = 0.67$ and $0.60$ are in the ordered state. Therefore, these $r_{\text{BODIPY}}$ values were similar to that of the DPPC vesicle (Fig. 4). Then, the $r_{\text{BODIPY}}$ was smaller in the DMPC/DHPC bicelle at $X_{\text{DMPC}} = 0.33$ than in the DMPC vesicles and the other DMPC/DHPC bicelles at $X_{\text{DMPC}} = 0.67$ and $0.60$. A DHPC molecule has much shorter chains than a DMPC molecule (Fig. 1), and acts as a ‘weak detergent’ against the bilayer membrane of long-chain phospholipids, such as DMPC molecules. Our previous report has shown that the DMPC/DHPC bicelles are disc-shaped at $X_{\text{DMPC}} = 0.60$, suggesting that DMPC molecules and DHPC molecules are separated in a DMPC/DHPC bicelle. Increasing the proportion of DHPC molecules is expected to eliminate the separation, resulting in smaller, disordered bicelle like a mixed micelle. It was presumed that the $r_{\text{BODIPY}}$ value at $X_{\text{DMPC}} = 0.33$ decreased because DHPC molecules constructed a bilayer membrane together with DMPC molecules.

The characteristics of the parameters of the molecular assembly evaluated by each probe are shown in Fig. 6. The phase state of a phospholipid molecular assembly has been reproducibly obtained by conventional membrane fluidity using DPH molecules. Recall that the threshold of the disordered state is $r_{\text{DPH}} = 0.12$. The heterogeneous phase of the OA bilayer membrane in an OA vesicle and an OA/CHAPSO bicelle has been observed in the improved packing-density evaluation using a Laurdan molecule. The fluorescent spectrum of Laurdan in molecular assemblies has three moments in the range of 400–600 nm; those moments show the hydrophobic (moment i), hydrophilic molecular assembly (moment ii), and more hydrophilic molecular assembly like a micellar assembly (moment iii), respectively. The packing density of molecular assemblies was calculated based on the area ratio of the deconvoluted moment i to ii, $A/A_i$. The threshold of packing density was defined by comparing the previously revealed phase states of DOPC and DPPC vesicles. In contrast, al-
though the exact anisotropy threshold of $r_{\text{BODIPY}}$ is currently unclear, the anisotropy differences detected by the BODIPY C12 molecule were attributable to differences in membrane fluidity of the center of the bilayer membrane.

3.2 Morphologies of OA/CHAPSO bicelles prepared at different $X_{\text{OA}}$ values

The morphologies of the OA/CHAPSO bicelles were observed in TEM images (Fig. 7). The initial OA vesicles were destroyed by mixing with CHAPSO solution. The OA/CHAPSO bicelles formed various shapes for the ratio of OA to CHAPSO. The assemblies seen in $X_{\text{OA}} = 0.70–0.30$ were bicelles made by the interaction of the initial OA vesicles and the CHAPSO molecules. A CHAPSO molecule has a hydrophobic steroid skeleton with three hydroxy groups on its surface and a hydrophilic tail (Fig. 1). The hydrophobic surface of the steroid skeleton adsorbs to the hydrophilic surface of an OA vesicle; thereby, the CHAPSO molecule acts as a spacer that destroys the OA vesicle.\textsuperscript{14, 26–28} The edge of the disrupted OA vesicle is exposed to aqueous solution and becomes covered by other CHAPSO molecules.\textsuperscript{14, 27, 28} As $X_{\text{OA}}$ decreased, the size of the OA/CHAPSO bicelles also decreased (Fig. 7), indicating that the CHAPSO molecules further destroyed the bilayer membrane derived from the OA vesicle.\textsuperscript{14} The micelles were dominant at $X_{\text{OA}} = 0.10$, and the presence of OA/CHAPSO bicelle was clearly unconfirmed in the TEM image (Fig. 7). As determined in DLS measurements (Fig. 8), the mode sizes of the OA/CHAPSO bicelles ranged from 135 nm at $X_{\text{OA}} = 0.70$ to 66.8 nm at $X_{\text{OA}} = 0.30$; meanwhile, the particle size distributions at $X_{\text{OA}} = 0.70$ and 0.30 obtained from the TEM images correlated with their DLS results.\textsuperscript{24, 25} After analyzing the packing densities of Laurdan molecules in bicelles, we previously reported that the bilayer membrane structures in OA/CHAPSO bicelles ($0.30 \leq X_{\text{OA}} \leq 0.70$) are derived from the OA vesicles.\textsuperscript{14, 24} The packing densities were slightly improved in the OA/CHAPSO bicelles obtained after mixing with CHAPSO molecules,\textsuperscript{14, 24} suggesting that the OA bilayer membrane derived from the OA vesicle included a heterogeneous coexistence of...

\begin{figure}[h]
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\includegraphics[width=\textwidth]{image1.png}
\caption{r values probed by BODIPY C12, pyromethene, and DPH on DOPC, and DPPC vesicles of 2 mM at 298 K. The size of the vesicles was adjusted to 100 nm.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image2.png}
\caption{r values probed by BODIPY C12 on DMPC vesicles (2 mM), and DMPC/DHPC bicelles of 20 mM at 289 K.}
\end{figure}
ordered and disordered phases. Here, the packing densities of the bicelles formed at $0.30 \leq X_{OA} \leq 0.70$ were not significantly different, suggesting that bilayer membrane structures existed even in the smaller bicelles. This result is probably explained by adsorption of CHAPSO molecules on the OA bilayer membranes. A single CHAPSO molecule adsorbed on the hydrophilic surface of an OA vesicle is linked to several OA molecules. Because the OA molecules beneath the CHAPSO molecule are difficult to fluidize, the OA bilayer membrane is thought to persist even after mixing the CHAPSO solution. From the previous report and the present TEM images, it was inferred that the anisotropic OA/CHAPSO bicelles formed by interactions between the OA bilayer membranes and the CHAPSO molecules.

### 3.3 Anisotropy of OA vesicle and OA/CHAPSO bicelles

The $r$ values in the OA vesicles are shown in Fig. 9a. Each $r_{DPH}$ value indicated a disordered phase in the OA vesicles. Similar results were obtained for the values of $r_{Pyrromethene}$ and $r_{BODIPY C12}$. As an OA molecule has an unsaturated chain, an OA vesicle tends to form a disordered bilayer structure (similarly to DOPC vesicles). The $r$ values remained similar after diluting the OA vesicles (Fig. 9a), indicating that the $r$ value of each probe was independent of OA concentration. After mixing with CHAPSO solution at $X_{OA} = 0.70$ and 0.30, the final OA concentrations were 6.0 and 14 mM, respectively.

**Figure 9b** compares the $r$ values after mixing the CHAPSO solutions with OA vesicles at different $X_{OA}$. In all cases, the $r$ value increased with decreasing $X_{OA}$. Here, in the OA vesicle and OA/CHAPSO bicelle, the $r_{DPH}$ values indicated only the disordered phase. The $r_{Pyrromethene}$ tended to increase with decreasing $X_{OA}$ as well as the $r_{DPH}$. However, as $X_{OA}$ decreased from 0.70 to 0.30, the change in $r_{BODIPY}$ was much more obvious than the changes in $r_{Pyrromethene}$ and $r_{DPH}$. This result correlated with our previous finding that when CHAPSO molecules are mixed with an OA bilayer membrane derived from an OA vesicle, the packing density of the OA bilayer membrane increases in $X_{OA} = 0.70$–0.30; consequently, the decrease in membrane fluidity and the increase in packing density correlated. DHPC molecules are inserted into a bilayer membrane to fluidize the bilayer membrane, whereas CHAPSO molecules are expected to adsorb on the hydrophilic groups of a bilayer membrane and act as spacers to fluidize the bilayer membrane (Fig. 2).
crease of the packing density around their hydrophilic groups and a decrease in membrane fluidity. BODIPY C12 can be employed for representing changes in membrane fluidity within the OA bilayer membrane of OA/CHAPSO bicelle. The higher \( r \) values of all probes in the CHAPSO micelle than in the OA vesicle can be explained by the internal structure of the CHAPSO micelle. The molecular-assembling morphology of CHAPSO molecules is thought to depend on the CHAPSO concentration in aqueous media\(^{30}\), and above \( \text{CMC}_{\text{CHAPSO}} \), a two-layer spherical structure is expected\(^{31}\). In the hydrophobic core of a CHAPSO micelle, the aliphatic groups of the inner layer interact with the steroid groups of the outer layer\(^{31, 32}\). This interaction within the core is expected to be stronger than the hydrophobic interaction between the unsaturated chains within the OA vesicle, and possibly suppresses rotation of the pyrromethene and DPH molecules inserted in the core. The prolate-ellipsoid shapes of CHAPSO micelles are also

Fig. 8  Size distributions of (a) 2 mM OA vesicle \( X_{\text{OA}} = 1.0 \) and (b–d) 20 mM OA/CHAPSO bicelles prepared at \( X_{\text{OA}} = 0.70, 0.50, \) and 0.30.

Fig. 9  \( r \) values probed by (circles) BODIPY C12, (squares) pyrromethene, and (triangles) DPH on (a) OA vesicles at 6, 14, and 20 mM and (b) OA/CHAPSO bicelles at 20 mM. The empty and solid symbols are the measurements at 1–2 days after preparation and after 2 weeks, respectively. All measurements were performed at 298 K.
thought to increase the r_{BODIPY} value. Moreover, all r values of the OA/CHAPSO bicelles remained almost unchanged for at least two weeks (Fig. 9b), suggesting that the OA/CHAPSO bicelles had dispersed in the aqueous solution without aggregation or precipitation.

4 Conclusion
The membrane fluidity of OA/CHAPSO bicelles was inferred from the anisotropy values of two fluorescent probe molecules, BODIPY C12 and pyrromethene. As the OA/CHAPSO bicelle has a heterogeneous bilayer membrane structure, its anisotropy could not be discussed using the conventional spectroscopic membrane-property evaluation method with DPH or Laurdan probes. The anisotropy value of pyrromethene, which has no carboxyl group, did depend on the membranes’ phase state, similarly to that of DPH. However, the core of BODIPY C12, which has a single chain with a terminal carboxyl group, was inserted in the center of the phospholipid bilayer membranes; consequently, the anisotropy of BODIPY C12 showed a smaller difference in the phase states of the phospholipid bilayer membranes than the other probes. Then, the anisotropy values of the probes in the OA vesicle were very similar, but were increased by mixing OA with CHAPSO solution. It suggested a decrease in membrane fluidity in more fluid bilayer structures such as OA/CHAPSO bicelles. Especially, the anisotropy of BODIPY C12 increased more significantly from \( X_{OA} = 0.70 \) to 0.30 than those of pyrromethene and DPH. The increased anisotropy values correlated well with the increase in membrane packing density. By combining the anisotropies of BODIPY C12 and pyrromethene, we could evaluate a membrane fluidity of the OA/CHAPSO bicelles. This technique is applicable to bicelles composed of more fluid bilayer structures and is useful for designing membrane materials from bicelle parents.

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Conflicts of Interest
The authors declare no conflicts of interest.

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