Microenvironment around Thi carbocyanine Dyes in Lysolecithin Micelles, Surfactant Micelles and Lecithin Liposomal Membranes

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The microenvironmental properties of lysolecithin micelles, surfactant (heptaethylene glycol dodecyl ether, cetyltrimethyl ammonium chloride, sodium dodecyl sulfate and dodecyl sulfobetaine) micelles and lecithin liposomal membranes in aqueous solutions were investigated by using dialkylthiacarbocyanine dyes as spectroscopic (absorption and fluorescence) probes. These probes give information concerning effective polarity and effective viscosity in the vicinity (microenvironment) of the probe in liposomal membranes and micelles. The microenvironment of the cyanine dyes in lysolecithin micelles have higher polarity (corresponding to that of ethanol) and lower viscosity (3—10 cP) than those of other surfactant micelles and lecithin liposomal membranes. The microenvironment provided by lecithin liposomal membranes has medium polarity (1-propanol) and the highest viscosity (10—65 cP) among the three kinds of amphiphilic molecular aggregates. Surfactant micelles provide microenvironments with a wide range of polarity (corresponding to ethanol, 1-propanol or 1-butanol) and lower viscosity (2—8 cP) than lecithin liposomal membranes. Thus, these molecular aggregates provide microenvironments of various polarities and viscosities, and a microenvironment suitable for solubilizing and stabilizing particular drugs can be provided by choosing appropriate molecular aggregates.

Keywords—microenvironment; thiacarbocyanine; lysolecithin; micelle; liposomal membrane; micropolarity; microviscosity

An important property of micelles and liposomal membranes is to provide less polar and more viscous environments for compounds solubilized in these molecular aggregates as compared with those in bulk water. Such a microenvironment provided by molecular aggregates not only contributes to improving the solubility of poorly soluble drugs,1 but also protects them against degradative processes such as hydrolysis2 and autoxidation.3 The hydrolysis of drugs is known to be affected by the polarity of the environment; e.g. the hydrolysis rate of indomethacin is markedly reduced by lowering the dielectric constant of the reaction mixture.4 It is also known that the membrane structure affects the efficiency of incorporation of nonpolar drugs, and the drugs tend to prefer more fluid membrane environments rather than more rigid ones.14 The efflux rate of nonpolar drugs from molecular aggregates has been suggested to be governed by the physical characteristics (particularly fluidity) of the micelles and the liposomal membranes.5 To select molecular aggregates suitable for the solubilization and the stabilization of drugs, experimental assessments of the polarity and viscosity provided by various micelles and liposomal membranes are important. In the present work, the microenvironments provided by lysolecithin micelles, surfactant micelles and lecithin liposomal membranes in aqueous solutions were investigated by using dialkylthiacarbocyanine dyes as spectroscopic probes. Dialkylthiacarbocyanine dyes were employed, because these compounds can probe the polarity and the viscosity in micelles or liposomal membranes simultaneously,6 and they are
also strong inhibitors of endogenous respiration of Ehrlich ascites tumor cells.\textsuperscript{7)}

**Experimental**

3,3'–Diyethyl-2,2'-thiacyrbocyanine iodide, 3-methyl-3'-octadecyl-2,2'-thiacyrbocyanine bromide (C\textsubscript{18–18}) and 3,3'-diotadecyl-2,2'-thiacyrbocyanine bromide (C\textsubscript{18–18}) were purchased from the Japan Research Institute for Photosensitizing Dye Co., Ltd. L-α-Lauroyl lysophosphatidylcholine (lysolecithin) (LysoPC), l-α-myristoyl lysolecithin (MlysoPC), l-α-palmitoyl lysolecithin (PlysoPC) were supplied by Sigma Chemical Company. L-α-Dimyristoyl phosphatidylcholine (lecithin) (DMPC), L-α-dipalmitoyl lecithin (DPPC) and L-α-distearoyl lecithin (DSPC) were also obtained from Sigma Chemical Company. Heptaethyl glycol dodecyl ether (HED) was obtained from Nikko Chemicals Co., Ltd. Sodium dodecyl sulfate (SDS) and cetyltrimethylammonium chloride (CTAC) were purchased from Nakarai Chemicals Ltd. Dodecyl sulfobetaine (DSB) was obtained from Serva Feinbiochemical GmbH. Tris(hydroxymethyl)aminomethane (Tris) was used as received. Water was doubly distilled from a quartz still. Cyanine dyes, lysolecithins and lecithins were stocked as chloroform–ethanol (1:1, v/v) solutions below 4°C.

For preparations of lysolecithin micelles and other surfactant micelles, stock solutions of cyanine dyes and of lysolecithins or surfactants were mixed and then the solvent was evaporated off and the residue was dried in a vacuum desiccator at room temperature for 24–48 h. Then, 20 nm Tris–HCl buffer solution (pH 7.0) was added. The values of critical micelle concentration (cmc) of lysolecithins in the aqueous buffer solution at 25°C of 5.0 x 10\textsuperscript{-4} M for LysoPC, 6.3 x 10\textsuperscript{-5} M for MlysoPC and 8.3 x 10\textsuperscript{-5} M for PlysoPC. The cmc values of surfactants in the aqueous buffer solution at 25°C are 8 x 10\textsuperscript{-5} M for HED, 8 x 10\textsuperscript{-5} M for SDS, 9.2 x 10\textsuperscript{-5} M for CTAC and 3.58 x 10\textsuperscript{-3} M for DSB.

Liposomal dispersions were prepared by the following procedure. Aliquots of stock solutions of lecithins and of cyanine dyes were mixed and dried to a thin film on the wall of a round-bottomed flask. A trace of remaining solvent was removed by drying for 24–48 h at room temperature under a vacuum. Removal of the lipid–cyanine dye film from the wall was accomplished by vortexing with 10 ml of the buffer solution. The lecithin solution was dispersed by sonication at about 50°C for 15 min using a Tomy Seiko model UR-200P. C\textsubscript{18–18} and C\textsubscript{18–18} are practically insoluble in water, and therefore, all cyanine dyes are incorporated into micelles or liposomal membranes.

Absorption spectra were obtained using a Shimadzu 180 spectrophotometer. To determine the wavelengths of the absorption maxima of the cyanine dyes in various micelles and liposomal membranes, the first derivative absorption spectra were measured with a Hitachi 220 spectrophotometer. Fluorescence spectra were obtained by the use of a Jasco FB-550 spectrophotometer. The excitation wavelength was 530 nm for both cyanine dyes. The absorbance of the cyanine dyes at the excitation wavelength was less than 0.05. The fluorescence yields of the cyanine dyes in various micelles and liposomal membranes were evaluated as the fluorescence intensities integrated over the spectra and divided by the absorbance at the excitation wavelength.

All measurements were carried out at 25°C.

**Results**

**Estimation of Micropolarity in the Vicinity of Cyanine Dyes**

Figure 1 shows the absorption and emission spectra of C\textsubscript{18–18} in methanol. In our previous work,\textsuperscript{10} it was demonstrated that C\textsubscript{18–18} and C\textsubscript{18–18} show large shifts in their absorption maxima in various aliphatic alcohols as solvents; the wave numbers at their absorption maxima (\(v_{\text{max}}\)) increase linearly with the dielectric constant (\(D\)) of the alcohols, as shown in Fig. 2. The dielectric constant in the vicinity of the chromophore of the dye molecules in various micelles and liposomal membranes, which has been defined as the effective dielectric constant (\(D_{\text{eff}}\)) in our studies, was estimated by the use of the relationship between the \(v_{\text{max}}\) values of the cyanine dyes in various molecular aggregates and the \(D\) values of aliphatic alcohols. The \(v_{\text{max}}\) values of the dyes in HED micelles as an example are shown by the arrows in Fig. 2. The above procedures led to values for \(D_{\text{eff}}\) of 18.5 for C\textsubscript{18–18} and 20.0 for C\textsubscript{18–18}. The values of \(v_{\text{max}}\) and \(D_{\text{eff}}\) of the dyes in other molecular aggregates are shown in Table I. The \(D_{\text{eff}}\) values of the dyes in various molecular aggregates are in the range of about 18 to 25, indicating that the effective micropolarity in the vicinity of the chromophore of the cyanine dyes in various micelles and liposomal membranes corresponds to that of ethanol, 1-propanol or 1-butanol. The micropolarity provided by the three kinds of aggregates decreased in the order of lysolecithin micelles \(\geq\) lecithin liposomal membranes \(\approx\) surfactant micelles.
TABLE I. Experimental Values for C\textsubscript{18--18} and C\textsubscript{18--18} in Various Micelles and Liposomal Membranes

<table>
<thead>
<tr>
<th>Dye</th>
<th>Surfactant or lipid</th>
<th>Aggregate</th>
<th>( \phi_i^{R} ) ( ^{a)} )</th>
<th>( D_{eff} ) ( ^{b)} )</th>
<th>( \phi_i^{0R} ) ( ^{c)} )</th>
<th>( k_i^{A} / k_B A \times 10^{-2} ) ( ^{(KcP^{-1})} )</th>
<th>( \eta_{eff} ) ( ^{e)} ) (cP)</th>
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<tr>
<td>C\textsubscript{18--18}</td>
<td>HED</td>
<td>Micelle</td>
<td>4.1</td>
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<td></td>
<td>SDS</td>
<td></td>
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<td>12.2</td>
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<tr>
<td></td>
<td>CTAC</td>
<td></td>
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<td>11.8</td>
<td>5.1</td>
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</tr>
<tr>
<td></td>
<td>DSB</td>
<td></td>
<td>3.3</td>
<td>22.6</td>
<td>11.7</td>
<td>5.0</td>
<td>2.7</td>
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<tr>
<td></td>
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<td>25.0</td>
<td>11.5</td>
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<tr>
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<td>12.2</td>
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<td>21.6</td>
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<tr>
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<td>Liposome</td>
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<td></td>
<td>DPPC</td>
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<td>63.4</td>
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\( a) \\phi_i^{R} \), relative fluorescence yield. \( b) D_{eff} \), effective dielectric constant. \( c) \phi_i^{0R} \), relative fluorescence yield when the relaxation via a twisting motion is absent. \( d) k_i(k_B A) \), a value increasing with the effective volume associated with the twisting motion. \( e) \eta_{eff} \), effective viscosity.

**Estimation of Microviscosity in the Vicinity of Cyanine Dyes**

The fluorescence yields of the cyanine dyes in various micelles and liposomal membranes were evaluated (\( \phi_i^{R} \)) relative to that of 3,3'-diethyl-2,2'-thiacarbocyanine in the aqueous phase. The \( \phi_i^{R} \) values of the cyanine dyes in various molecular aggregates increased with
increasing concentration of surfactants or lipids at a constant concentration (0.5 μM) of the cyanine dyes. Figure 3 presents plots of the $\phi^R$ values versus the ratio of the concentration of C$_{1-18}$ to that of lysolecithins as an example, yielding linear relationships. This is because the dilution of the cyanine dyes with increasing concentration of the surfactants or the lipids interferes with fluorescence quenching (self-quenching) due to the association of cyanine dye molecules in these molecular aggregates. To prevent the contribution of self-quenching to the fluorescence yield, the fluorescence yield of the cyanine dyes in the monomer state (at infinite dilution of the cyanine dyes) should be adopted as the fluorescence yield reflecting the polarity and viscosity in the vicinity of the dyes. Therefore, in this study, the fluorescence yields of the cyanine dyes in the lysolecithin micelles and the lecithin liposomal membranes were evaluated by extrapolation of the linear plot to the $\phi^R$ axis in Fig. 3. In surfactant micelles, the
fluorescence yields of the cyanine dyes at the surfactant concentration of 400 mM were used. The fluorescence yields of the dyes in various micelles and liposomal membranes evaluated by the above procedures are presented in Table I.

Figure 4 shows the fluorescence yields of the cyanine dyes as a function of the D values of straight-chain aliphatic alcohols and those of the cyanine dyes in various molecular aggregates. The fluorescence yields are larger in solvents of lower polarity. Furthermore, C_{18-18} having two long alkyl chains, has greater fluorescence yields than C_{1-18} in all molecular aggregates and solvents studied here. The fluorescence yield in molecular aggregates is larger than that in a solvent where the cyanine dyes are exposed to the same dielectric constant as that in the molecular aggregates. The microenvironmental effects on the fluorescence yields of dyes presumably include polarity and also viscosity factors. These are usually associated with each other in a complex way.

Most carbocyanine dyes exhibit intense twisting motions along the polymethine chain in the electronically excited state. This is because the conjugated π electronic structure is broken in the excited state, allowing twisting motions of the substituent groups at each end and destruction of the initial planar structure. The capability for such intramolecular motion, which may be strongly affected by the viscosity in the vicinity of the dye molecule, is associated with the internal conversion (radiationless relaxation) process of the singlet excited state to the ground state, and therefore, with fluorescence yield.

In our previous studies, it was found that the following relation holds between the relative fluorescence yield of the cyanine dyes (\(\phi^R\)) and the viscosity (\(\eta\)) of the medium,

\[
\frac{1}{\phi^R} = \frac{1}{\phi^R_{\text{rel}}} \frac{k_B A}{k_B A T} \frac{k_T}{k_T \eta}
\]

Here, \(A\) is a parameter which decreases with increase of the effective volume associated with the twisting motion, \(k_T\) is the rate constant for radiative (fluorescence) relaxation of the singlet excited state, \(k_B\) is the Boltzmann constant, and \(T\) is absolute temperature. Also, \(\phi^R_{\text{rel}}\) is the relative fluorescence yield when the relaxation via the twisting motion is absent and is expressed as \(\phi^R_{\text{rel}} = \alpha k_T / (k_R + k_{\text{isc}} + k_T)\), where \(k_R\) is the rate constant of the usual internal conversion which is independent of the viscosity in the vicinity of the dye molecule, \(k_{\text{isc}}\) is the rate constant for the intersystem crossing of the singlet excited state and \(\alpha\) is a proportionality constant. The first and the second terms on the right-hand side of Eq. 1 express the contributions of the polarity and viscosity of the medium to the fluorescence yield, respectively. In our previous studies, Eq. 1 was examined in relation to the viscosity

![Fig. 5](image-url)
change of a single solvent with change of temperature, and it was found that plots of the reciprocal of $\phi_l^R$ for $C_{1-18}$ and $C_{18-18}$ in straight-chain aliphatic alcohols versus $T/\eta$ give straight lines. The slope and the intercept of the lines allow $k_{R}A/k_{L}$ and $1/\phi_l^R$ to be evaluated. Figure 5\textsuperscript{10} shows the values of $k_{L}((k_{R}A)$ and $\phi_l^R$ as a function of the $D$ values for $C_{1-18}$ and $C_{18-18}$. The greater the polarity of the solvent, the smaller is $\phi_l^R$ and the larger is $k_{L}((k_{R}A)$.

In the same solvent, the $k_{L}((k_{R}A)$ value for $C_{18-18}$ is about twice as large as that for $C_{1-18}$.

Rearrangement of Eq. 1 leads to

$$\eta = \frac{T \phi_l^R \phi_l^R}{(\phi_l^R - \phi_l^R) k_{L}((k_{R}A)}$$

When Eq. 2 is applied to micellar and liposomal membrane systems, the $\phi_l^R$ and $k_{L}((k_{R}A)$ values in the solvent where the cyanine dyes are exposed to the same effective polarity as in the particular molecular aggregates should be used. Therefore, in the case of HED micelles, for example, the values of $\phi_l^R$ and $k_{L}((k_{R}A)$ of the cyanine dyes in alcohols of $D = 18.5$ for $C_{1-18}$ and $20.0$ for $C_{18-18}$, are assigned as those in the HED micelles (see Fig. 5). Introduction of the observed $\phi_l^R$ value in the HED micelles into Eq. 2 yields a value for effective viscosity ($\eta_{eff}$) in the HED micelles. Table I shows the $\eta_{eff}$ values obtained by such a procedure together with the values of $\phi_l^R$ and $k_{L}((k_{R}A)$ in the micelles and the liposomal membranes. The $\eta_{eff}$ values in the lysolceithin micelles and the other surfactant micelles are 2—10 cP and the values in the liposomal membranes are 10—65 cP.

**Discussion**

In order to evaluate micropolarity in micelles and liposomal membranes, the wave numbers of the absorption maxima of solubilized cyanine dyes were measured and their dependence on solvent polarity was examined. The reasons for employing aliphatic alcohols to calibrate the micropolarity in micellar phases have already been discussed in detail.\textsuperscript{12} As seen from Table I, the chromophores of the dye molecules solubilized in the micelles and the liposomal membranes are accommodated in a remarkably less polar environment than in the aqueous phase ($D = 78.5$), but they seem to be in a more polar environment than in a liquid hydrocarbon ($D = 2—5$). These results indicate that the chromophore of the cyanine dye is located in the surface region of the micelles and the liposomal membranes.
The microviscosity ($\eta_{\text{eff}}$) is 2–10 cP in the lysocleithin and surfactant micelles and 10–65 cP in the lecithin liposomal membranes. Values of microviscosity in the range of 8–40 cP have been estimated in various micelles by nuclear magnetic resonance relaxations, fluorescence depolarization and intramolecular eximer formation rate studies. By similar methods, microviscosity in lecithin liposomal membranes was estimated to be 40–200 cP. Liposomal bilayers are not flexible and provide considerably more rigid interiors than micelles. It is known that microviscosity in micelles and liposomal membranes is dependent on the kind of molecular motion associated with the method of measurement and on the location of the probe in the molecular aggregates. Here, the motion associated with the dynamic internal conversion is twisting with a slight angle along the polymethine chain of the dye molecule, and it is influenced by the motions of the hydrocarbon chains of surfactant and lipid molecules around the long alkyl chains (octadecyl groups) of the cyanine dye molecules. This is supported by the fact that in the same solvent the value of $k_e/(k_B A)$, which increases with the effective volume associated with the twisting motion, for C$_{18}$–18 having two long chains is about twice as large as that for C$_{18}$–18 having only one long chain.

Figure 6 shows the effective dielectric constant ($D_{\text{eff}}$) and effective viscosity ($\eta_{\text{eff}}$) in the vicinity of the cyanine dyes in various micelles and liposomal membranes. The three kinds of molecular aggregates give distinct groups of microenvironment (the dotted lines in Fig. 6 indicate the borders of the three kinds of molecular aggregates). The microenvironment of the cyanine dyes provided by lysocleithin micelles is one of higher polarity and lower viscosity. That provided by lecithin liposomal membranes has medium polarity and the highest viscosity among the three kinds of the aggregates. Surfactant micelles provide a microenvironment having a wide range of polarity and lower viscosity. Thus, micelles and liposomal membranes provide microenvironments of various polarities and viscosities.

If the type of microenvironment suitable for solubilizing and stabilizing a drug is known, it should be possible to provide a suitable microenvironment by choosing an appropriate molecular aggregate.

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References