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

MASAYUKI NAKAGAKI,[a] HIROAKI KOMATSU,[*,a] and TETSUROU HANDA[b]

*Faculty of Pharmaceutical Sciences, Kyoto University,[a] Yoshidashimoadachi-cho,
Sakyo-ku, Kyoto 606, Japan and Gifu Pharmaceutical University,[b]
Mitahorahigashi, Gifu 502, Japan*

The microenvironments provided by lysolecithin micelles, surfactant (heptaethylene glycol dodecyl ether, cetyltrimethylammonium 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 (micro-environment) 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 hydrolysis[2] and autooxidation.[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.[1d] 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 lyso-lecithin 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.[7]

Experimental

3,3'-Diethyl-2,2'-thiacarbocyanine iodide, 3-methyl-3'-octadecyl-2,2'-thiacarbocyanine bromide (C_{1-18}) and 3,3'-dioctadecyl-2,2'-thiacarbocyanine bromide (C_{18-18}) were purchased from the Japan Research Institute for Photosensitizing Dye Co., Ltd. L-α-Lauroyl lysophosphatidylcholine (lysolecithin) (LlysoPC), 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. Heptaethylene 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 mM 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[8] are 5.0×10^{-4} M for LlysoPC, 6.3×10^{-5} M for MlysoPC and 8.3×10^{-6} M for PlysoPC. The cmc values of surfactants in the aqueous buffer solution at 25 °C[9] are 8×10^{-5} for HED, 8×10^{-3} M for SDS, 9.2×10^{-4} M for CTAC and 3.58×10^{-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_{1-18} and C_{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 spectrofluorimeter. 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_{18-18} in methanol. In our previous work,[10] it was demonstrated that C_{1-18} and C_{18-18} show large shifts in their absorption maxima in various aliphatic alcohols as solvents; the wave numbers at their absorption maxima ($\tilde{\nu}_{max}$) 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_{eff}) in our studies, was estimated by the use of the relationship between the $\tilde{\nu}_{max}$ values of the cyanine dyes in various molecular aggregates and the D values of aliphatic alcohols. The $\tilde{\nu}_{max}$ 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_{eff} of 18.5 for C_{1-18} and 20.0 for C_{18-18}. The values of $\tilde{\nu}_{max}$ and D_{eff} of the dyes in other molecular aggregates are shown in Table I. The D_{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 ≥ lecithin liposomal membranes ≈ surfactant micelles.
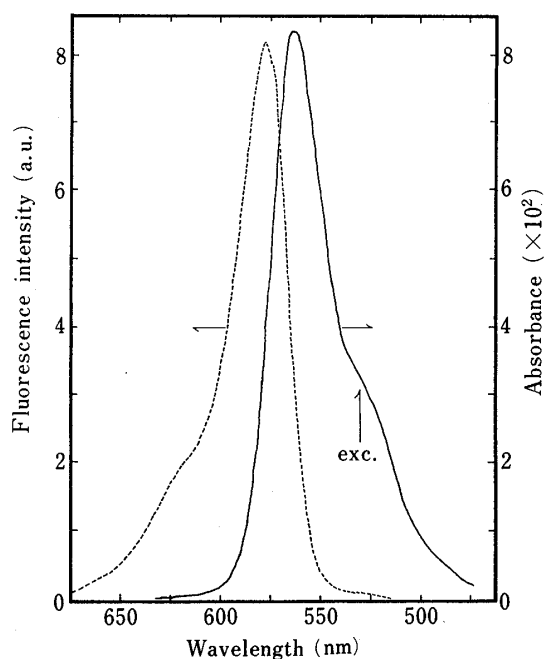
Fig. 1. Absorption and Emission (Excited at 530 nm) Spectra of 0.5 μM C_{1-18} in Methanol at 25 °C

The chemical structures of probe dyes (C_{1-18} and C_{18-18}) are also shown. The fluorescence intensity of the emission spectra is not corrected.
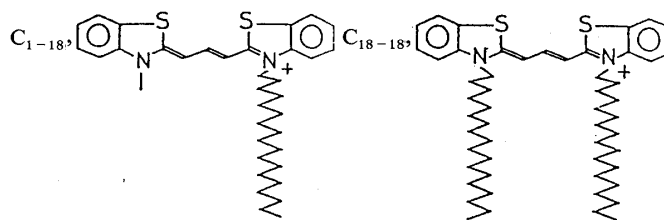
TABLE I. Experimental Values for C_{1-18} and C_{18-18} in Various Micelles and Liposomal Membranes

Dye	Surfactant or lipid	Aggregate	$\phi_f^{R\ a)}$	$D_{eff}^{\ b)}$	$\phi_f^{OR\ c)}$	$\dfrac{k_f^{\ d)}}{k_B A} \times 10^{-2}$ (KcP^{-1})	$\eta_{eff}^{\ e)}$ (cP)
C_{1-18}	HED	Micelle	4.1	18.5	12.5	4.2	4.5
	SDS		4.5	19.3	12.2	4.1	5.2
	CTAC		3.0	22.5	11.8	5.1	2.4
	DSB		3.3	22.6	11.7	5.0	2.7
	LlysoPC		3.8	25.0	11.5	6.2	2.9
	MlysoPC		4.3	23.5	11.6	5.5	3.7
	PlysoPC		5.2	22.0	11.7	4.9	5.7
	DMPC	Liposome	6.5	18.9	12.2	4.1	10.0
	DPPC		8.2	20.5	12.0	4.5	17.0
	DSPC		8.8	21.6	11.8	4.8	21.4
C_{18-18}	HED	Micelle	6.2	20.0	14.2	8.2	5.0
	SDS		8.4	18.0	14.5	7.0	8.6
	CTAC		4.5	20.0	14.2	8.2	2.4
	DSB		5.1	23.0	11.6	10.4	2.6
	LlysoPC		7.0	24.5	9.3	11.6	7.1
	MlysoPC		8.5	23.0	11.7	10.3	9.1
	PlysoPC		9.0	22.7	12.0	10.1	10.4
	DMPC	Liposome	9.5	21.0	13.4	8.8	10.9
	DPPC		12.3	21.4	13.1	9.1	63.4

a) ϕ_f^R, relative fluorescence yield. b) D_{eff}, effective dielectric constant. c) ϕ_f^{OR}, relative fluorescence yield when the relaxation via a twisting motion is absent. d) $k_f/(k_B A)$, a value increasing with the effective volume associated with the twisting motion. e) η_{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 (ϕ_f^R) relative to that of 3,3'-diethyl-2,2'-thiacarbocyanine in the aqueous phase. The ϕ_f^R values of the cyanine dyes in various molecular aggregates increased with
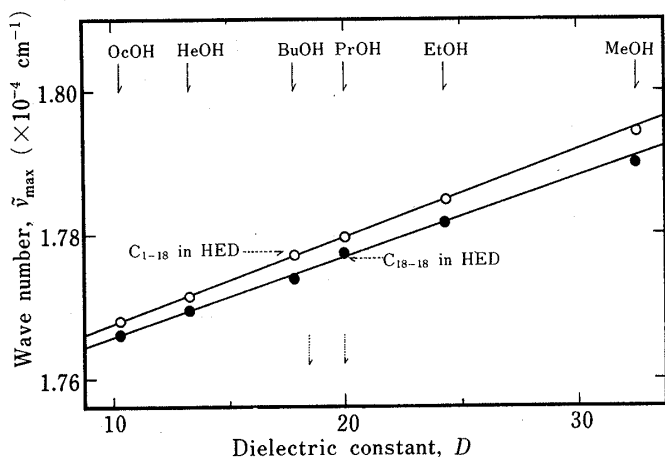
Fig. 2. Relationship of the Wave Number of Absorption Maxima ($\tilde{\nu}_{max}$) for C_{1-18} and C_{18-18} with the Dielectric Constant (D) of Solvents[10]

MeOH, methanol; EtOH, ethanol; PrOH, 1-propanol; BuOH, 1-butanol; HeOH, 1-hexanol; OcOH, 1-octanol. $\bigcirc$, C_{1-18}; ●, C_{18-18}.
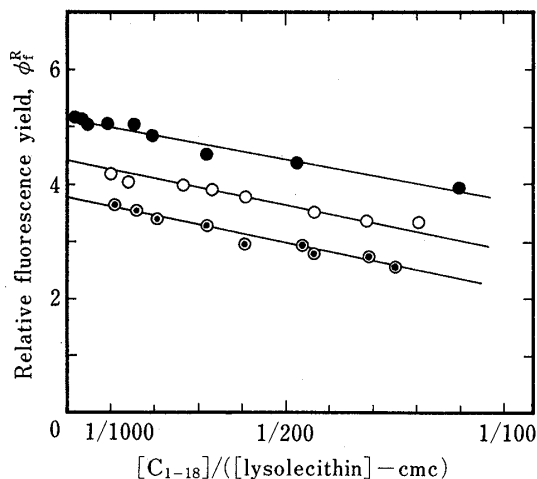

Fig. 3. Increments of Fluorescence Yield (ϕ_f^R) of C_{1-18} with Increasing Concentration of Lysolecithins
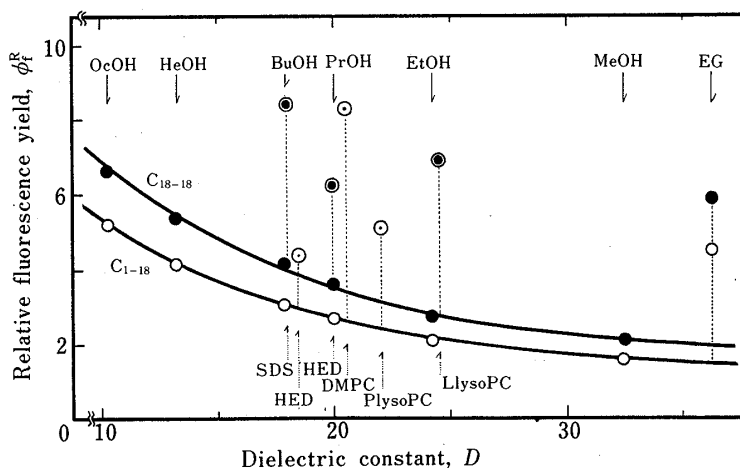
$\bigcirc$, MlysoPC; ●, PlysoPC; ◉, LlysoPC.

Fig. 4. Relationship of Fluorescence Yield (ϕ_f^R) for C_{1-18} and C_{18-18} with Dielectric Constant (D) of Solvents

($\bigcirc$) and (●), C_{1-18} and C_{18-18} in solvent; (◉) and (◉), C_{1-18} and C_{18-18} in sodium dodecyl sulfate (SDS) and heptaethylene glycol dodecyl ether (HED) micelles, and dimyristoyl lecithin liposomal membrane (DMPC). EG, in ethyleneglycol solution.

increasing concentration of surfactants or lipids at a constant concentration (0.5 μM) of the cyanine dyes. Figure 3 presents plots of the ϕ_f^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 ϕ_f^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[10] 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.[11] 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.[11]

In our previous studies,[6] it was found that the following relation holds between the relative fluorescence yield of the cyanine dyes (ϕ_f^R) and the viscosity (η) of the medium,

$$\frac{1}{\phi_f^R} = \frac{1}{\phi_f^{OR}} + \frac{k_B A}{k_f}\frac{T}{\eta} \tag{1}$$

Here, A is a parameter which decreases with increase of the effective volume associated with the twisting motion, k_f 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, ϕ_f^{OR} is the relative fluorescence yield when the relaxation via the twisting motion is absent and is expressed as $\phi_f^{OR} = \alpha k_f/(k_a + k_{isc} + k_f)$, where k_a is the rate constant of the usual internal conversion which is independent of the viscosity in the vicinity of the dye molecule, k_{isc} is the rate constant for the intersystem crossing of the singlet excited state and α 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,[6,10] Eq. 1 was examined in relation to the viscosity
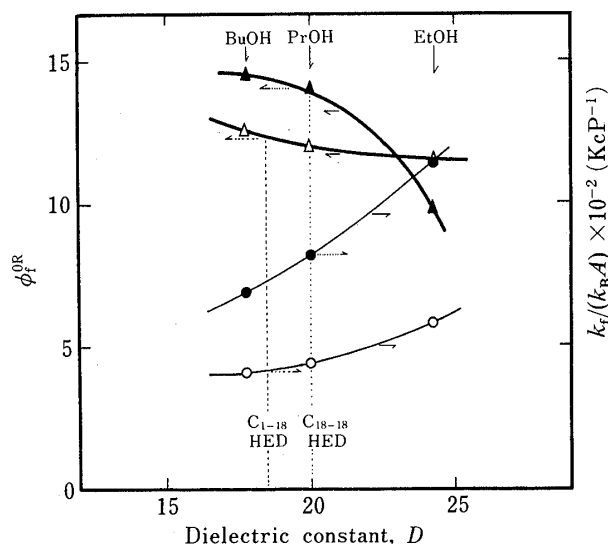

Fig. 5. ϕ_f^{OR} and $k_f/(k_B A)$ Shown as a Function of Dielectric Constant (D)[10]

ϕ_f^{OR}, the relative fluorescence yield when the relaxation via intrarotation (twisting) motion is absent; $k_f/(k_B A)$, the value increasing with the effective volume associated with the twisting motion. $\bigcirc, \triangle$; C_{1-18}. $\bullet, \blacktriangle$; C_{18-18}.

change of a single solvent with change of temperature, and it was found that plots of the reciprocal of ϕ_f^R for C_{1-18} and C_{18-18} in straight-chain aliphatic alcohols versus T/η give straight lines. The slope and the intercept of the lines allow $k_B A/k_f$ and $1/\phi_f^{OR}$ to be evaluated. Figure 5[10] shows the values of $k_f/(k_B A)$ and ϕ_f^{OR} 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 ϕ_f^{OR} and the larger is $k_f/(k_B A)$. In the same solvent, the $k_f/(k_B A)$ value for C_{18-18} is about twice as large as that for C_{1-18}.

Rearrangement of Eq. 1 leads to

$$\eta = \frac{T\phi_f^{OR}\phi_f^R}{(\phi_f^{OR} - \phi_f^R)\dfrac{k_f}{k_B A}} \tag{2}$$

When Eq. 2 is applied to micellar and liposomal membrane systems, the ϕ_f^{OR} and $k_f/(k_B A)$ 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 ϕ_f^{OR} and $k_f/(k_B A)$ 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 ϕ_f^R value in the HED micelles into Eq. 2 yields a value for effective viscosity (η_{eff}) in the HED micelles. Table I shows the η_{eff} values obtained by such a procedure together with the values of ϕ_f^R and $k_f/(k_B A)$ in the micelles and the liposomal membranes. The η_{eff} values in the lysolecithin 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.[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.
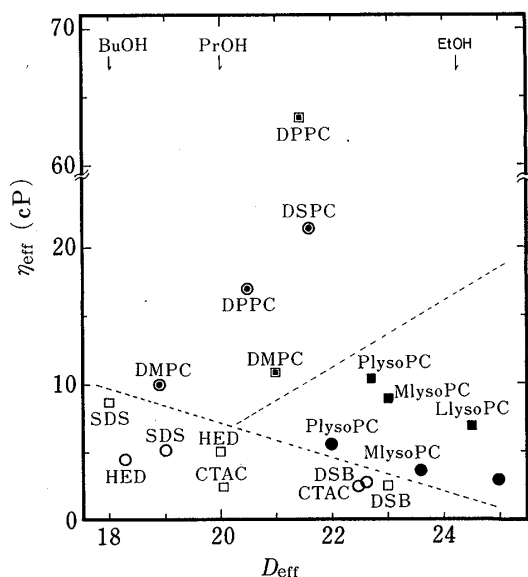

Fig. 6. The Relation between Effective Dielectric Constant (D_{eff}) and Effective Viscosity (η_{eff}) in the Vicinity of the Cyanine Dyes (C_{1-18} and C_{18-18}) in Various Micelles and Liposomal Membranes

○, ●, ◉ ; C_{1-18}. □, ■, ▣; C_{18-18}.

The microviscosity (η_{eff}) is 2—10 cP in the lysolecithin 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,[13] fluorescence depolarization[14] and intramolecular eximer formation rate[15] studies. By similar methods, microviscosity in lecithin liposomal membranes was estimated to be 40—200 cP.[16] 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.[17] Here, the motion associated with the dynamic internal conversion is twisting with a slight angle along the polymethine chain of the dye molecule,[18] and it 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 $k_f/(k_BA)$, 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_{1-18} having only one long chain.

Figure 6 shows the effective dielectric constant (D_{eff}) and effective viscosity (η_{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 lysolecithin 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.

References

1) *a*) R. L. Julian, "Liposomes," ed. by M. J. Ostro, Marcel Dekker, Inc., New York and Basel, 1983, p. 53; *b*) E. J. Fendler and J. H. Fendler, *Adv. Phys. Org. Chem.*, **8**, 271 (1970); *c*) S. Riegelman, *J. Pharm. Sci.*, **49**, 339 (1960); *d*) D. Stamp and R. L. Juliano, *Can. J. Physiol. Pharmacol.*, **57**, 535 (1979); *e*) J. B. D'Silva and R. E. Notari, *J. Pharm. Sci.*, **71**, 1394 (1982).
2) *a*) H. Nogami, S. Awazu, and M. Iwatsuru, *Chem. Pharm. Bull.*, **11**, 1251 (1958); *b*) P. B. Sheth and E. L. Parrot, *J. Pharm. Sci.*, **56**, 983 (1967); *c*) J. A. Hamid and E. L. Parrot, *ibid.*, **60**, 901 (1971).
3) M. I. Amin and J. T. Bryan, *J. Pharm. Sci.*, **62**, 1768 (1973).
4) H. Krasowka, *Int. J. Pharmaceut.*, **4**, 89 (1979).
5) R. L. Juliano and D. Stamp, *Biochim. Biophys. Acta*, **586**, 137 (1979).
6) T. Handa, H. Komatsu, K. Matsuzaki, and M. Nakagaki, *Nippon Kagaku Kaishi*, **1984**, 8.
7) E. Okimasu, J. Akiyama, N. Shiraishi, and K. Utsumi, *Physiol. Phys.*, **11**, 425 (1970).
8) M. Nakagaki, H. Komatsu, and T. Handa, *Chem. Pharm. Bull.*, **34**, 4479 (1986), and the 106th Annual Meeting of the Pharmaceutical Society of Japan, Chiba, April 1986, p. 724 (3P 11-4).
9) P. Mukerjee and K. J. Mysels, "Critical Micelle Concentrations of Aqueous Surfactant Systems," National Standards Reference Data Series, Vol. 36, National Bureau of Standards, Washington D.C., 1971.
10) M. Nakagaki, H. Komatsu, and T. Handa, *Bull. Chem. Soc. Jpn.*, **58**, 3197 (1985).
11) D. Dempster, T. Morrow, R. Rankin, and G. F. Thompson, *J. Chem. Soc., Faraday Trans. 2*, **68**, 1479 (1972); R. H. Baker, M. Gräzel, and R. Steiger, *J. Am. Chem. Soc.*, **102**, 847 (1980).
12) P. Mukerjee and J. R. Cardinal, *J. Phys. Chem.*, **82**, 1620 (1978); P. Mukerjee, C. Ramachandran and R. Pyter, *ibid.*, **86**, 3189 (1982).
13) F. M. Menger and J. M. Jetkunica, *J. Am. Chem. Soc.*, **100**, 688 (1978).

14) M. Shinitzky, A. C. Dianoux, C. Gilter, and G. Weber, *Biochemistry*, **10**, 2106 (1971).
15) N. J. Turro, W. Aikawa, and A. Yekta, *J. Am. Chem. Soc.*, **101**, 774 (1079).
16) J. R. Lakowiz, F. G. Prendergast, and D. Hogen, *Biochemistry*, **18**, 508 (1979); K. Kano and J. H. Fendler, *Chem. Phys. Lipids*, **23**, 189 (1979).
17) J. H. Fendler, "Membrane Mimetic Chemistry," John Wiley and Sons, Inc., New York, 1982, p. 223.
18) J. Jaraudias, *J. Photochem.*, **13**, 35 (1980).