Effect of the Phase Transition in Liposomes on the Fluorescence of Amphiphilic Cyanine Dyes

Kaoru Onuki, Kazue Kurihara, Yoshinori Toyoshima,† and Mitsunori Sukigara*

Institute of Industrial Science, The University of Tokyo, 7—22—1, Roppongi, Minato-ku, Tokyo 106

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The dependence of fluorescence intensity of amphiphilic cyanine dyes on the thermotropic fluid-solid phase transition of lipid bilayer membranes has been investigated. *N,N'*-dioctadecyl oxacarbocyanine iodide or *N,N'*-dioctadecyl thiacarbocyanine bromide was incorporated into single lamellar liposomes, and the fluorescence intensity of each dye was observed to be modified considerably by the phase transition of the lipid bilayers. An amphiphilic cyanine dye with single long alkyl chain, *N*-octadecyl-*N'*-methyl thiacarbocyanine bromide was also incorporated in the lipid bilayers, but in this case, the phase transition showed little effect on the fluorescence intensity. These results were interpreted in terms of the effect of the bilayer membrane fluidity and rigidity on the rotational motion of the incorporated cyanine dyes. The phase transition parameters were obtained by applying those amphiphilic dyes as the fluorescence probe of the phase transition of the liposomes.

The fluidity of the hydrocarbon chain in a lipid bilayer membrane has been known to play an important role in the functions of the biological membrane[1,2]. The fluid-solid phase transitions observed in many kinds of lipid membranes are associated with an abrupt change in the membrane characteristics, such as NMR relaxation time[3,4] and permeability,[5] and many methods have been applied for detecting the phase transition, *e.g.* the calorimetry which directly measures the enthalpy change of transition,[6-8] ESR spin label method by which the solubility or the mobility change of the probes in a membrane is detected,[9,10] and light scattering method.[11] Fluorescence probes were also used widely.[12] Fluorescence probes so far used are mainly lyophobic fluorescent materials such as 1,6-diphenyl-1,3,5-hexatriene (DPH) which are localized in the hydrocarbon chain region of the membrane, and its fluorescence anisotropy reflects the microviscosity of that region.[13] Also, the 5-(dimethylamino)-1-naphthylsulfonyl group or 9-anthracenecarbonyl group which covalently binds to the alkyl chain of lipid was used for detecting the microviscosity of the chains[14,15].

A cyanine dye, composed of two long *N*-alkyl chains and a chromophore, has an amphiphilic molecular structure and can be incorporated into a bilayer membrane as a constituent.[16] We reported previously the effect of the phase transition on the aggregation of the dye in the vesicle.[17] Calzaferry *et al.*[18] reported that twisting around the conjugated hydrocarbon bond plays an important role in the nonradiative relaxation of excitation energy. So it is expected that a change in the fluorescence intensity of a cyanine dye incorporated into a lipid bilayer will reflect the difference of the microviscosity of the bilayer. We examined the temperature dependence of the fluorescence intensity of cyanine dyes, such as *N,N'*-dioctadecyl oxacarbocyanine iodide (DSOCC), *N,N'*-dioctadecyl thiacarbocyanine bromide (DSSCC), and *N*-octadecyl-*N'*-methyl thiacarbocyanine bromide (MSSCC) incorporated into a single bilayer vesicle composed of dimyristoyl phosphatidylcholine (DMPC) or dipalmitoyl phosphatidylcholine (DPPC).

† Permanent address: Faculty of Integrated Arts and Sciences, Hiroshima University, Higashisendamachi, Hiroshima 730.

Experimental

DMPC and DPPC were purchased from Sigma Chem. Co. and the cyanine dyes were purchased from the Japanese Research Institute for Photosensitizing Dyes Co. Ltd. and used without further purification. The purity of the reagents was checked by thin layer chromatography. KCl and tris-(hydroxymethyl)aminomethane (tris) of analytical grade were used.

Phosphatidylcholine colyophilized under vacuum with a dye of a desired composition from a benzene solution was suspended in 10 ml of buffered 0.1 M KCl aqueous solution (0.1 M KCl in 0.01 M tris–HCl at pH 7.5). The electrolyte concentrations in the dispersion were kept constant throughout the experiments. The suspension was ultrasonically irradiated (Otake Seisakusho OT-5202 Sonicator) for 20 min at 20 kHz under an argon atmosphere. The sonication temperature was kept about 5 °C above the phase transition temperature of the lipid. After sonication the sample was centrifuged at 13000 r.p.m. for 10 min to remove undispersed reagents.

The phosphatidylcholine concentration in the vesicle dispersion was checked by the Fiske-Subbarow method.[19] The lipid concentration was 1—3 mg/dm^3 throughout the experiments. Concentration of DSOCC, DSSCC, and MSSCC in the vesicle dispersion were estimated from the absorbances at 492 nm, 560 nm, and 560 nm respectively attributed to the monomer bands, using the molecular extinction coefficients determined in methanol. The dye concentration in the dispersion was below 7×10^{-7} mol/dm^3 throughout the experiments. Thus the molar ratio of lipid to dye was 4600—7000, or the local concentration of the dye was less than 1 molecule/vesicle throughout the experiments.

Absorption measurements were carried out using a spectrophotometer, Beckman 25. Fluorescence measurements were performed on a spectrofluorophotometer, Hitachi MPF-2A under argon atmosphere. The temperature was monitored with a thermocouple dipped in the sample cell. The decrease of excitation light intensity caused by the light scattering by the vesicles had little effect on the fluorescence measurement.

Results and Discussion

The fluorescence spectra of the dyes, DSOCC, DSSCC, and MSSCC, in vesicles were respectively quite similar to those in methanol solutions. The fluorescence intensity of DSOCC and that of DSSCC

in the vesicles were two to six times as large as those of the dyes in methanol at 20 °C while the fluorescence intensity of MSSCC was twice the intensity of the same dye in methanol. These facts are interpreted qualitatively in terms of the difference of effective viscosity of the media affecting the freedom of motion of the dyes. The fluorescence of DSOCC and that of DSSCC in vesicles were enhanced as the temperature was lowered, and there are two points, T_1 and T_2 ($T_1 > T_2$), where the increment of the fluorescence intensity changes. Those temperatures are in good agreement with the upper and the lower limits of the phase transition temperature range of each vesicle, suggesting that the temperature dependence of the fluorescence intensity reflects the phase transition of the vesicles. On the other hand, the same measurement of MSSCC did not show any inflection point in the experimental temperature range. If we know the equation which gives the temperature dependence of fluorescence intensity in each phase, we may estimate the fraction of phase transition at any temperature in the region of phase separation.

The fluorescence quantum yield, Φ, is given in the following form.

$$\Phi = \frac{k_f}{k_f + k_1 + k_2 \exp(-E/kT)} \tag{1}$$

where k_f is the rate constant of the fluorescent process, $[k_1 + k_2 \exp(-E/kT)]$reflects the rate constants of the nonradiative processes, and E is the activation energy of a temperature dependent nonradiative porcess. Eq. 1 is rewritten into the following form.

$$\ln\left[\frac{1}{\Phi} - \left(1 + \frac{k_1}{k_f}\right)\right] = \ln\frac{k_2}{k_f} - \frac{E}{kT} \tag{2}$$

We cannot directly apply this equation to our experimental results, because we cannot determine the quantity k_1/k_f at present. The fluorescence quantum yields of oxacarbocyanine and thiacarbocyanine in methanol were respectively reported to be 0.053 and 0.048 at room temperature,[20] and we assume the following temperature dependence of the fluorescence intensity, I_f,

$$\ln I_f = A + \frac{B}{T} \tag{3}$$

where A and B are constants. The above approximation means that the temperature dependent nonradiative process is the dominant path for dissipation of excitation energy. We tested the applicability of Eq. 3 by plotting the temperature dependence of fluorescence intensity of SOCC in methanol in the range, 10—50 °C, showing a good linear relationship between $\ln I_f$ and $1/T$. The plots according to Eq. 3 for DSOCC and DSSCC are shown in Figs. 1 and 2, respectively. These plots clearly show that each $\ln I_f$ *vs.* $1/T$ curve is divided into three parts, *i.e.*, low temperature part, high temperature part, and middle part. The low temperature part and the high temperature part correspond to the monophase regions, and the Arrhenius relation holds as expected. The temperature dependence of the fluorescence intensity of MSSCC incorporated into the DPPC vesicle is shown in Fig. 3. In this case, the fluorescence intensity of MSSCC does not reflect the phase transition. This result suggests that the temperature dependent nonradiative process is the twisting motion of the

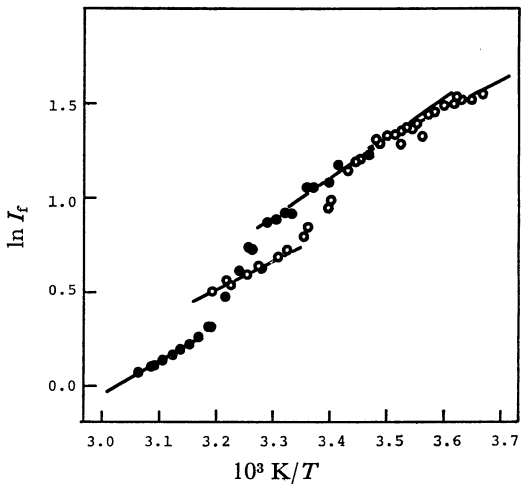

Fig. 1. Logarithm of the fluorescence intensity of DSOCC *vs.* reciprocal of temperature. (○); DMPC vesicles, (●); DPPC vesicles.

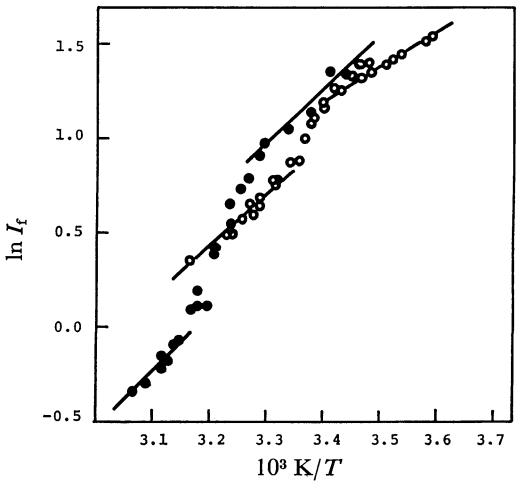

Fig. 2. Logarithm of the fluorescence intensity of DSSCC *vs.* reciprocal of temperature. Notation is the same as in Fig. 1.

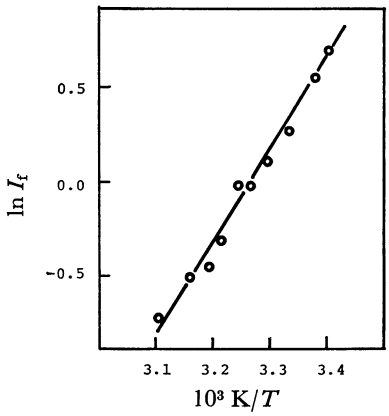

Fig. 3. Logarithm of the fluorescence intensity of MSSCC in DPPC vesicles *vs.* reciprocal of temperature.

chromophore around the bridging bond in the excited singlet state and the suppression of this motion by the membrane lipid moiety will increase the fluorescence intensity of dioctadecyl carbocyanines incorporated into the membrane. Thus, the fluorescence intensity of dioctadecyl cyanines will reflect the phase transition of the lipid bilayers. The observed fluorescence intensity, I_f, in the region of a phase transition may be given in the following form,

$$I_f(T) = I_s(T)\cdot\theta + I_{fl}(T)\cdot(1-\theta) \quad (4)$$

where $I_{fl}(T)$ and $I_s(T)$ are the fluorescence intensity of the dye in the fluid phase region and the solid phase region, respectively, at temperature T, and θ is the fraction of the solid phase region at T. From Eq. 4 we get the following expression for θ.

$$\theta = \frac{I_f(T)-I_{fl}(T)}{I_s(T)-I_{fl}(T)} \quad (5)$$

Temperature dependence of θ is shown in Figs. 4 and 5.

It is possible to calculate the cluster size of cooperative transition from the data in Figs. 4 and 5, if we assume that the solubility of the amphiphilic cyanine dye in the lipid bilayer is not affected by the physical state of the bilayer. The phase transition phenomenon can be regarded as a quasi-first-order reaction with the equilibrium constant, K.

$$K = \frac{\theta}{1-\theta} \quad (6)$$

Combining Eq. 6 with van't Hoff equation, the following equation is obtained.[21]

$$\left(\frac{d\theta}{dT}\right)_{T_m} = \frac{\Delta H_{v.H.}}{4RT_m}, \quad (7)$$

where T_m is the temperature at the center of the transition and $\Delta H_{v.H.}$ is the so-called van't Hoff enthalpy of the transition. Thus, we can calculate the van't Hoff enthalpy from the slope of the curve in Figs. 4 and 5 at $\theta=0.5$. The values of the van't Hoff enthalpy obtained with DSOCC fluorescence were 66.8 kcal/mol and 90.7 kcal/mol for DMPC and DPPC vesicles, respectively. The calorimetrically obtained enthalpy of transition has not been reported for single bilayer vesicles, though the values for multilamellar liposomes have been reported as listed in Table 1. Assuming that those values are also applicable to the phase transition of single bilayer vesicles, the cluster size of the cooperative transition was estimated. The phase transition parameters obtained in the present study are listed in Table 2 together with those obtained by other methods, *e.g.* ESR, fluorescence depolarization, and the differential scanning calorimetry (DSC). The centers of the transition temperatures obtained in the present study are in good agreement with those of the other methods, although values of the cooperativity obtained in this work are smaller than those reported previously. Although the reason for such differences is not clear at the present stage, we may conclude that the fluorescence intensity of the amphiphilic cyanine dyes with two long alkyl chains which are incorporated in bilayer liposomes is significantly modified by the phase transition of the bilayers through the suppression of twisting motion of the chromophores around the bridging chain in the excited singlet state.

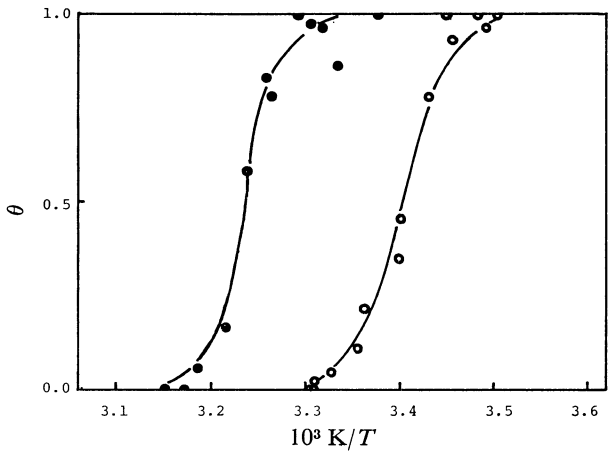

Fig. 4. Mole fraction of solid phase obtained with the DSOCC fluorescence *vs.* reciprocal of temperature. Notation is the same as in Fig. 1.

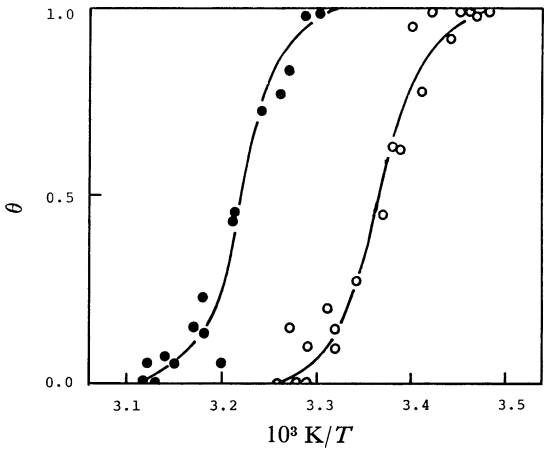

Fig. 5. Mole fraction of solid phase obtained with the DSSCC fluorescence *vs.* reciprocal of temperature. Notation is the same as in Fig. 1.

Table 1. Heat of transition of multilamellar liposomes of DMPC and DPPC

Lipid	Heat of transition ΔH_{cal} (kcal/mol)	
DMPC	6.64[a]	6.26[b]
DPPC	8.66[a]	9.69[b]

a) Phillips *et al.*[6] b) Hintz *et al.*[7]

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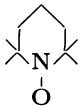
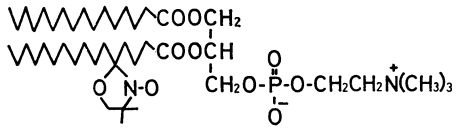
Table 2. Phase transition parameters of DMPC and DPPC

Method	Lipid	Transition temperature		$\frac{\Delta H_{v.H.}}{\Delta H_{cal}}$	Reference
		T_m (°C)	Range[a] (°C)		
DSOCC fluorescence	DMPC	20.3	16.0—30.0	12	This work
	DPPC	36.0	27.3—42.2	12	
DSSCC fluorescence	DMPC	23.5	16.1—30.9	11	This work
	DPPC	37.6	30.0—45.4	11	
DPH[b] fluorescence depolarization	DMPC	20.9±0.5	14.3—27.4		Lenz *et al.*[13]
	DPPC	36.4±0.5	29.4—40.6		
ESR (Tempo 1)[c]	DMPC	22.4		21	Marsh *et al.*[10]
	DPPC	36.4		23	
ESR (Tempo 2)[d]	DMPC	19.7		14	Marsh *et al.*[10]
	DPPC	36.0		35	
Differential scanning calorimetry	DPPC	36.9±0.1			Suurkuusk *et al.*[8]

a) Temperature range of the transition. b) 1,6-Diphenyl-1,3,5-hexatriene.

c) 

d) 

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