Journal of Fluorescence, Vol. 8, No. 1, 1998

Fluorescence of Organic Dyes in Lipid Membranes: Site of Solubilization and Effects of Viscosity and Refractive Index on Lifetimes

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Received October 27, 1997; accepted February 29, 1998

The fluorescence decay of several organic dye molecules intercalated in egg phosphatidylcholine lipid membrane vesicles is consistent with the existence of two or three prominent lifetime components rather than a single continuous distribution of lifetimes. The major lifetime components are identified with different sites of solubilization in the membrane. The variation of the lifetime of the membrane-bound dye was studied as a function of the sucrose concentration, which varied the viscosity and refractive index of the aqueous solution. The combined effect of viscosity and refractive index on the lifetime of the dye was used to identify the site of solubilization of the dye in the membrane. The study was useful to identify dye molecules on the surface which are exposed to the aqueous phase, for which the fluorescence lifetime increased systematically with sucrose (viscosity effect). More importantly, it was possible in a few cases to identify the dye molecules which are oriented in the membrane phase, and the fluorescence lifetime decreased systematically with sucrose (refractive index effect). Anomalous values of order parameters determined from the refractive index effect are explained in terms of an orientational distribution of the linear dye molecule weighted in favor of mutually orthogonal orientations.

KEY WORDS: Lifetimes; membrane; vesicles; egg phosphatidylcholine; viscosity; refractive index; order parameter.

INTRODUCTION

Fluorescence of organic dye molecular probes is widely used in the investigation of the physical properties of lipid membranes in model and biological systems [1–3]. The structure and dynamics of the dye molecule bound to the membrane are of great interest because of the usefulness of the fluorescence property in a wide variety of biological applications [4,5]. One or more fluorescence properties (spectra, quantum yield, lifetime, and anisotropy) of the molecule are sensitive to variations in the structure and/or dynamics of the membrane and hence their usefulness in the investigation of the biomembranes. A large number of applications are based on the increase in the fluorescence quantum yield of the membrane-bound dye. The exact molecular mechanism for the change in fluorescence property in some applications (for example, the action of dyes that are sensitive to the membrane potential [6–8]) is not clearly understood. Identification of the solubilization sites for the dye in the membrane and its local structure (site of solubilization, surface vs core, and orientation with respect to the interface) is a prerequisite for an understanding of the molecular mechanism for the change in the fluorescence parameters. Ideally, one would like to use direct structure-determining techniques such as NMR for this purpose. Unfortunately, such techniques cannot be used

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1053-0509/98/0300-0081$15.00/0

when the concentration of the dye is in the micromolar range. In the absence of an independent technique that is useful for determining the structure of the dye molecule at low concentrations, one has to use fluorescence from the same dye as the probe for the investigation of solubilization site.

Fluorescence of dye molecules incorporated in membranes is perturbed by the physical properties of the external aqueous medium such as the viscosity and refractive index. For example, the fluorescence intensity, lifetime, and anisotropy of a dye which is bound in the surface region of the membrane will be sensitive to the viscosity of the aqueous medium. The effect of the refractive index of the aqueous medium on the membrane-bound, oriented dye molecule (diphenyl hexatriene; DPH) has also been observed in recent experiments [9,10]. Thus, it is possible to use the effects of the viscosity and refractive index of the aqueous medium on the fluorescence property of a membrane-bound molecule to obtain information about its location and orientation. DPH is the only fluorophore which is reported to exhibit the refractive index effect. One of the objectives of this study is to identify fluorophores which are oriented in the membrane and exhibiting an unambiguous effect of the refractive index on the fluorescence lifetime.

The site of solubilization and the local structure for the fluorophore in the membrane phase are known for several frequently used fluorescent probes such as DPH and TMA-DPH. The site of solubilization could also be inferred from the structure of the fluorophore itself. For example, polar or ionic fluorophores are unlikely to be located in the core region. However, even for such hydrophilic probes the location on the surface/interface region and the extent of exposure to solvent cannot be guessed from the structure alone. In addition, multiple sites of solubilization for a dye in the membrane are likely but difficult to substantiate without direct experimental evidence. Time-resolved fluorescence of 15 organic dye molecules bound to lipid membrane vesicles was studied. The effects of the viscosity and refractive index of the aqueous medium on the fluorescence lifetimes are interpreted in terms of the site of solubilization/local structure and orientation of the organic dye molecule in the lipid matrix.

MATERIALS AND METHODS

Fluorescence measurements were carried out on 15 organic dyes (chemical structures shown in Fig. 1) incorporated in egg PC (L-α-phosphatidylcholine from fresh egg yolk; Sigma Chemical Co., USA) vesicles. The dyes used were Nile red (Nile blue A oxazone; Exciton Inc., USA), R6G (rhodamine 6G; Exciton Inc.), BODIPY 611/627 [3,5-bis-(2-thienyl-4,4-difluoro-4-bora-3a,4a-diaza-s-indacene; Molecular Probes Inc., USA], MC540 (merocyanine 540; Sigma Chemical Co.), RH421 [*N*-(4-sulfobutyl)-4-(4-(4-dipentylamino)phenyl) butadienyl)pyridinium, inner salt; Molecular Probes Inc.], FM4-64 [*N*-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl)pyridinium dibromide; Molecular Probes Inc.], DiA [4-di-16-ASP; (4-(4-dihexadecylamino)styryl)-*N*-methylpyridinium iodide; Molecular Probes Inc.], DPH (1,6-diphenyl-1,3,5-hexatriene; Molecular Probes Inc.) TMA-DPH [1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate; Molecular Probes Inc.], $DiOC_2(5)$ (DODCI; 3,3'-diethyloxadicarbocyanine iodide; Exciton Inc.), $DiSC_2(3)$ (3,3'-diethylthiacarbocyanine iodide; Molecular Probes Inc.), $DiSC_2(5)$ (3,3'-diethylthiadicarbocyanine iodide; Molecular Probes Inc.), $DiSC_3(5)$ (3,3'-dipropylthiadicarbocyanine iodide; Molecular Probes Inc.), $DiIC_{18}(3)$ (DiI; 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; Molecular Probes Inc.), and $DiIC_{18}(5)$ (DiD, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate; Molecular Probes Inc.).

The sonicated egg PC liposomes were prepared in pH 7.4 buffer (10 m*M* CH_3COONa, 10 m*M* NaH_2PO_4, 10 m*M* MES [2(*N*-morpholino) ethanesulfonic acid] and 150 m*M* NaCl) as described in Ref. 11. The sonicated vesicles are of a single bilayer with diameters in the range of 200–400 Å, with nearly 70% of the vesicles having diameters ~300 Å. The lipid concentration used in these experiments was about 0.1 mg/ml (~0.14 m*M*). The dyes were added from the stock solutions made in ethanol to the vesicles and kept overnight. The final samples contain ~1% (v/v) ethanol. The dye-to-lipid ratio was kept at approximately 1:500. All measurements were carried out in air-saturated solutions at room temperature (25°C).

The steady-state fluorescence intensity and anisotropy measurements were made using either a Shimadzu RF540 or a SPEX Fluorolog 1681 T format spectrofluorophotometer. The steady-state anisotropy (r_{ss}) is defined as

$$r_{ss} = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \tag{1}$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities measured with the emission polarizer kept parallel and perpendicular to the excitation polarizer. The time-resolved fluo-

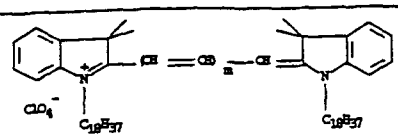
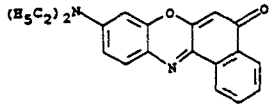
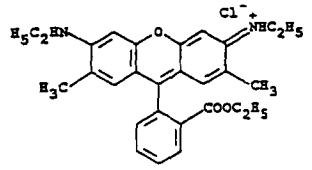
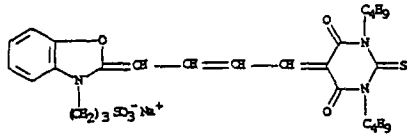
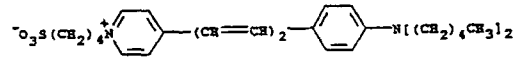
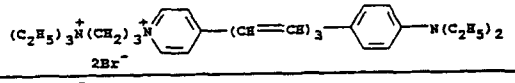
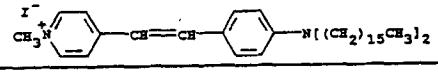
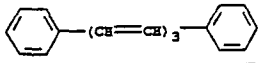
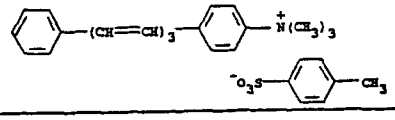
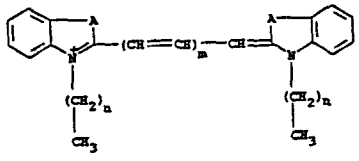
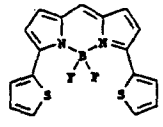
(1) m=1 $DiIC_{18}(3)$ (2) m=2 $DiIC_{18}(5)$	
(3) Nilered	
(4) Rhodamine 6G	
(5) Merocyanine 540	
(6) RH421	
(7) FM4-64	
(8) DiA	
(9) DPH	
(10) TMA-DPH	
(11) A=O m=2 n=1 $DiOC_2(5)$ (12) A=S m=1 n=1 $DiSC_2(3)$ (13) A=S m=2 n=1 $DiSC_2(5)$ (14) A=S m=2 n=2 $DiSC_3(5)$	
(15) BODIPY 611/627	

Fig. 1. Structures of different dyes with the abbreviated names used in the text.

rescence measurements were made using a high-repetition rate (800-kHz) picosecond dye laser (rhodamine 6G) coupled with a time-correlated single-photon counting (TCSPC) setup described elsewhere [12], using a microchannel plate photomultiplier (Hamamatsu 2809). The sample was excited with vertically polarized light and the fluorescence decay was collected with an emission polarizer kept at the magic angle (~54.7°) with re-

Table I. Fluorescence Lifetimes and Amplitudes of Organic Dyes in Ethanol and Water

Dye	Ethanol		Water						
	τ	χ^2	τ_1	τ_2	τ_3	α_1	α_2	α_3	χ^2
$DilC_{18}(3)$	0.31	1.15	0.1	0.66	1.84	0.76	0.19	0.05	0.98
$DilC_{18}(5)$	1.26	1.02	0.1	0.51	3.6	0.77	0.19	0.04	1.2
Nilered	3.58	1.09	0.59	3.18	—	0.9	0.1	—	1
Rhodamine 6G	3.83	1	3.98	—	—	1	—	—	1.18
Merocyanine 540	0.49	0.96	0.14	1.41	—	0.88	0.12	—	1.18
RH421	1.11	1.01	0.14	1.12	—	0.96	0.04	—	0.8
FM4-64	0.36 (0.85), 0.82 (0.15)	0.76	0.08	0.8	—	0.98	0.02	—	0.84
DiA	0.12	0.95	0.17	0.75	2.34	0.78	0.17	0.05	0.9
DPH	4.82	0.85	0.17	2.25	10	0.53	0.42	0.05	1.21
TMA DPH	0.09 (0.99), 1.85 (0.01)	0.89	0.05	0.74	3.3	0.96	0.03	0.01	1.08
$DiOC_2(5)$ (DODCI)	1.07	0.82	0.65	—	—	1	—	—	0.98
$DiSC_2(3)$	0.19	0.7	0.13	1.1	—	0.97	0.03	—	1.14
$DiSC_2(5)$	1.38	1.07	0.77	2.25	—	0.95	0.05	—	0.96
$DiSC_3(5)$	1.44	0.95	0.9	—	—	1	—	—	0.95
BODIPY 611/627	5.56	1.08	1.69	4.45	—	0.7	0.3	—	1.19

spect to the excitation polarizer for measuring lifetimes. The instrument response function (IRF) was recorded using a nondairy creamer scattering solution. The full width at half-maximum (FWHM) of the IRF is about 200 ps. The typical peak count in the emission decay for fluorescence intensity measurements was about 10,000.

The experimentally measured fluorescence decay data are a convolution of the instrument response function with the intensity decay function. The intensity decay data were fitted to the appropriate equations by iterative reconvolution procedure using the Levenberg–Marquardt algorithm for optimization of the parameters [12]. The fluorescence decays of all the dyes in vesicles were analyzed by the maximum-entropy method (MEM) as well as discrete exponential analysis.

In discrete exponential analysis, the intensity decay function was fitted to the multiexponential function as

$$I(t) = \sum_i \alpha_i \exp(-t/\tau_i) \qquad (2)$$

where α_i and τ_i are the amplitudes and the lifetimes. The goodness of the fits was judged by the χ^2 value (close to 1) and the random residual distribution. The average lifetime was calculated by the equation $\langle\tau\rangle = \Sigma_i\alpha_i\tau_i/\Sigma_i\alpha_i$.

In the MEM, the decays were analyzed for the model of distribution of lifetimes [13,14]. In this, the intensity decay function is

$$I(\mathrm{t}) = \int_0^{\tau_{max}} \alpha(\tau) \exp(-t/\tau)d\tau \qquad (3)$$

where $\alpha(\tau)$ is the distribution function which must be determined. In the analysis, the above integral is approximated as a multiexponential function of about 150 discrete lifetime values τ_i uniformly spaced in $\log(\tau)$ space (in our case, 10 ps to 20 ns), where the amplitudes α_i represent a continuous, smooth function. The optimum distribution is the one which fits the data with a χ^2 value close to 1.0 and maximizes the Shannon–Jaynes entropy [14].

RESULTS AND DISCUSSION

Fluorescence Lifetimes in Membranes

The fluorescence decay of all the dye molecules, except FM464, investigated in this study is single exponential (or nearly so) in dilute solutions ($<10\,\mu M$) in ethanol (Table I). Observation of single-exponential decay for the dye in ethanol confirms that the dye is pure and that the photophysics of fluorescence is due to a single structurally distinct species.

The fluorescence decay of the dyes in water [containing ethanol 2% (v/v)] is predominantly single exponential for water-soluble charged dyes [rhodamine 6G, RH421, FM464, DODCI, $DiSC_m(n)$] and multiexponential for other neutral dyes (Table I). The fluorescence emission and excitation spectra are consistent with the presence of a single monomer species for those dyes for which the fluorescence decay is single exponential. The dyes which exhibited multiexponential decay in the aqueous solutions are presumed to have multiple species including aggregates. All the dyes (charged and neutral

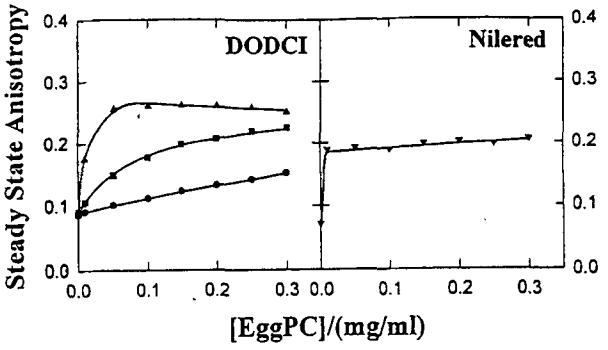


Fig. 2. Variation of steady-state anisotropy of Nile red (λ_{ex} = 570 nm, λ_{em} = 615 nm) and DODCI [(●) λ_{ex} = 570 nm, λ_{em} = 590 nm, (■) λ_{ex} = 570 nm, λ_{em} = 690 nm, (▲) λ_{ex} = 580 nm, λ_{em} = 690 nm] in aqueous solutions as a function of lipid concentration.

dyes) listed in Table I are readily solubilized in membrane. There is no evidence for the formation of aggregates in membranes for a [dye] <10 μM. The readiness of solubilization is indicated by the increase in the quantitative parameters such as fluorescence anisotropy for the dye molecule which is incorporated in the membrane.

Figure 2 shows the typical variation of the steady-state fluorescence anisotropy (r_{ss}) for two dyes (Nile red and DODCI) as a function of the lipid concentration at a fixed dye concentration. In both cases, the fluorescence anisotropy increases with the concentration of lipid but the rate of increase is different. For DODCI, the anisotropy variation depends upon the excitation and emission wavelengths as well. The difference in the trends for the two dyes can be understood from the fact that Nile red is not soluble in water, whereas DODCI is water soluble. Even at a low concentration of the lipid, the dye Nile red is solubilized only in the lipid phase and the anisotropy attains the limiting maximum value. In the case of water-soluble dyes such as DODCI, the variation of fluorescence anisotropy with the concentration of lipid and the wavelength dependence are attributable solely to the partitioning of the dye between the aqueous and the lipid phase. At short excitation and emission wavelengths (570 and 590 nm), the fluorescence of the dye in water is selectively excited and detected. But at long wavelengths of excitation and emission (580 and 690 nm), the fluorescence is due predominantly to the membrane-bound dye and the plot of r_{ss} vs lipid concentration resembles that of Nile red.

The fluorescence decay of all the dyes bound to the membrane is either two or three exponential (except R6G, which is single exponential). The values of lifetimes and amplitudes for the dyes and the wavelengths of excitation and emission are given in Table II. The fluorescence of each dye (except R6G) consists of a long-lifetime component and a short-lifetime component. In a few cases there was a middle-lifetime component as well. The long lifetime in the membrane is comparable to or longer than the lifetime in ethanol. In several cases the fraction of the long-lifetime component was the major one or a significant fraction. The existence of multiple lifetimes was also confirmed by analysis of the decay data by the MEM.

The fluorescence decays of the membrane-bound dye were subjected to analysis by the MEM to obtain the distribution of lifetimes. The MEM is an unbiased method which does not assume any a priori model for the excited-state kinetics or lifetimes. It assumes that the lifetimes are distributed with equal probability (amplitude) in a range (typically, 10 ps to 20 ns in our case), and the shape of the distribution (amplitude vs. lifetime) is obtained as the final outcome of analysis [14]. Figure 3 shows the results of MEM analysis of fluorescence decays for all the dyes bound to the membrane. The fluorescence of the dyes in the membrane is not due to a single continuous distribution of lifetimes. The distribution shows one or more well-resolved peaks for all the dyes, and for most dyes the width of the prominent peak is relatively sharp. A comparison of the average lifetime (results not shown) for each prominent peak in the multipeaked distribution of lifetimes with the lifetimes (Table II) obtained by discrete exponential analysis shows a one-to-one correspondence. It is clear from the above results and discussion that the fluorescence decay of the membrane-bound dye can be associated with at least two spectroscopically distinct species; however, only one is prominent in several cases. Excited-state reaction of the dye molecule in a single site in the membrane leading to a fluorescent product would be indicated by a negative amplitude for the short-lifetime component. This has not been observed. Hence, the two species ought to be the dye molecule incorporated in two sites in the membrane.

Effect of Refractive Index and Viscosity on Lifetimes

The viscosity and refractive index affect the fluorescence lifetime of a dye in a homogeneous medium (pure or mixed solvent). The mechanism by which the viscosity modifies the lifetime is different from that of the refractive index. The fluorescence lifetime, τ_f, is equal to $(k_r + k_{nr})^{-1}$, where k_r and k_{nr} are the radiative and nonradiative rates of the excited singlet state of the

Table II. Fluorescence Lifetimes and Amplitudes of Organic Dyes in Egg PC Vesicles

Dye	λ_{ex}	λ_{em}	τ_1	τ_2	υ_3	α_1	α_2	α_3	χ^2
$DiIC_{18}(3)$	570	650	0.13	0.48	1.51	0.63	0.31	0.06	1.04
$DiIC_{18}(5)$	600	750	0.34	1.29	—	0.63	0.37	—	1.02
Nilered	570	615	0.72	3.71	—	0.19	0.81	—	1.11
Rhodamine 6G	300	630	4.11	—	—	1	—	—	1.07
Merocyanine 540	570	680	0.62	1.69	—	0.21	0.79	—	1.08
RH421	570	700	0.55	1.72	—	0.14	0.86	—	0.99
FM4-64	570	690	0.1	0.57	1.13	0.69	0.21	0.1	0.93
DiA	570	630	0.19	0.59	1.74	0.71	0.26	0.03	0.98
DPH	300	500	1.79	5.92	9.61	0.14	0.62	0.24	0.98
TMA DPH	300	470	0.91	3.67	6.11	0.35	0.51	0.14	1.05
$DiOC_2(5)$ (DODCI)	580	690	0.66	1.74	—	0.43	0.57	—	0.96
$DiSC_2(3)$	570	690	0.3	1.04	—	0.37	0.63	—	0.94
$DiSC_2(5)$	630	760	0.98	2.03	—	0.29	0.71	—	0.9
$DiSC_3(5)$	630	760	1.09	2	—	0.16	0.84	—	1.02
BODIPY 611/627	610	730	2.75	5.35	—	0.18	0.82	—	1.18

dye. The refractive index of the solvent modifies the value of k_r according to the Strickler–Berg equation [15]:

$$k_r = 2.88 \times 10^{-9} n_0^2 \int \frac{(2\nu_0 - \nu)^2}{\nu} \varepsilon_\nu d\nu \qquad (4)$$

where n_0 is the refractive index of the medium, ε_ν is the molar extinction coefficient at frequency ν (cm^{-1}), and ν_0 is the frequency of the S_0–S_1 transition. The viscosity modifies the value of k_{nr} [15–17]. The nonradiative rate k_{nr} is a sum of two intramolecular rates (assuming that intermolecular nonradiative quenching is absent): internal conversion (k_{ic}) and intersystem crossing (k_{isc}). The viscosity effect on the two intramolecular rates differs from dye to dye but the nonradiative rates decrease with increasing viscosity because the frequency of collisions between the excited state and the solvent decreases with increasing viscosity. Thus, an increasing viscosity tends to increase the lifetime, whereas an increasing refractive index has the opposite effect of decreasing the lifetime. The combined effect of viscosity and refractive index on the fluorescence lifetime of the dyes is therefore unpredictable but easily determined experimentally.

The radiative rate of an excited state in a homogeneous medium depends upon the refractive index of the medium as given by Eq. (4). However, the radiative rate for molecules bound to optically thin layers (such as lipid membranes) is predicted to depend on the refractive index of the external medium in a unique way determined also by the orientation of the transition moment of the molecule with respect to the interface [9,18], as given by Eq. (5).

$$k_r = \frac{4\omega^3}{3\hbar c^3} f^2 |\mu|^2 n_0 \left(\sin^2\theta + \frac{n_0^4}{n_1^4}\cos^2\theta\right) \qquad (5)$$

where ω is the circular frequency of fluorescence light, $\hbar$ is the Planck's constant, c is the speed of light, f is a factor which accounts for the difference between the local electric field experienced by the dye and the macroscopic field inside the layer, μ is the matrix element of the emission electric dipole operator, n_0 and n_1 are the refractive indices of the aqueous medium present on both sides of the bilayer and the bilayer, and θ is the angle between the molecular emission dipole and the normal to the surface of the bilayer. According to the above equation, the radiative rate of a molecule increases with the refractive index of the aqueous medium, approximately as $(n_0)^m$, where the exponent $m = 5$ for perpendicular orientation of the transition moment with respect to the membrane surface, $m = 1$ for the dye oriented parallel to the surface, and values of m are intermediate for other orientations of the dye. Thus, for those dye molecules with long lifetimes for which the nonradiative rate is negligible, one could observe the effect of the refractive index on the fluorescence lifetime.

The viscosity and refractive index of the aqueous solution can be increased by the addition of an organic solute [19]. Sucrose was chosen in our study as the solute because the membrane vesicles were stable. Figure 4 shows the change in the fluorescence decays for the two cases (DiA and DODCI) with the presence of sucrose (44.4%) in the aqueous phase. The relative viscosity of the sucrose solution is increased from 1 to 10.74 and the refractive index is increased from 1.334

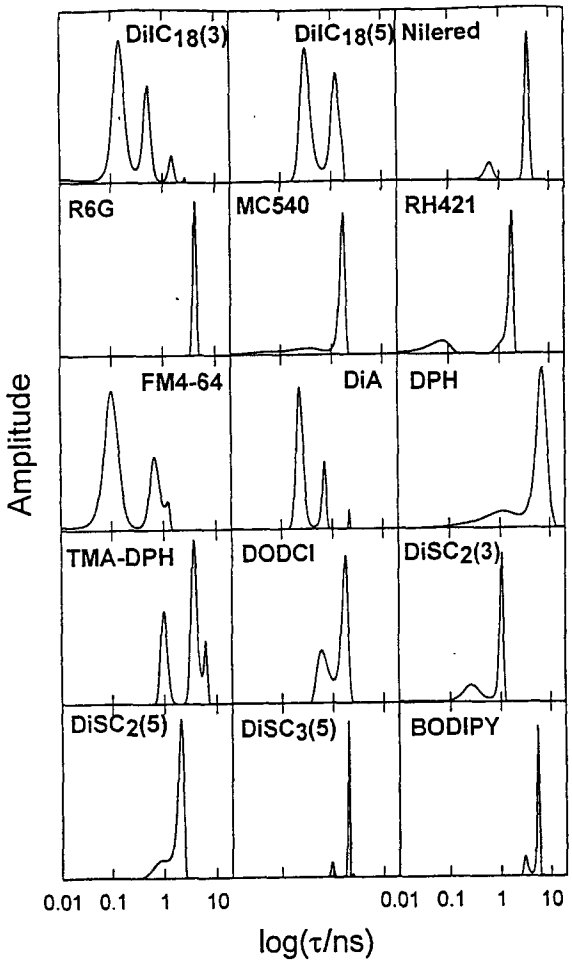


Fig. 3. Distribution of fluorescence lifetimes obtained by analysis by the maximum-entropy method for the fluorescence decay of 15 dyes in egg PC membranes. The excitation and emission wavelengths are given in Table II.

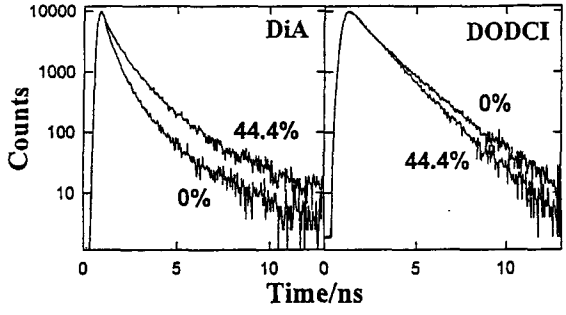


Fig. 4. Fluorescence decay of DiA (λ_{ex} = 570 nm, λ_{em} = 630 nm) and DODCI (λ_{ex} = 580 nm, λ_{em} = 690 nm) in membranes with and without sucrose in the aqueous solution.

to 1.413. In the case of DiA, the fluorescence decay becomes slower, whereas the decay is faster for DODCI.

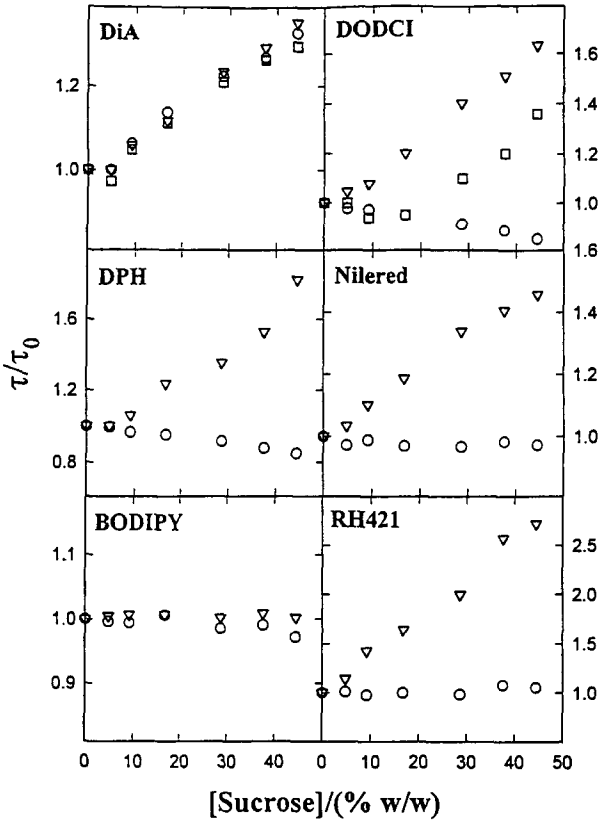


Fig. 5. Variation of the fluorescence lifetime ratio (τ/τ_0) for the dye with the concentration of sucrose. (▽) Dye in aqueous solution. (○) Dye bound to the membrane (long-lifetime or major component). (□) Dye bound to the membrane (short-lifetime component).

The foregoing discussion on the effect of the viscosity and refractive index on the fluorescence property of the dye suggests that the viscosity effect is dominant for DiA, whereas the refractive index effect is dominant for DODCI. The quantitative variations of the lifetimes (τ/τ_0) of major lifetime components are plotted as a function of sucrose concentration in Fig. 5 for DiA, DODCI, and a few other dyes. The results are discussed further in the next section.

Site of Solubilization

There are three regions in the membrane phase in which an organic dye molecule is expected to have different degrees of solvent exposure. These are the surface region, in which the dye is exposed to the solvent; the interface region, in which the exposure to the solvent is limited; and the core region. The core region can be anywhere from the center of the bilayer to the interface. Figure 6 shows a schematic of the membrane structure

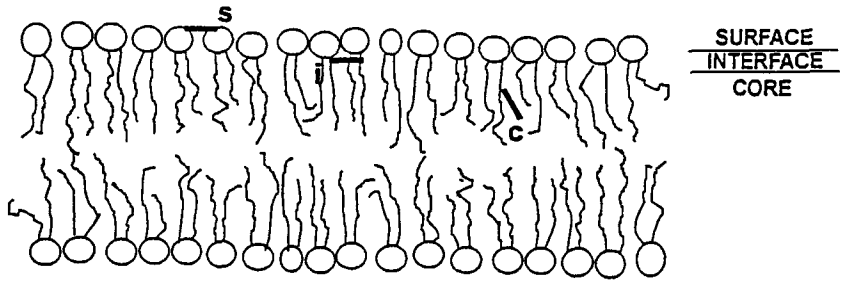


Fig. 6. Cartoon of the structure of the membrane and interfacial region. The site of solubilization for the dyes is indicated as the surface (S), interface (I), or core (C) region of the membrane.

Table III. Site of Solubilization of Major Lifetime Components of Organic Dyes in Egg PC Membranes

Dye No.	Dye	τ_w	% change	τ_1	% change	Site	τ_2	% change	Site
1	$DiIC_{18}(3)$	0.10	+80	1.51	x	Interface	0.13	+64	Surface
2	$DiIC_{18}(5)$	0.10	+14	1.29	x	Interface	0.34	+13	Surface
3	Nilered	0.59	+46	3.71	0	Core	—		—
4	Rhodamine 6G	3.98	−4	4.11	−5	—	—		—
5	Merocyanine 540	0.14	+143	1.69	+5	Interface	—		—
6	RH421	0.14	+171	1.72	0	Interface	—		—
7	FM4-64	0.08	+213	1.13	−5	Interface	0.10	+117	Surface
8	DiA	0.17	+36	0.59	+33	Surface	0.19	+30	Surface
9	DPH	0.17	+82	9.61	−15	Core	—		—
10	TMA DPH	0.05	+300	6.11	x	Interface	—		—
11	$DiOC_2(5)$ (DODCI)	0.65	+63	1.74	−14	Core	0.66	x	Interface
12	$DiSC_3(3)$	0.13	+208	1.04	+12	Core	0.30	0	Interface
13	$DiSC_2(5)$	0.77	+57	2.03	−7	Core	0.98	x	Interface
14	$DiSC_3(5)$	0.90	+59	2.00	−10	Core	—		—
15	BODIPY 611/627	4.60	0	5.35	−3	—	—		—

and the three sites of solubilization, marked S (surface), I (interface), and C (core).

The effect of the refractive index and viscosity of the aqueous medium on the fluorescence lifetime of the membrane-bound dye depends upon the site of solubilization. If the dye is solubilized in the interior region (say, between the two leaves of the bilayer), then it is expected to be insulated from the variation in some of the physical properties of the aqueous medium. The effect of the viscosity on the lifetime is based on a collisional mechanism between the solvent molecules and the excited state of the dye. Therefore, the viscosity of the aqueous medium should have no effect on the lifetime of a dye molecule bound in the interior region of the membrane. However, a dye molecule bound in the surface region of the membrane which is exposed to the aqueous phase is still subjected to the collisions of solvent molecules and a viscosity effect is observable. On the other hand, the effect of the refractive index on the radiative rate is an optical phenomenon and this effect would be observable even for a molecule bound in the interior region of the membrane. Thus, identification of the site of solubilization (surface vs interior region) of a membrane-bound dye molecule is possible from the variation of the lifetime of the membrane-bound dye in sucrose solutions.

The fluorescence decay of DiA in egg PC membrane indicates three lifetime components: 0.19, 0.59, and 1.74 ns. The value of the two lifetimes of the major components (0.19 and 0.59 ns) increase by 30 and 33% in the presence of sucrose (44.4%, w/w) (Fig. 4 and Table III). The variations of the two lifetimes with sucrose are shown in Fig. 5. A comparison of the values of lifetimes (0.17, 0.75, and 2.34 ns) measured for DiA in water (Table I) and the variation of the lifetime (0.17 ns) with sucrose (triangles in Fig. 5) suggest that the dye in the membrane is exposed to the aqueous solution and the increase in lifetime with sucrose is a viscosity effect. Therefore, we conclude that the site of solubilization of the two major components of DiA in membrane is on

the surface exposed to water. This conclusion is consistent with the structure of the dye, where the chromophore is attached to a long alkyl chain.

The fluorescence decay of DODCI in membrane consists of two major components: 0.66 and 1.74 ns. The lifetime of the short component (0.66 ns) shows a decrease followed by a marginal increase and the lifetime of the long component (1.74 ns) decreases by 14% with sucrose (44.4%). The opposite trends of variation of these two components indicate that the site of solubilization for these two species are different. The short component shows the refractive index effect followed by viscosity effect at high sucrose concentrations, and hence its site is inferred to be the interfacial region, where the exposure to water is partial. On the other hand, the long component shows a refractive index effect and hence its site is inferred to be the core region of the membrane.

The change of lifetime of the major components of other membrane-bound dyes in sucrose (44.4%) are given in Table III. The variations are also shown in Fig. 5 for a few dyes. The change in lifetime with sucrose was unambiguously positive for several dyes: $DiIC_{18}(3)$ (0.13 ns), $DiIC_{18}(5)$ (0.34 ns), and FM464 (0.1 ns). The site of solubilization for these species (lifetime components) is inferred to be the surface region. For a few cases the change in lifetime with sucrose (Fig. 5 and Table III) is clearly negative due to the refractive index effect. These are DPH (9.61 ns), $DiSC_2(5)$ (2.03 ns), and $DiSC_3(5)$ (2.0 ns). The species associated with these lifetime components are therefore inferred to be in the core region. All the molecules which show a refractive index effect are linear dye molecules and these cases are discussed further in the next section. The fluorescence lifetimes of some components of the above dyes and several other membrane-bound dyes do not fall into the above two categories. For example, the 0.66-ns-lifetime component of DODCI in the membrane shows a decrease followed by a marginal increase with sucrose compared to that of DODCI in water (Fig. 5). A dye molecule which is placed in the interfacial region of the membrane is expected to exhibit a relatively lesser viscosity effect than its counterpart which is exposed to water. The lifetime components which show such trends are marked x in Table III. Finally, the fluorescence lifetimes of BODIPY and R6G are not sensitive to viscosity like all the other dyes (Fig. 5). Even if these dye molecules are present on the surface, it is not possible to observe the viscosity effect. The site of solubilization cannot be assigned for these dyes. The lifetimes of these dyes bound to the membrane show a marginal decrease which is consistent with the refractive index effect.

Orientation of Some Linear Dye Molecules Inside the Membrane

As discussed earlier, the dependence of the radiative rate of a membrane-bound fluorophore on the refractive index of the medium external to the membrane (here an aqueous medium) depends on its orientation with respect to the interface, according to Eq. (5). Such effects can be observed only for dyes which have a linear structure and in which the long axis of the molecule is preferentially oriented with respect to the membrane interface. Theory predicts that the fluorescence lifetime (inverse of the sum of radiative and nonradiative rates) decreases with the increase in refractive index of the aqueous solution when the radiative rate dominates over the nonradiative rate. This dependence can be used to extract information about the probe orientation in the bilayer membrane. Of the 15 organic dye molecules used in this study, only 4 dyes, DPH, DODCI [$DiOC_2(5)$], $DiSC_2(5)$, and $DiSC_3(5)$, in the egg PC membrane showed a decrease in the fluorescence lifetime of the membrane-bound dye with an increase in the sucrose concentration. It may be noted that this effect is observed only for the long-lifetime component, for which the nonradiative rate is negligible. This is unambiguously the refractive index effect which is expected for the oriented dye molecules in the membrane. The variation of the fluorescence lifetime with the external refractive index has been reported in the literature [9] for DPH but not for the dicarbocyanine dye molecules.

Exact information about the orientation of these dye molecules (more specifically, the order parameters) can be obtained if the nonradiative rate for that species (the long-lifetime component) inside the bilayer membrane is known. For all four dye molecules, the fluorescence decay is due to two or three species. This makes it impossible to determine the nonradiative rate for one species alone based on fluorescence quantum yield measurements in membranes. The nonradiative rate is therefore assumed to be negligible for the long-lifetime component of DPH and dicarbocyanine dyes in the egg PC membrane. This is a reasonable assumption because the long-lifetime value is comparable to its value in highly viscous glycerol, where the quantum yield approaches unity.

Figure 7 shows the decrease in fluorescence lifetime with the refractive index of the aqueous medium in which the egg PC vesicles were made. The experimental data have been fitted to $\tau \sim (n_0)^{-m}$, and the value of m for each dye is indicated in the respective plots. Among the three dicarbocyanines, DODCI shows maximum variation in the fluorescence lifetime with the external re-

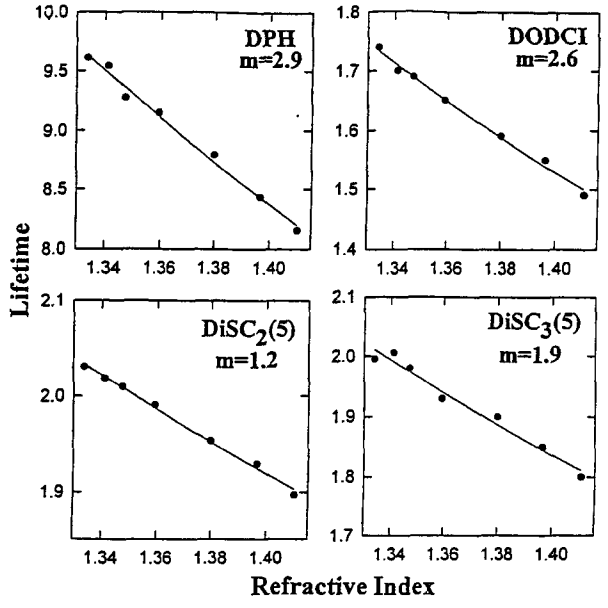


Fig. 7. Variation of the long fluorescence lifetime of the four dyes DPH, DODCI, $DiSC_2(5)$, and $DiSC_3(5)$ in egg PC vesicles with the external refractive index. The values of m obtained by fitting $\tau/\tau_0 = (n_0)^{-m}$ (see text for details) are indicated in the plots.

fractive index [m = 2.6 for DODCI, m = 1.2 for $DiSC_2(5)$, and m = 1.9 for $DiSC_3(5)$]. A value of m = 5 indicates that the dye is oriented normal to the membrane [$\theta = 0$ in Eq. (5)] and m = 1 indicates an orientation parallel to the surface [$\theta = \pi/2$ in Eq. (5)]. The observed values of m for the four dyes suggest that they are oriented at different angles.

It is useful to discuss briefly the orientation of DPH in the bilayer membrane. Toptygin and Brand [9] determined the second-rank order parameter $\langle P_2 \rangle$ for DPH in DPPC by the lifetime method [Eq. (5)] and the anisotropy method [$\langle P_2 \rangle_a = (r_\infty/r_0)^{1/2}$]. The values of order parameters determined by the two methods were in total disagreement (0.285 and 0.91). The two experimental values could be reconciled only if one were to assume that the orientation angle of DPH is 43° to the membrane normal. It was suggested that the lipid chains may be oriented at such angles, thereby aligning DPH at the same angle. In the absence of independent experimental evidence, the question of the orientation of DPH in the bilayer membrane was left open.

Following the approach of Toptygin and Brand [9], we calculated the order parameter $\langle P_2 \rangle_\tau$ for the four linear dyes using the experimental results (τ vs n_0) and the value of the refractive index for the bilayer membrane n_1 = 1.406, assuming that $k_{nr} = 0$. The calculated values of $\langle P_2 \rangle_\tau$ are 0.257 (DPH), 0.141 (DODCI), −0.137 [$DiSC_3(5)$], and −0.422 [$DiSC_2(5)$]. It may be noted that $\langle P_2 \rangle_\tau$ is negative for $DiSC_2(5)$ and $DiSC_3(5)$. The order parameters $\langle P_2 \rangle_a$ determined from the anisotropy data [$\langle P_2 \rangle_a = (r_\infty/r_0)^{1/2}$] are 0.267 (DPH), 0.766 (DODCI), 0.424 [$DiSC_3(5)$], and 0.320 [$DiSC_2(5)$]. The average orientation θ of the dye molecule with respect to the membrane normal is calculated using eq. (6) [9].

$$\frac{1}{2}(3\cos^2\theta - 1) = \frac{\langle P_2 \rangle_\tau}{\langle P_2 \rangle_a} \tag{6}$$

The calculated values of average orientation angles for these linear dyes in the egg PC membrane are 9° for DPH, 48° for DODCI, and 70° for $DiSC_3(5)$. The orientation angle cannot be calculated for $DiSC_2(5)$ using the above equation. The variation of the orientation angle for the linear dyes is difficult to explain in terms of the orientation of the lipid chain or any other reasonable model.

A simplified version of the model proposed by van der Heide *et al.* [20] for the orientational distribution satisfactorily explains the above experimental results: (a) the negative values for $\langle P_2 \rangle_\tau$ for some dyes and (b) the difference in the values of order parameters determined by the two methods. In this model, the orientational distribution of the linear dye molecules in the core region (that is, the fraction with the long-lifetime component) consists of two populations which are oriented about the parallel ($\theta = 0$) and the perpendicular ($\theta = \pi/2$) axes with respect to membrane normal. The potential barrier for intermediate orientations is sufficiently high that the net population in the intermediate orientations is small. The order parameter determined by the lifetime method (refractive index effect) is with respect to the membrane normal and given by Eq. (7).

$$\langle P_2 \rangle_\tau = f\langle P_2 \rangle_{\theta=0} + (1 - f)\langle P_2 \rangle_{\theta=\pi/2} \tag{7}$$

where f is the fraction of molecules oriented about θ = 0, and $\langle P_2 \rangle_{\theta=0}$ and $\langle P_2 \rangle_{\theta=\pi/2}$ are the order parameters for the two populations. In the extreme case of fixed orientations at $\theta = 0$ and $\theta = \pi/2$, $\langle P_2 \rangle_{\theta=0} = 1$ and $\langle P_2 \rangle\theta=\pi/2 = -0.5$. Negative values for $\langle P_2 \rangle_\tau$ are therefore possible if $f < \frac{1}{3}$. The values of f calculated for this extreme case are 0.504 (DPH), 0.427 (DODCI), 0.242 [$DiSC_3(5)$], and 0.052 [$DiSC_2(5)$]. However, realistic values for f (expected to be larger than the limiting values calculated above) can be calculated only when an accurate angular distribution is experimentally determined. This work is in progress. According to this model the order parameter determined from the anisotropy experiment will always be positive and greater than or

equal to $\langle P_2 \rangle_\tau$, irrespective of the value of f in the above model.

CONCLUSIONS

The fluorescence lifetimes of several organic molecules in aqueous solutions are sensitive to the viscosity of the medium. The site of solubilization of these dyes when bound to biomembranes can be identified (surface, interface, or core region) from the effect of the viscosity and refractive index of the aqueous medium on the lifetime. Additional information on the orientation of the dye in the core or interfacial region with respect to the membrane normal can be obtained in favorable cases from the effect of the refractive index on the fluorescence lifetime. Steady-state and time-resolved fluorescence of 15 organic fluorophores were studied in aqueous egg PC vesicles. Multiple fluorescence lifetime (two or three) of the dye bound to the membrane were determined. The site of solubilization of major lifetime components for the dyes were determined from the variation of fluorescence lifetimes with sucrose in the aqueous solution. In a few cases (linear dye molecules), the decrease in fluorescence lifetime with sucrose was identified to be the effect of the refractive index on the radiative rate of oriented dye molecules in the membrane phase. The experimental results favor a model of orientational distribution in mutually orthogonal directions in the membrane.

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