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LIPOSOME FUSION AS MONITORED BY A FLUORESCENCE QUENCHING TECHNIQUE†

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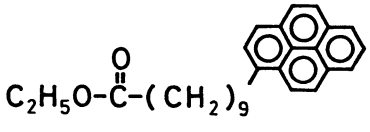
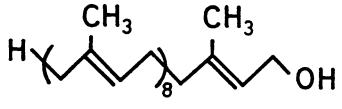
The fluorescence emission from ethyl ω-(1-pyrenyl)decanoate (EP) is quenched by 4-dodecyl-*N*,*N*-dimethylaniline (DDA) in liposomal bilayers according to the collisional quenching mechanism without heteroexcimer formation. This phenomenon was utilized to monitor liposome fusion between two small and single-walled vesicles. When a set of two liposomes, F-liposome containing the fluorophore EP and Q-liposome containing the quencher DDA, was incubated and passively sensitized by Ca(II) ion or a lipophilic agent (solanesol), significant quenching of EP fluorescence was observed. This was ascribed to the encounter of the fluorophore and quencher in the bilayers upon liposome fusion.

Although fusion of liposomes can be monitored by a variety of techniques such as NMR and turbidity,[1-5] a simpler and more sensitive method is still desired. In this work, we have devised a novel fluorescence quenching technique for studying fusion between single-walled liposomes. It has been previously shown that the fluorescence from aromatics such as anthracene and pyrene is quenched with *N*,*N*-dimethylanilines by either dynamic or static mechanisms.[6-8] We employed ethyl ω-(1-pyrenyl)decanoate (EP) as a fluorophore[9] and 4-dodecyl-*N*,*N*-dimethylaniline (DDA) as a quencher.[10] Since both probes carry a long alkyl moiety, they must be tightly intercalated in liposomal bilayers. Thus, the quenching of EP fluorescence would occur only when the fluorophore and quencher encounter following fusion of a set of two independent vesicles, F-liposome carrying EP and Q-liposome containing DDA. F- and Q-liposomes were prepared separately from egg yolk lecithin according to the method described previously.[13-15]



EP



Solanesol

When an ethanolic solution of DDA was added to an F-liposome suspension, a time-dependent quenching of the fluorescence from EP was observed.[17] This is

caused by the rapid adsorption of DDA onto the vesicle surface and its subsequent permeation and/or diffusion into liposomal bilayers. The microscopic environments around EP and DDA in the liposomal bilayers were deduced to be close to the polarity of carbon tetrachloride and ethanol, respectively. They were estimated using a ruler obtained from the correlation between the emission maxima of fluorescence and the parameter of solvent polarity, $E_T(30)$.[18] This means that the fluorophore is located in the deep hydrophobic domain of bilayers and the quencher is close to the membrane surface of liposomes. Under the present conditions, no heteroexcimer emission was observed in the liposomal bilayers.[19] The fluorescence intensity in the equilibrated state at a given DDA concentration was obtained by re-sonication of the resulting mixture. The quenching data followed the Stern-Volmer mechanism:

$$I_0/I = 1 + k_q\tau_0[Q] \quad (1)$$

where I_0 and I stand for the fluorescence intensity at 377 nm in the absence and presence of DDA, respectively, and k_q refers to the Stern-Volmer quenching constant. Since the fluorescence lifetime of EP, τ_0, in the present system was 223 ± 1 ns,[20] k_q was consequently evaluated as $(7.6 \pm 0.5) \times 10^{11}$ M^{-1} s^{-1}. This value seems unusually large for a probe situated in a relatively viscous environment.[6,7] However, if an appropriate correction is made for the "effective" concentration of DDA in liposomal bilayers, the value becomes reasonable. Since DDA is very hydrophobic, it hardly diffuses out of the liposomal bilayers.[21] If we assume, then, that a single-walled and small vesicle is composed of 3,000 molecules of lecithin[22] and has an average diameter of 250 Å with 50 Å bilayer thickness,[22,23] we can estimate that 3.1×10^{-4} M lecithin will occupy 1.2×10^{-3} cm^3 as bilayers in 3.0 ml

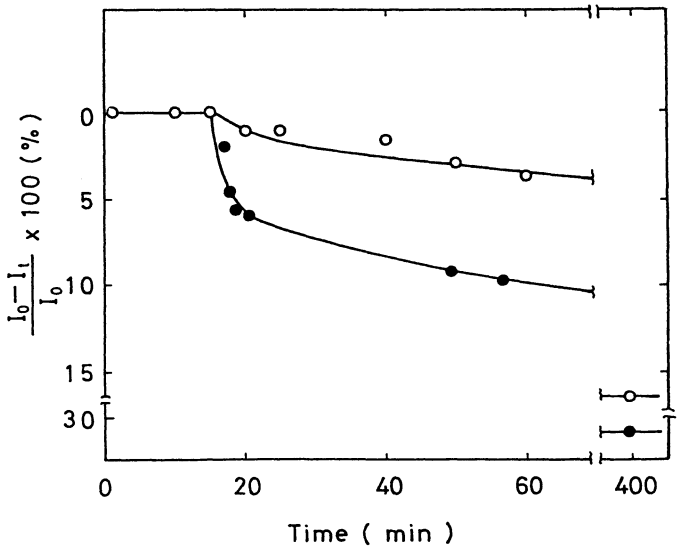


Fig. 1. Fusion between F-liposome (egg lecithin, 1.02×10^{-4} M; EP, 3.9×10^{-7} M) and Q-liposome (egg lecithin, 1.31×10^{-5} M; DDA, 2.1×10^{-6} M) as mediated by 4.7×10^{-5} M Ca(II) ion (○) and 4.3×10^{-7} M solanesol (●). Liposomes were incubated at 25.0 °C in 0.1 M aq. NaCl solution.

of liposome suspension. Therefore, if DDA is localized only in the bilayer, DDA must be 2.5×10^3 times effectively concentrated compared with the case where it is homogeneously dispersed in the aqueous solution. If this correction is taken into account, the k_q-value obtained above reduces to 3.0×10^8 M^{-1} s^{-1}, which is absolutely acceptable.

When a mixture of F- and Q-liposome suspensions was incubated at 25.0 °C, no quenching of the fluorescence from EP was observed for at least 1 h. This is good evidence that the intervesicle exchange of both hydrophobic probes must be extremely slow on the present time scale.[21,24] Upon addition of Ca(II) ion, a slow, but obvious quenching of EP fluorescence was observed as seen in Fig. 1. Many examples have been presented to show that when liposomes contain acidic lipids Ca(II) ion is a potent inducer of liposome fusion.[25,26] Solanesol causes more significant quenching than the Ca(II) ion. Formation of larger liposomes was confirmed by an electron microscopic observation of a liposome mixture after the fusion experiment. Figure 1 suggests also that the mechanism of fusion may be different between the two inducers. In the case of Ca(II) induced fusion, the key event leading to membrane fusion is the isothermic phase separation of acidic lipids caused by Ca(II) ion.[27] In view of the fact that long chain fatty acids increase fluidity of membranes to result in fusion,[28,29] solanesol may be acting in a similar way. This conclusion was reached also in our previous study in which liposome fusion was monitored by the leakage of an amphiphilic dye marker.[13] The result obtained on liposome fusion in the present study is consistent with that obtained by the marker release technique, substantiating the validity of the fluorescence quenching technique. The new method is sensitive and reliable, and should find wide use in the study of a dynamic process of cell or model membrane fusion.

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† Liposomal Membranes. VIII.

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10) DDA was prepared as follows. *p*-Dimethylaminoundecylphenone was synthesized first by the Friedel-Crafts acylation of *N*,*N*-dimethylaniline with dodecanoyl chloride and aluminum chloride in carbon disulfide;[11] yield, 15%; mp 73.0-

73.8 °C. Anal. Found: C, 78.97; H, 11.10; N, 4.54%. Calcd for $C_{20}H_{33}NO$: C, 79.15; H, 10.96; N, 4.61%. It was reduced with lithium aluminum hydride and aluminum chloride in ether to afford DDA in 65% yield;[12] mp 26-27 °C. NMR ($CDCl_3$); δ, 0.90 (t, J = 7 Hz, 3H, CH_3), 1.28 (s, 20H, $-(CH_2)_{1\overline{0}}-$), 2.52 (t, J = 7 Hz, 2H, $-CH_2-$), 2.91 (s, 6H, $N-CH_3$), 6.75 (d, J = 9 Hz, 2H, phenyl), 7.13 (d, J = 9 Hz, 2H, phenyl). Anal. Found: C, 83.34; H, 12.44; N, 4.73%. Calcd for $C_{20}H_{35}N$: C, 82.98; H, 12.18; N, 4.84%.

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15) The protocol for F- and Q-liposomes was as follows: egg lecithin, 15 mg; EP, 24 μg; DDA, 113 μg. Liposome concentration was determined as inorganic phosphate according to the Allen's procedures.[16] The actual concentration of EP in liposomal bilayers was spectrophotometrically determined from the absorption band at 345 nm (ε_{345} = 38,300 M^{-1} cm^{-1}), while that of DDA in Q-liposome was from the fluorescence intensity (E_x = 305 nm and E_m = 360 nm) with the use of rhodamine B as a standard. They are given in the caption to Fig. 1.

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