Biochimica et Biophysica Acta, 647 (1981) 155–158
Elsevier/North-Holland Biomedical Press

BBA Report

BBA 71536

DELAYED LUMINESCENCE OF PYRENE IN PHOSPHATIDYLCHOLINE DISPERSIONS

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(Received April 27th, 1981)

Key words: Delayed luminescence; Pyrene: Phosphatidylcholine; Phase transition

Delayed luminescence of pyrene in phosphatidylcholine dispersions was studied over the temperature range 5–60°C. It was found that the pyrene delayed-luminescence spectrum is similar to its fluorescence spectrum. The transient behavior of the luminescence was also studied and was found to show a drastic change when the lipid matrix undergoes phase transition. This method will be a new tool for membrane studies, especially for the detection of the phase transition.

The biological significance of the thermotropic phase transition in biomembranes is now well recognised [1,2]. Several physicochemical techniques such as scanning calorimetry, infrared and Raman spectroscopy, magnetic resonance spectrometry, X-ray diffraction and fluorescence spectroscopy have been applied to detect the phase transition. Pyrene has been used as a membrane probe since it has a characteristic excimer emission and long fluorescence lifetime [3]. Since triplet excited molecules are expected to have longer lifetimes than singlet excited molecules, they will undergo translation and rotation and encounter other molecules more extensively while they are excited; thus we expect that phosphorescence will be more sensitive to a subtle change of environment of the probe molecules. However, as shown in this report, delayed luminescence other than phosphorescence was observed with pyrene in lipid dispersions more easily and found to be sensitive to the phase transition of the phospholipid dispersions.

We describe the detection of fluorescence-like delayed luminescence of pyrene in dipalmitoylphosphatidylcholine and dimyristoylphosphatidylcholine dispersions, including the transient behaviour and its temperature dependences.

Dipalmitoylphosphatidylcholine (DPPC) and dimyristoylphosphatidylcholine (DMPC) were obtained from Sigma. Their purity was checked by thin-layer chromatography using chloroform/methanol/water (130 : 50 : 8, v/v) as a solvent and both DPPC and DMPC were found to show a single spot. Zone-refined pyrene was a gift from N.I. Wakayama of National Chemical Laboratory for Industry (Ibaraki).

The phospholipid was dissolved in chloroform/methanol (1 : 1, v/v). Pyrene was dissolved in hexane. Portions of the two solutions were mixed and evaporated to dryness. 50 mM potasium phosphate buffer, pH 7.0, was introduced and submitted to sonication to prepare a lipid dispersion. All the lipid dispersions were prepared at a concentration of 1 mg phospholipid in 2.0 ml solution. Sonication was performed at temperatures above the phase transition until the dispersions became clear. The molar ratio of pyrene to phospholipid was 1 : 1000 in each preparation. Then it was transferred into a quartz cell equipped with a stopcock.

Ultra-high purity nitrogen gas (over 99.999%)

Abbreviations: DPPC, dipalmitoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine.

was bubbled into the dispersion for more than 30 min, and the cell was sealed with the stopcock shortly before the measurement.

Emission from the lipid dispersion excited by a nitrogen laser was studied as illustrated in Fig. 1. The time interval between the laser excitation and the onset of the recording by the multichannel analyzer was determined with the simultaneous use of the photomultiplier, the oscilloscope, a delay generator and the multichannel analyzer to be approx. 4.8 μs.

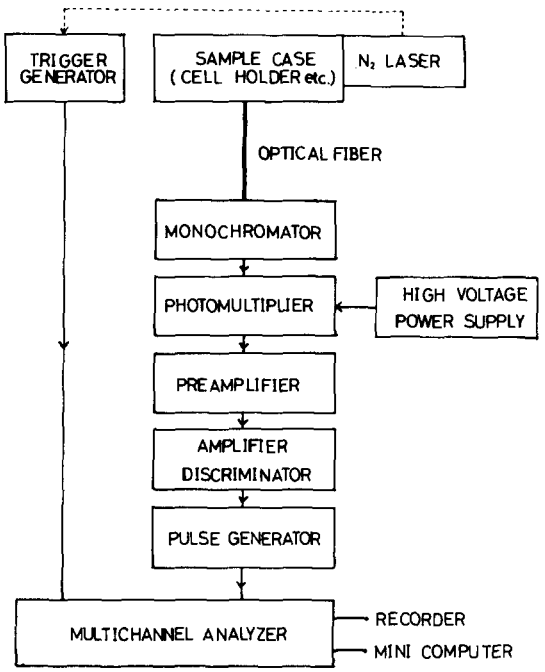

Fig. 1. Block diagram of the apparatus. Radiation from a self-made nitrogen laser (337.1 nm) (Blumelein-type discharge laser, output energy $\lesssim$ 1 mJ/pulse, halfwidth <10 ns) was used as a pulsed light source and fed through a glass filter onto a quartz cell containing a lipid dispersion. The temperature of the cell was regulated by circulating temperature-controlled water around the cell container. The emitted light was passed through an optical fiber cable (Oki Electric Cable Co., Ltd.), a gelatin filter (SC 37; Fuji Photo Film Co., Ltd.) and a monochromator (Nikon G-250) onto a photomultiplier (RCA 8850). The photoelectric pulse signal from the photomultiplier was amplified, discriminated, transformed into a sequence of positive pulses and finally fed into a multichannel analyzer (Canbera 8100) in its multichannel scaling mode. The electromagnetic radiation accompanying the pulse discharge was picked up, shaped and used to trigger an oscilloscope or the multichannel analyzer.

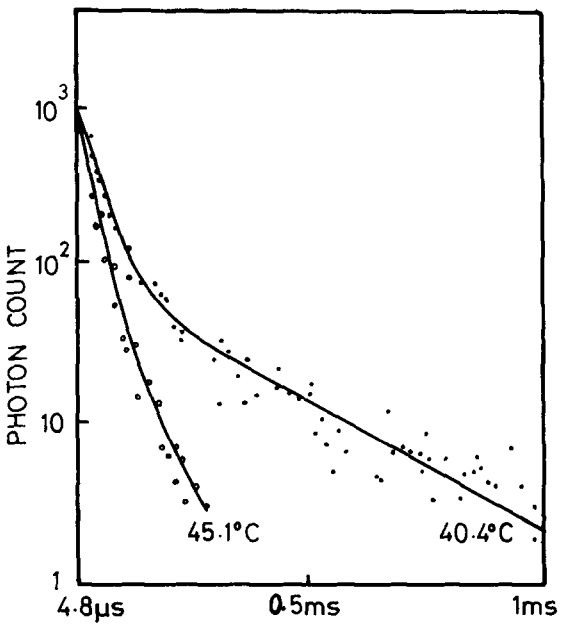

Fig. 2. Typical time courses of the pyrene delayed luminescence (480 nm) in DPPC at 40.4°C and 45.1°C. Solid lines represent the calculation based on the following parameters:
40.4°C: $a = 0.91$, $\tau_1 = 36$ μs, $\tau_2 = 260$ μs,
45.4°C: $a = 0.95$, $\tau_1 = 31$ μs, $\tau_2 = 92$ μs.

The detected light intensity was carefully adjusted to less than 20 pulses per 10 μs to insure that the pulse pair resolution limit of the instruments does not affect the results significantly.

The spectrum of the delayed luminescence was obtained by integrating the total counts of the emitted light recorded in the multichannel analyzer at the respective wavelength.

Typical time courses of the emitted light intensity at 480 nm of pyrene in DPPC dispersions at 40.4°C (below T_c) and 45.1°C (above T_c) are shown in Fig. 2. Similar behavior is obtained at 390 nm.

The results were analyzed by a least-squares method, assuming that the decay characteristics of the light intensity $I(t)$ is described by the following equation,

$$I(t) = I(0) \cdot (ae^{-t/\tau_1} + (1 - a)e^{-t/\tau_2})$$

where τ_1 and τ_2 are lifetimes of the shorter and longer components respectively (Table IA).

The spectrum of the emitted light of pyrene in

TABLE I

PARAMETERS OF THE TRANSIENT BEHAVIOR OF THE DELAYED FLUORESCENCE IN PYRENE-DPPC (A) AND PYRENE-DMPC (B)

Parameters are analyzed using the following equation:

$I(t) = I(0) \cdot (ae^{-t/\tau_1} + (1-a)e^{-t/\tau_2})$

by a least-squares procedure. χ^2 is a chi-squared value for the fit.

(A) DELAYED FLUORESCENCE LIFETIMES OF PYRENE-DPPC

T(°C)	a	τ_1(μs)	τ_2(μs)	χ^2
25.0	0.92	48	430	0.92
30.0	0.92	44	370	1.27
35.1	0.92	40	310	1.01
40.4	0.91	36	260	1.21
45.1	0.95	31	92	0.89
50.3	0.99	31	170	0.79
55.1	0.98	30	110	0.86
60.0	0.98	28	116	0.91

(B) DELAYED FLUORESCENCE LIFETIMES OF PYRENE-DMPC

T(°C)	a	τ_1(μs)	τ_2(μs)	χ^2
5.5	0.91	54	560	1.14
10.6	0.90	55	480	1.19
15.8	0.90	54	390	1.40
18.2	0.92	52	350	1.22
21.0	0.92	50	300	1.72
24.6	0.99	56	1540	2.57
27.0	1.00	38	–	1.92
30.5	1.00	35	–	1.64
35.6	1.00	31	–	2.09

DPPC at 30°C is shown in Fig. 3. This is similar to the normal fluorescence spectrum except that the ratio, excimer emission (480 nm)/monomer emission (390 mm), is higher.

Similar results were obtained with pyrene in DMPC (Table 1B). In this case, the longer component diminished at approx. 25°C and above, in good correspondence with T_c (=22°C) of DMPC.

It is also noted that τ_1 decreased remarkably at $T > T_c$.

The spectra of the τ_1 and τ_2 components were also studied by integrating the τ_2 component separately and no significant difference was found.

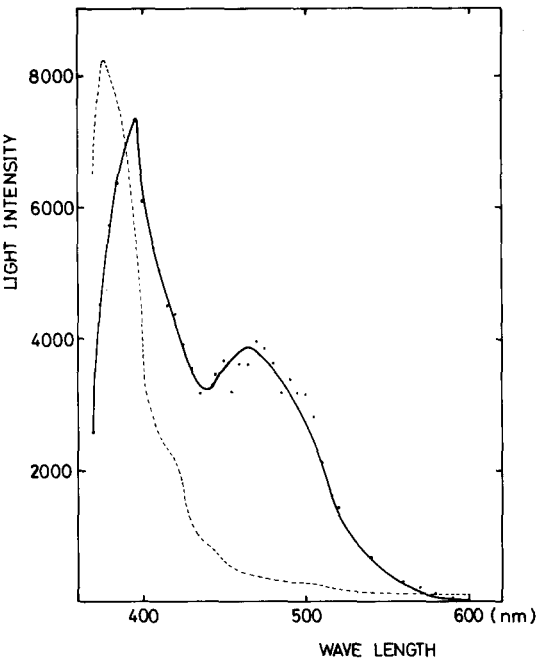

Fig. 3. Spectrum of delayed luminescence of pyrene in DPPC at 30°C (uncorrected). This corresponds to total intensity of more than 4.8 μs after the excitation. [DPPC] 0.5 mg/ml potasium phosphate buffer (50 mM, pH 7.5), [pyrene]/[DPPC] = 1/1000. The broken line shows the steady-state emission spectrum of the same sample, excited with a xenon arc lamp at 337 nm and detected with the same optical and photoelectric system.

It is shown in this study that delayed luminsescence of pyrene in phospholipid dispersions can easily be detected.

The spectrum of the luminescence is similar to that of normal fluorescence. Thus we may call it delayed fluorescence. The fact that the ratio of excimer emission/monomer emission is higher than that of the normal fluorescence suggests that this delayed fluorescence may be due to the triplet-triplet annihilation mechanism [4]. In a recent study, Meyer and Nickel [5] obtained a much longer lifetime of delayed fluorescence of pyrene in hexane at 25°C. They employed a freeze-pump-thaw technique to eliminate traces of oxygen and took further precautions in the purification of the solvent and in sealing off. These are indeed desirable steps to study intrinsic molecular properties, but impractical for biological systems in aqueous buffer solution. Bubbling with ultra-pure nitrogen gas gave

reproducible results and proved to be practical, though it is not certain if the results obtained in this way correspond to the oxygen-free system. However, this does not devalue the delayed-luminescence study in biological applications. In fact, the transient behavior of delayed fluorescence of pyrene in both DPPC and DMPC is strongly dependent on the properties of the lipid phase. At $T > T_c$, the slowest component ($\tau \approx 300$ μs) almost disappeared, whereas at $T < T_c$ it is quite evident, as Fig. 1 shows. This fact indicates that delayed luminescence is quite sensitive to the subtle change of the environment and that further application is expected.

Room-temperature phosphorescence of aromatic hydrocarbons in aqueous micellar solutions was reported by Kalyanasundaram et al. [6] by Turro et al. [7], Humphry-Baker et al. [8] and more recently by Love et al. [9]. They estimated the phosphorescence lifetime of pyrene in sodium dodecyl sulfate micelles in the presence of thallous nitrate to be over 50 ms [6]. They had bubbled their solutions with ultra-high purity nitrogen gas for deoxygenation.

If we assume that the delayed fluorescence we observed is due to the singlet excited molecules generated by the triplet-triplet interaction mechanism, the triplet state lifetime in our system will be two orders of magnitude shorter than their estimation. The reasons of this difference may possibly be attributed to

(1) the difference of the systems;
(2) the difference in the concentration of quenchers;
(3) the difference of the exciting light intensity.

The first two may be almost inevitable when our interests are focussed on biological applications.

The third reason may deserve some further consideration.

Since we used a nitrogen laser as an exciting light, the photon density of the exciting light in our system will be much higher. Consequently, the concentration of the triplet excited molecules, n_T, shortly after the excitation may be much higher. Then, n_T may obey the following equation, $dn_T/dt = -\gamma n_T^2$ with the omission of monomolecular decay term, $-\beta n_T$, where γ is the triplet-triplet annihilation rate constant. Thus, the apparent triplet-state lifetime may be shortened. In this case, analysis of the transient behavior of the delayed fluorescence by the sum of two exponential terms will be only tentative.

Emitted light intensity was shown to be proportional to the exciting light intensity instead of the square of it (data not shown). This, together with the absence of phosphorescence in our system, is consistent with the above-mentioned mechanism.

These conditions facilitate the observation of delayed fluorescence even when the mono-molecular decay, $-\beta n_T$, is considerably large and the phosphorescence is very weak. Thus delayed fluorescence studies with hydrophobic probes in physiological conditions have a vast potency, as is exemplified in this study.

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