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Studies of alcohol-induced interdigitated gel phase in phosphatidylcholine multilamellar vesicles by the excimer method

Masahito Yamazaki, Motoi Miyazu and Tsutomu Asano

Department of Physics, Faculty of Science, Shizuoka University, Shizuoka (Japan)

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We have developed a new spectroscopic method (excimer method) for detecting the interdigitated gel phase ($L_{\beta I}$) in phospholipid vesicles. This method is based on the disappearance of an excimer fluorescence peak of pyrenephosphatidylcholine (pyrene-PC) in the $L_{\beta I}$ phase. Using this method we have studied the phase transition from gel phase ($L_{\beta'}$) to $L_{\beta I}$ phase of dipalmitoylphosphatidylcholine multilamellar vesicles in the presence of ethanol or ethylene glycol (EG). In both the cases of ethanol and EG, a sharp decrease in the ratio of excimer to monomer fluorescence intensity (E/M) of pyrene PC appeared at the same concentration of the transition from $L_{\beta'}$ to $L_{\beta I}$ as determined by the X-ray diffraction method or the scanning density method. After the transition to the $L_{\beta I}$ phase, E/M values became very low. This excimer method enables us to detect the $L_{\beta I}$ phase in unilamellar vesicles of phospholipids, which can hardly be studied by other methods such as X-ray diffraction.

Introduction

Recently, interdigitated structures in phospholipid membranes have been studied and have attracted much attention [1]. In this structure, lipid molecules from opposing monolayers are fully interpenetrated or interdigitated and the terminal of the alkyl chain faces aqueous phase. In the presence of ethanol [2–4], ethylene glycol (EG) [5], and under high pressure [6], multilamellar vesicles (MLV) of phosphatidylcholine (PC) can form the interdigitated gel phase ($L_{\beta I}$ phase). In order to detect and study the interdigitate gel structure, X-ray diffraction and neutron diffraction have been used extensively. However, these methods cannot be applied to the detection of the interdigitated gel structure in unilamellar vesicles, owing to their low sensitivity.

The ultimate purpose of this work was to develop a new, highly sensitive spectroscopic method (excimer method) to detect the $L_{\beta I}$ phase in unilamellar phospholipid vesicles and to study the phase transition between $L_{\beta I}$ phase and $L_{\beta'}$ phase. The excimer method is based on the spectroscopic properties of a fluorescent pyrene probe. The emission spectra of pyrene and its derivatives typically show two components: one is due to the excited pyrene monomer A* and the other, at longer wavelengths, is attributed to the excited dimer (excimer) $(AA)^*$ formed on the collision of an excited monomer A* with a ground-state pyrene A. The ratio of the excimer to monomer fluorescence intensities (E/M) is proportional to the collision frequency of pyrenes [7,8]. In this report, we present the new excimer method and examine it in studies of ethanol- or EG-induced $L_{\beta I}$ phase in the MLV of DPPC.

Materials and Methods

1,2-Dipalmitoyl-L-α-phosphatidylcholine (DPPC) and 1,2-dioleoyl-L-α-phosphatidylcholine (DOPC) were purchased from Sigma Chemical Co. 1-Palmitoyl-2-pyrenedecanoyl-L-α-phosphatidylcholine (pyrene-PC) was purchased from Molecular Probes Inc. Ethanol and EG were purchased from the Wako Chemical Co. The molecular structure of pyrene PC is shown in Fig. 1(a) (inset).

Abbreviations: DPPC, 1,2-dipalmitoyl-L-α-phosphatidylcholine; MLV, multilamellar vesicle; DOPC, 1,2-dioleoyl-L-α-phosphatidylcholine; Pipes, piperazine-*N*,*N*'-bis(2-ethanesulfonic acid); $L_{\beta'}$, tilted chain bilayer gel phase; $L_{\beta I}$, interdigitated gel phase, E/M, the ratio of excimer to monomer fluorescence intensity; EG, ethylene glycol; pyrene-PC, 1-palmitoyl-2-pyrenedecanoyl-L-α-phosphatidylcholine.

Correspondence: M. Yamazaki, Department of Physics, Faculty of Science, Shizuoka University, Shizuoka 422, Japan.

MLVs were prepared by adding the appropriate amounts of Pipes buffer (10 mM Pipes (pH 7.5), 140 mM NaCl) to the dry mixture of DPPC (or DOPC) and pyrene-PC. Then the mixture was heated for 10 min at 50°C (20°C for DOPC). During the incubation time, the suspension was vortexed several times for 0.5 min. Ethanol or EG solution was added to the preformed MLV solution, and the mixture was incubated at 50°C for 1 h (ethanol) or 2 h (EG) to attain equilibrium.

For fluorescence measurements, a Hitachi F3000 spectrofluorimeter was used. The excitation wavelength of pyrene-PC was 347 nm and emission wavelengths were 376 nm for monomer fluorescence and 481 nm for excimer fluorescence. Excitation bandpass and emission bandpass were 3 nm and 1.5 nm, respectively. The ratio of excimer to monomer fluorescence intensities (E/M) was calculated. The fluorescence intensities for calculation of E/M were obtained by their time averaging for 0.5 min. Emission spectra were average ones of four measurements. The concentrations of DPPC in the samples for the measurement of fluorescence were 74 μM. X-ray diffraction data were recorded using a position-sensitive proportional counter method as described before [5]. The concentrations of lipid were determined by phosphate analysis [9].

Results and Discussion

An emission spectrum of DPPC MLV containing pyrene-PC is shown in Fig. 1(a). There is a large peak of excimer fluorescence around 481 nm. In 15% (w/v) ethanol (Fig. 1(b)), the peak of excimer fluorescence almost disappeared and the monomer peak intensity increased to 2.6-times that in the absence of ethanol. The fluorescence intensity ratio (E/M) was plotted as a function of ethanol concentration. As shown in Fig.

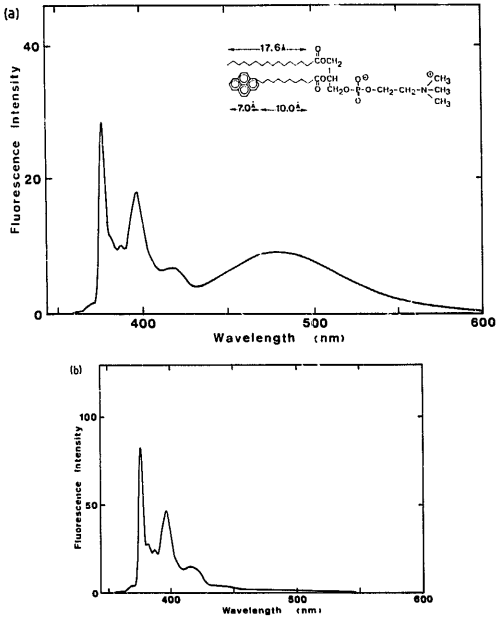

Fig. 1. Emission spectra for pyrene-PC in the DPPC MLV in the absence of ethanol (a) and in 15% (w/v) ethanol (b) at 23°C. Excitation wavelength was 347 nm. The pyrene-PC concentration was 2.8 mol% of the amount of total lipid. The inset in (a) shows a structural formula of 1-palmitoyl-2-pyrenedecanoyl-L-α-phosphatidylcholine (pyrene-PC).

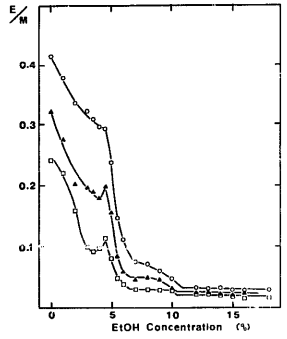

Fig. 2. Ratio of excimer to monomer fluorescence intensities (E/M) of pyrene-PC in DPPC MLV in the various concentrations of ethanol. E/M was determined at 23°C as explained in Materials and Methods. The pyrene PC concentration was 4.1 mol% (○), 2.8 mol% (▲) and 1.4 mol% (□) of the total amount of lipid.

2, irrespective of pyrene-PC concentration, the E/M value at 23°C decreased sharply at 4.5% (w/v) ethanol. This concentration was almost equal to that of the transition from $L_{\beta'}$ to $L_{\beta I}$ (C_t) at 23°C, determined by the scanning density meter ($C_t = 4.5\%$) [10]. The repeating period (spacing) of DPPC MLV at 23°C rapidly decreased from 65 Å to 50 Å around 4.6% (w/v), which indicated the phase transition from $L_{\beta'}$ to $L_{\beta I}$ phase as discussed in detail in the previous paper [5]. Above 12% (w/v) ethanol, there was no peak of excimer, although there was a residual small fluorescence intensity at 481 nm due to the monomer fluorescence.

High concentration of EG also induced the $L_{\beta I}$ phase in DPPC MLV. A temperature-EG concentration phase diagram was determined by use of the X-ray diffraction method [5]. The value of E/M at 19°C was plotted as a function of EG concentration (Fig. 3). A sharp decrease in E/M began at 33% (w/v) EG, irrespective of pyrene-PC concentration. This EG concentration corresponds to that of the transition from $L_{\beta'}$ to $L_{\beta I}$ ($C_t = 33\%$) at 19°C obtained from the phase diagram in the previous paper [5].

As a control experiment, interactions of DOPC MLV with ethanol or EG were investigated using this excimer method. DOPC MLV showed its main transition at −22°C, and was in the liquid crystalline phase at room temperature. Therefore, ethanol or EG could not induce $L_{\beta I}$ phase. In accordance with this fact, the E/M value at 18°C did not show a sharp decrease, but increased gradually with an increase in ethanol concentration (Fig. 4). In the case of EG, E/M values did not change below 48% (w/v).

In both the cases of ethanol and EG, the onset concentrations of the sharp decrease of E/M corresponded to the transition concentration from $L_{\beta'}$ to $L_{\beta I}$. After the transition, the monomer intensity is twice as large as that in the absence of ethanol (or EG) and E/M values became very low, showing the reduction of the excimers in the MLV. Supposing that the reduction of the excimers occur by excluding pyrene PC molecules from the membrane in $L_{\beta I}$ phase, most of the molecules would exist in the aqueous phase. In the case of Figs. 2 and 3, the resulting concentrations of the pyrene-PC in the aqueous phase could be 1–3 μM, which are higher than the critical bilayer concentration (cbc) for pyrene-PC ($1.5 \cdot 10^{-7}$ M) [11]. E/M values of MLV composed for 100% pyrene-PC were measured at 17°C in the absence of ethanol and in 15% (w/v) ethanol as 48($\pm$7) (concentration range of pyrene-PC is from 0.33 μM to 13 μM) and 3.4($\pm$0.3) (from 0.05 μM to 17 μM), respectively, which shows good agreement with the value of Somerharju et al. [12]. The above consideration and the experimental results suggest that the pyrene-PC molecules expelled from the membrane into the aqueous phase could form bilayer membranes and could show the E/M values of 3.4 in 15% (w/v) ethanol. Our experimental values ($E/M = 0.024$) were much smaller than 3.4, which excludes the possibility of expelling pyrene-PC molecules from the MLV in the $L_{\beta I}$ phase.

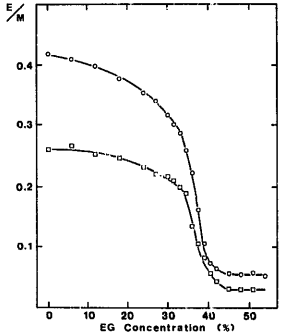

Fig. 3. Ratio of excimer to monomer fluorescence intensities (E/M) of pyrene-PC in DPPC MLV in the various concentrations of EG. E/M was determined at 19°C as explained in Materials and Methods. The pyrene-PC concentrations were 4.1 mol% (○) and 1.4 mol% (□) of the total amount of lipid.

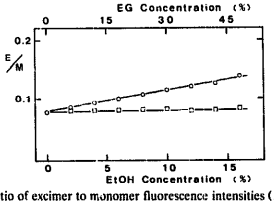

Fig. 4. Ratio of excimer to monomer fluorescence intensities (E/M) of pyrene-PC in DOPC MLV in the various concentrations of ethanol and EG. E/M was determined at 18°C as explained in Materials and Methods. The pyrene-PC concentration was 2.8 mol% of the total amount of lipid. Ethanol (○) and EG (□).

Structural change during the transition from $L_{\beta'}$ to $L_{\beta I}$ may account for the reduction of the E/M value. An excimer is formed when an excited pyrene molecule collides with a ground-state pyrene molecule under the condition where their aromatic rings are oriented parallel to each other and most of the areas of two pyrene rings are superposed [7,8]. In gel-state DPPC bilayers, the pyrene-PC molecules form small clusters, which do not consist of pure pyrene-PC but of aggregates of pyrene-PC and DPPC [12]. The cluster formation explains that E/M values in gel state are much higher than that expected from a diffusion-controlled process [7]. As shown in a structure model (Fig. 5(a, c)), two pyrene molecules attached to the hydrocarbon chain can easily collide with each other in $L_{\beta'}$ phase. On the other hand, in $L_{\beta I}$ phase, a pyrene molecule of DPPC existing on one side of monolayer of $L_{\beta'}$ cannot collide with other pyrene molecules on the opposite side, even when they are next to each other, because of the mismatching of two pyrene rings shown in Fig. 5(b). Thereby, in $L_{\beta I}$ phase, the effective pyrene concentration in the membrane is reduced to half compared with the $L_{\beta'}$ phase. Considering that the E/M value is proportional to the pyrene concentration [7,8,13], it is evident that the E/M value of $L_{\beta I}$ phase is about half that of $L_{\beta'}$ phase.

However, our E/M value after the transition were less than 10% of the original value before transition. This discrepancy could be explained by the structural model (Fig. 5(c, d)) on the basis of the slow diffusion of the lipids in the gel phase. Experiments of surface isotherm of the neat pyrene PC monolayer indicated that the pyrene moiety did not appreciably influence the packing properties of the alkyl chains [12]. Thereby, we assume that the alkyl chains of phospholipids form a hexagonal lattice. The two-dimensional arrangement of the phospholipid molecules in a bilayer membrane of the gel phase MLV in Fig. 5(c) is based on the model of Hatta et al. [15]. In Fig. 5(c, d), an open circle represents an alkyl chain of the lipid whose head group is in front of the paper (figure) and a closed circle represents an alkyl chain of the lipid whose head group is behind the paper. According to this model, in one dimension (e.g. horizontal direction in Fig. 5(d)) pyrene molecules of pyrene-PC cannot collide with other pyrene molecules on the same side by interstition of DPPC (or pyrene-PC) molecules on the opposite side, because of the low diffusion coefficient of lipids in gel phase. This effect may reduce E/M values from a half of that in $L_{\beta'}$ phase to very low values. From a two-dimensional point of view, there remains a possibility that two pyrenedecanoyl chains come to a neighboring position and make an excimer. However, this possibility is very much more unlikely than that of excimer formation in $L_{\beta'}$ phase, as discussed above. Therefore, in $L_{\beta I}$ phase, pyrene molecules can hardly collide with each other and, as a result, there are very few excimers.

As shown in Fig. 4, E/M of the DOPC MLV did not sharply decrease, corresponding to no transition to $L_{\beta I}$ phase. In the case of ethanol, E/M values in-

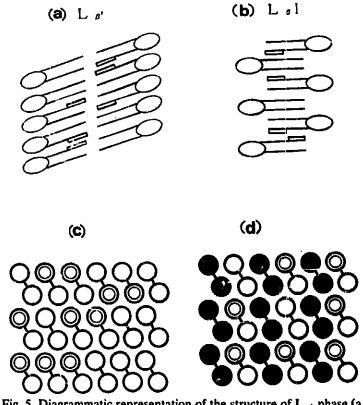

Fig. 5. Diagrammatic representation of the structure of $L_{\beta'}$ phase (a and b) and $L_{\beta I}$ phase (b and d). (a) and (b) are one-dimensional, and (c) and (d) are two-dimensional diagrammatic representations. In (a) and (b), a small rectangle represents a pyrene molecule attached a hydrocarbon chain of lipid. In (c) and (d), each dumbbell indicates a phospholipid molecule; a circle denotes the average cross-section of the alkyl chain of the lipid and the bar connecting the two circles denotes both the glycerol backbone and the polar head group. A double circle represents a pyrenedecanoyl chain. An open circle and a closed circle represent an alkyl chain of the lipid whose head group is in front of the paper and that whose head group is behind the paper, respectively.

creased gradually with an increase in concentration. It is well known that ethanol increases the membrane fluidity in low concentrations, like other anaesthetic molecules [14]. Increase in membrane fluidity increases E/M values because the excimer formation is a diffusion-controlled process in the liquid crystalline phase [7].

A fluorescence method for detecting phase transition of DPPC using 1,6-diphenyl-1,3,5-hexatriene (DPH) was developed by Nambi et al. [4]. The fluorescence intensity of DPH in the DPPC MLV was reduced by over 50% when the $L_{\beta'}$ to $L_{\beta 1}$ transition occurred, showing low sensitivity. By this DPH method, the transition may be detected by the change of fluorescence intensity, but there are many factors involved in the reduction of fluorescence intensity and, moreover, $L_{\beta 1}$ phase cannot be distinguished from other phases by the DPH fluorescence intensity alone. On the other hand, the excimer method using pyrene-PC enables us to distinguish between the $L_{\beta 1}$ phase and other phases by observing the excimer peak.

In both the cases of ethanol and EG, at low concentrations below that of the transition, a small decrease in E/M values was observed. The penetration of ethanol (or EG) into the membrane surface may cause the decrement of collisions between pyrene rings.

We are now studying the induction of $L_{\beta 1}$ phase in unilamellar vesicles of DPPC by ethanol (or EG) and the properties of this phase transition using this excimer method (Yamazaki et al., unpublished results).

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