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$C_{12}E_7$-DPPC mixed systems studied by pyrene fluorescence emission

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Abstract The lipid/surfactant mixed interactions between the lipids dipalmitoylphosphatidylcholine (DPPC) or egg phosphatidylcholine (EggPC) and the non-ionic surfactant $C_{12}E_7$ [$C_{12}H_{25}(OCH_2CH_2)_7OH$] were studied by the use of the fluorescence properties of pyrene, namely the excimer to monomer emission intensity ratio, I_E/I_M. Previously, the behaviour of the $C_{12}E_7$/water system was also monitored. It was found to exhibit a significant preassociation of pyrene in ground state, which is more pronounced in micelles than in premicellar aggregates. In mixed systems, pyrene has proved to detect the changes from mixed bilayers to mixed micelles. The temperature influence in lipid/surfactant interactions was also studied. It was found that the pyrene I_E/I_M ratio is sensitive to the phase transition of DPPC. Pyrene microcrystallites are probably present in the gel phase region, justifying the enhancement of I_E/I_M in the DPPC/$C_{12}E_7$ system at low temperatures.

Keywords Non-ionic surfactants · Dipalmitoylphosphatidylcholine · Egg phosphatidylcholine · Lipid/surfactant interactions · Pyrene emission · Ground-state aggregation

Introduction

The surfactant micelles have an ability to solubilise insoluble or only sparingly soluble materials in aqueous media by incorporating them into the micellar interior with the formation of mixed micelles. Phospholipids, and other constituents of biomembranes, can also be solubilised by surfactant micelles. Due to this property, surfactants are widely used as molecular tools in membranology [1]. The applications of surfactants in membranology are based on the transformation from vesicles to mixed micelles (or reverse direction) occurring in aqueous surfactant/phospholipid mixtures [2, 3]. Understanding of the transformation phenomenon should be helpful to achieve these practical purposes and, hence, great efforts have been developed so far to elucidate the pathway and mechanism of the transformation between vesicles and mixed micelles [4, 5]. The surfactant action on the phase transition of vesicle membranes has been studied by the use of several techniques for various surfactant and phospholipid species [6, 7].

Among the entire range of biophysical and spectroscopic methods, several techniques have been used to elucidate the properties of lipid vesicles and vesicle/surfactant interactions: cryotransmission electron microscopy [4], differential scanning calorimetry [6], light scattering [5], and absorption and fluorescence spectroscopy [8]. Fluorescence spectroscopy is probably the technique with the highest sensitivity for the study of lipid vesicles, biomembranes and lipid/surfactant interactions.

Since lipids are not fluorescent, study of the fluorescence of lipid vesicles is possible by introducing a fluorescence probe into the lipid environment. Among all probes used so far, pyrene (and its derivatives) stands unique, owing to its useful and versatile properties. These include the sensitivity of the emission spectrum's vibronic

structure to the polarity of the environment [9, 10] and its ability to form excimers, observed for the first time in 1954 [11].

In this work, we use pyrene's spectroscopic properties (excimer formation and the ratio of first to third peak emission intensity, I_1/I_3) to obtain information about the structural changes induced by different concentration ratios of the phospholipid dipalmitoylphosphatidylcholine (DPPC) and the non-ionic surfactant $C_{12}E_7$ [$C_{12}H_{25}(OCH_2CH_2)_7OH$]. The influence of temperature in these structures is also investigated.

Experimental

Materials Samples of polyoxyethylene 7 lauryl ether ($C_{12}E_7$) and of dipalmitoylphosphatidylcholine (DPPC) from Sigma were used as received. Pyrene (Koch Light, >99% pure) was zone refined (100 steps). Solutions were prepared using Milli-Q grade water.

Sample preparation The samples for the surfactant/water system were prepared by addition to water of the required amount of surfactant. Pyrene (2×10^{-7} M) was introduced by injection of a stock solution in ethanol. The samples were placed in an ultrasonic bath for mixing and left to stabilise.

DPPC was deposited by evaporation at 50 °C of a stock solution in ethanol. Then, the required amount of surfactant solution in water was added, followed by the injection of the probe. The samples were placed in an ultrasonic bath for mixing and left to stabilise.

Fluorescence measurements Steady-state excitation and emission spectra were recorded using a Spex Fluorolog 212 Spectrofluorimeter. The spectra were corrected for the instrumental response of the system. The temperature was maintained (± 0.2 °C) using a recirculating water supply connected to a water jacket on the cuvette holder.

Results and discussion

Before studying the influence of the phospholipid DPPC in mixed phospholipid/$C_{12}E_7$ systems, the behaviour of the fluorescent probe was monitored in the surfactant/water system.

For pyrene 2×10^{-7} M, it was found a pronounced decrease in the I_1/I_3 ratio (first to third peak monomer emission intensity ratio) until the critical micellar concentration (6.9×10^{-5} M) [12] is attained (Fig. 1a), resulting from the polarity decrease [9] experienced by the hydrophobic pyrene molecules as premicellar aggregates are formed, as already observed in other micellar systems [10, 13]. The cmc value corresponds to the inflection point of I_1/I_3 vs. log[$C_{12}E_7$] plot [10].

The excimer (500 nm) to monomer (372 nm) fluorescence intensity ratio, I_E/I_M, plotted in Fig. 1b, exhibits a significant increase, followed by a pronounced decrease. The peak in the I_E/I_M vs. log[$C_{12}E_7$] plot is located slightly after the cmc value. It should be noted that the pyrene concentration is the same in all the surfactant solutions. As the surfactant concentration is increased

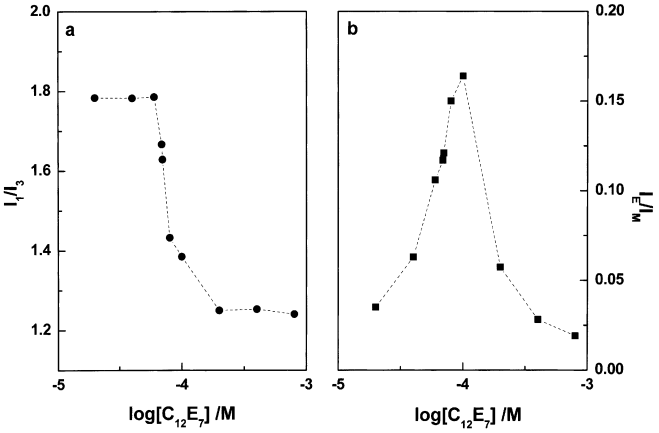


Fig. 1 (**a**) Fluorescence intensity ratio of pyrene first and third vibronic bands, I_1/I_3 ($\lambda_{exc} = 337$ nm), as a function of $C_{12}E_7$ concentration. (**b**) Excimer (500 nm) to monomer (372 nm) emission intensity ratio, I_E/I_M, of pyrene (2×10^{-7} M) as a function of $C_{12}E_7$ concentration ($\lambda_{exc} = 337$ nm)

through the cmc transition, the number of full-sized micelles increases. In this case, pyrene moves from premicellar aggregates to full-sized micelles, causing a rise in average occupancy of probe molecules in micelles, increasing therefore the I_E/I_M ratio [13]. After cmc, the number of full-sized micelles increases even further. Then, the average occupancy lowers and also the probability of excimer formation, decreasing I_E/I_M.

Assuming a radius of 40 Å for full-sized micelles [14], and a surface area per headgroup [12] of 61 Å^2, an aggregation number of 330 can be obtained (number of heads necessary to fill the micelle surface). From this value, pyrene average local concentrations of 6×10^{-3} M and 5.1×10^{-4} M can be estimated for surfactant solutions with concentrations 6.9×10^{-5} M (cmc) and 8×10^{-4} M, respectively. The probability (from a Poisson distribution) of having two or more pyrenes per micelle is 0.248 for cmc and 0.0032 for [$C_{12}E_7$] = 8×10^{-4} M. For premicellar aggregates ([$C_{12}E_7$] = 2×10^{-5} M), considered as spheres of a few surfactant molecules and water, a radius of 8 Å and a surface area per "hydrated" head of 80 Å^2 were estimated, giving an aggregation number of 10. In these conditions, the pyrene average local concentration is 0.078 M and the probability of having two or more probes per aggregate is 0.0047. Therefore, the I_E/I_M variation with [$C_{12}E_7$] agrees with the calculated probability of having two or more pyrenes per aggregate.

The temperature influence in the behaviour of this system was monitored by the variation of the pyrene excimer to monomer emission ratio, I_E/I_M, for several $C_{12}E_7$ concentrations. It was found that the I_E/I_M ratio decreases monotonically with temperature (Fig. 2a) for low surfactant concentrations (below 10^{-4} M) and does not follow the usual curve observed in low-viscosity

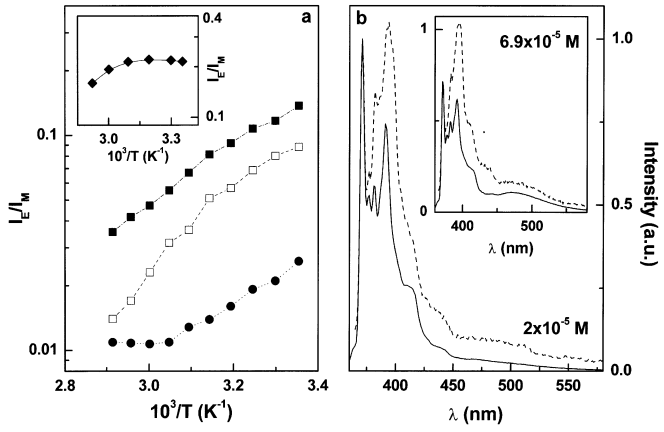
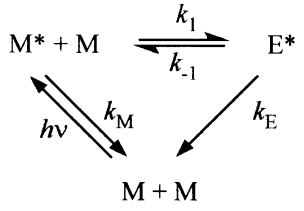


Fig. 2 (**a**) Excimer (500 nm) to monomer (372 nm) emission intensity ratio, I_E/I_M, for pyrene (2×10^{-7} M) as a function of reciprocal temperature ($\lambda_{exc} = 337$ nm), for several $C_{12}E_7$ concentrations: (●) 2×10^{-5} M; (□) 6.9×10^{-5} M; (■) 1×10^{-4} M. Insert shows I_E/I_M for pyrene in tetradecane. (**b**) Fluorescence spectra of pyrene (2×10^{-7} M) in a 2×10^{-5} M $C_{12}E_7$ solution, for $\lambda_{exc} = 337$ nm (—) and for $\lambda_{exc} = 350$ nm (- - -). Insert shows similar spectra for a 6.9×10^{-5} M $C_{12}E_7$ solution (room temperature). The spectra were normalised at 372 nm

solvents [15], with a maximum near or above room temperature (insert of Fig. 2a). The I_E/I_M ratio for pyrene in alkanes [16] shows the expected behaviour, resulting from the classical Birks scheme [15],



$$\frac{I_E}{I_M} \propto \frac{k_1}{k_{-1} + k_E} \qquad (1)$$

where k_1 and k_{-1} are the rate constants for excimer formation and dissociation, k_M and k_E are the rate constants for monomer and excimer deactivation, respectively. In the high-temperature region ($k_E << k_{-1}$), this scheme predicts a linear behaviour of $\ln(I_E/I_M)$ with 1/T [15].

For the $C_{12}E_7$/water system, it is clear that other photophysical processes involving pyrene molecules are playing an important role.

Fig. 2b shows the emission spectra of pyrene 2×10^{-7} M for the solution 2×10^{-5} M in $C_{12}E_7$, at room temperature, for two excitation wavelengths (337 nm and 350 nm). Insert of Fig. 2b displays the same spectra for $[C_{12}E_7] = 6.9 \times 10^{-5}$ M (cmc). In the spectra obtained with $\lambda_{exc} = 350$ nm, we can see the emission from an excited dimer preassociated in ground state. This ground state dimer absorbs a larger fraction of the excitation light at 350 nm, but not at 337 nm. In fact, the pyrene dimer to monomer relative absorption is higher at $\lambda_{exc} = 350$ nm, since the pyrene $S_1 \leftarrow S_0$ transition in the long wavelength region is symmetry forbidden, with a very low molar absorption coefficient. The Raman scattering of water (which should appear at 398 nm by excitation at 350 nm) is too small to justify the rise in emission intensity around 390 nm. This ground state aggregation was already observed for pyrene in several systems, especially in aqueous media [17–19]. The presence of a preassociated excited dimer strongly affects the I_E/I_M values and their variation with temperature, justifying the rather different behaviour observed in surfactant/water systems, compared to the one in homogeneous solvent. From spectra in Fig. 2b, it is clear that the emission from the dimer is stronger in the more concentrated $C_{12}E_7$ solution.

In order to investigate what happens well above the cmc, the I_E/I_M variation with temperature was recorded for a solution 8×10^{-4} M in $C_{12}E_7$ and in pure surfactant (Fig. 3a). The behaviour of I_E/I_M with temperature for these two systems is completely different from that observed in low $C_{12}E_7$ concentrations, the I_E/I_M now rising with temperature.

Fig. 3b shows the emission spectra for $\lambda_{exc} = 337$ nm and 350 nm for these two solutions. For $[C_{12}E_7] = 8 \times 10^{-4}$ M, a very strong dimer emission (relative to monomer) can be detected by excitation at 350 nm. This fact shows that, in this case, the excited dimer suffers a much slower conversion to excimer, indicating a lower mobility of the probe in micelles than in premicellar aggregates. This fact reflects the higher compactness of the surfactant molecules in micelles. Therefore, the major part of the excimers seems to come from the usual diffusion controlled process, and the I_E/I_M plot approaches that in a homogeneous solvent [15]. With

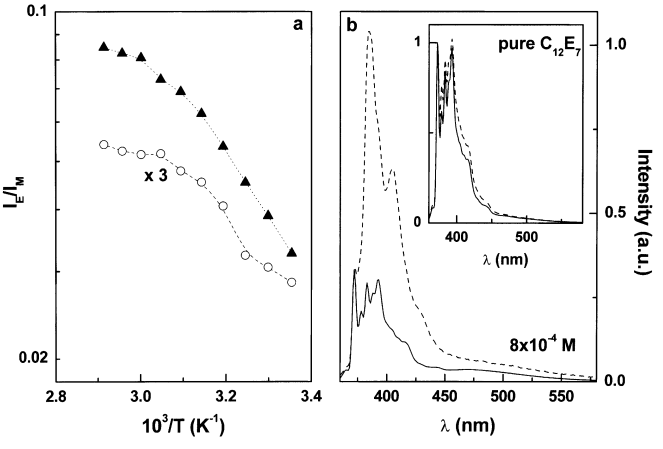


Fig. 3 (**a**) Excimer (500 nm) to monomer (372 nm) emission intensity ratio, I_E/I_M, for pyrene (2×10^{-7} M) as a function of reciprocal temperature ($\lambda_{exc} = 337$ nm), in a 8×10^{-4} M $C_{12}E_7$ solution (○) and for pyrene (10^{-3} M) in pure surfactant (▲). (**b**) Fluorescence spectra of pyrene (2×10^{-7} M) in a 8×10^{-4} M $C_{12}E_7$ solution, for $\lambda_{exc} = 337$ nm (—) and for $\lambda_{exc} = 350$ nm (- - -). Insert shows similar spectra for pyrene (10^{-3} M) in pure $C_{12}E_7$ (room temperature). The spectra were normalised at 372 nm

increasing temperature, the significant rise of the excimer dissociation rate (much higher than that of the excimer formation rate) and the highly compact medium surrounding pyrene in micelles may contribute to the occurrence of pyrene geminate pairs, which can recombine faster than the initial excited monomers [20]. This phenomenon will contribute to a further rise in I_E/I_M values with temperature.

In pure surfactant (insert of Fig. 3b), almost no dimer emission is observed. It is expected that some aggregation in the ground state is present, but the dimer suffers a fast conversion to excimer, with the corresponding decrease of the dimer emission. Therefore, the I_E/I_M plot with temperature exhibits almost no influence of pyrene aggregation, with a predictable maximum at high temperature, due to the significant viscosity of $C_{12}E_7$.

For the characterisation of lipid/micelle interactions, an important parameter is the surfactant molar ratio, x_s [7]:

$$x_s = \frac{[C_{12}E_7]}{[C_{12}E_7] + [DPPC]} \quad (2)$$

We will consider the usual three-stage model of solubilisation of liposomes by surfactants:

1. Mixed bilayers exist up to a critical surfactant concentration, x_{sat};
2. Between x_{sat} and x_{sol}, there are saturated bilayers and saturated mixed micelles with, respectively, x_{sat} and x_{sol} surfactant content;
3. Above x_{sol}, there are only mixed micelles.

Heerklotz et al. [21] obtained values of $x_{sat} = 0.45$ and $x_{sol} = 0.75$ for the POPC/$C_{12}E_7$ system at 25 °C. These two quantities both increase with temperature, while the difference between them decreases (reduction of the coexistence zone).

In Fig. 4a, the I_E/I_M ratio is plotted as a function of x_s for DPPC/$C_{12}E_7$ and EggPC/$C_{12}E_7$ systems at room temperature. According to our experimental conditions, as x_s increases the lipid content decreases, while the surfactant remains constant. Thus, on the assumption that the structure of the aggregates does not change (size and aggregation number of amphiphilic molecules), we would expect an increase of I_E/I_M with x_s, as the local concentration increases. The expected tendencies are plotted in Fig. 4a as solid lines, considering as constant structure the one corresponding to the lowest value of x_s (almost pure lipid bilayer aggregates).

For $x_s < 0.3$, it can be seen that there are no detectable changes in structure for both systems. In this region, the lipid bilayers are incorporating surfactant molecules, with a corresponding rise in the structure size. This would lead to a decrease in I_E/I_M, but there is an opposing factor: the fluidisation of the aggregate. In fact, surfactant molecules have a lower tail volume than the lipid ones, but the surfactant headgroup is larger. Thus, the accommodation of $C_{12}E_7$ molecules in the lipid layer results in a lower compactness of the structure in the region below the headgroup [21]. A value for x_{sat} near 0.3 can be estimated.

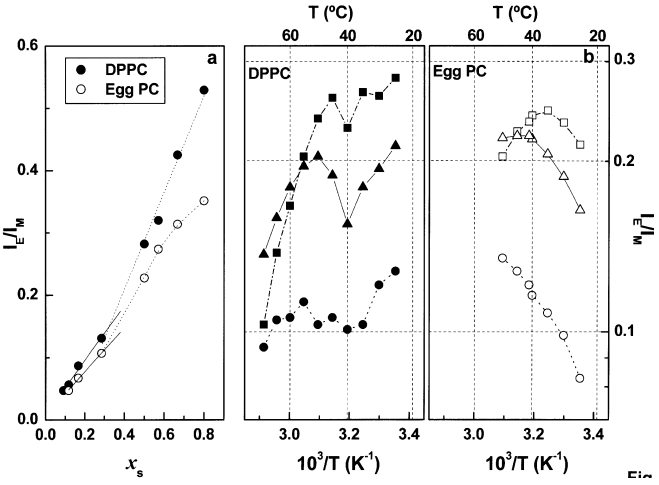


Fig. 4 (**a**) Excimer (500 nm) to monomer (372 nm) pyrene emission intensity ratio, I_E/I_M, in lipid/$C_{12}E_7$ mixed systems, as a function of surfactant molar ratio ($\lambda_{exc} = 337$ nm). (**b**) Excimer (500 nm) to monomer (372 nm) pyrene emission intensity ratio, I_E/I_M, as a function of reciprocal temperature ($\lambda_{exc} = 337$ nm), for DPPC/$C_{12}E_7$ and EggPC/$C_{12}E_7$ systems. DPPC: (■) $x_s = 0.67$; (▲) $x_s = 0.5$; (●) $x_s = 0.29$. EggPC: (□) $x_s = 0.67$; (△) $x_s = 0.5$; (○) $x_s = 0.29$.

For $x_s > 0.3$, a huge increase in I_E/I_M is observed. This corresponds to the second situation described above (between x_{sat} and x_{sol}). The system has now an increasing population of mixed micelles with x_{sol} fixed composition and a decreasing population of mixed bilayers with a x_{sat} surfactant composition. The I_E/I_M ratio feels this transition, as both the average local concentration and the probability of having two or more pyrene molecules per aggregate increase.

In DPPC the amount of excimer is always higher than for EggPC. At room temperature, DPPC bilayers are in the gel phase, whereas EggPC is in the liquid crystalline phase. Galla and Sackmann [22] observed that when DPPC vesicles change from the liquid crystalline to gel phase, a marked increase in I_E/I_M occurs. This behaviour was interpreted by considering that the pyrene solubility in the gel phase is very low, which results in the formation of microcrystallites with high yield of excimer formation.

The effect of temperature on these mixed aggregates was also investigated using pyrene monomer/excimer fluorescence. In Fig. 4b we can see that in the EggPC/$C_{12}E_7$ system there is a normal behaviour of I_E/I_M vs. temperature. The pronounced shift of the maximum with the lipid content is in agreement with an increase in the viscosity of the bilayer [16]. As EggPC is always in the liquid crystalline phase, it will mix almost homogeneously with $C_{12}E_7$, making a "fluid" of intermediate

viscosity, irrespective of the aggregate structure (mixed micelle or mixed bilayer).

For the DPPC/$C_{12}E_7$ system, it is expected that the DPPC phase transition influence the results. In fact, near 40 °C a minimum in the I_E/I_M variation with temperature occurs. This compares well with the known value of 41 °C for the phase transition of pure DPPC [22]. After the phase transition, similar trends to those of the EggPC/$C_{12}E_7$ system are observed, with slight shifts, which can be attributed to changes in the fluidity of the surfactant/lipid "fluid". Further, when the lipid content is higher ($x_s = 0.5$ and $x_s = 0.29$), the excimer formation is more efficient in the EggPC/$C_{12}E_7$ than in DPPC/$C_{12}E_7$ system. This can also be explained by the higher viscosity of the DPPC bilayer. In the phase transition region (30–40 °C), the increase in I_E/I_M with decreasing temperature is again understandable by the presence of pyrene microcrystallites. When the value of x_s is 0.67, the presence of mixed micelles plays an important role. The DPPC content in these micelles should be ~20% ($1-x_{sol}$). Considering 400 molecules of $C_{12}E_7$ per micelle, we estimate that there are 100 molecules of DPPC per mixed micelle. It is expected that the packing of the lipid molecules in the micelle would be more favourable if they form small domains, because of the significant difference in tail volume and headgroup surface area of DPPC and $C_{12}E_7$. These domains would also exhibit a phase transition, which can be observed in our experimental results (Fig. 4b). Regarding ground-state pyrene association in these systems, a very weak emission from a preassociated dimer is observed in fluorescence spectra (with long wavelength excitation). Furthermore, the amount of dimer does not change appreciably with the phase transition of the lipid.

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