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# Fluorescence characteristics of pyrene and phosphatidylethanolamine-bound pyrene incorporated into lipid vesicles solubilized in media of differing NaCl concentrations

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We used the excimer/monomer ratio of pyrene (PY) and *N*-(1-pyrenesulfonyl)dipalmitoyl-L-α-phosphatidylethanolamine (DPPE-PY) fluorescence intensities ($I_E/I_M$), and the polarity ratio I/III to investigate the state of the polar head group region of small, unilamellar phosphatidylcholine vesicles (SUV-PC) solubilized in media of differing NaCl concentrations. PY or DPPE-PY excimer formation resulting from vesicles' collisions is not affected by the presence of monovalent ions. In addition, the ionic strength does not alter the dielectric environment in the neighborhood of PY incorporated into SUV-PC. Since $I_E/I_M$ of both PY and DPPE-PY is insensitive to variations in the ionic strength, we conclude that the probes' diffusion in SUV-PC, and consequently the membrane fluidity, are independent of NaCl concentration at least up to 0.5 M. The vesicles' concentration in the aqueous solution was the only factor which induced a rise of $I_E/I_M$. To explain the results in the context of the transient-fusion model developed previously (G.P. L'Heureux and M. Fragata, Biophys. Chem. 30 (1988) 293) and the hypothesis of repulsive hydration forces, we postulate a heterogeneous distribution of dehydrated domains, or contact areas, on the outer surfaces of colliding vesicles.

## 1. Introduction

The bilayer membrane is the basic structure of two-chain amphiphile phospholipids in water. This arrangement confers special characteristics to the interface spanning the region from the bulk water near the polar head group of the lipid to its glyceryl moiety. There have been several reports showing that monovalent ions are able to adsorb at this water-lipid interface and to affect by the same token its properties (see ref. 1 and references therein). Nevertheless, the mechanisms underlying the mode of action of the ions are not well understood. One of the matters which deserves to be clarified is the relation of the ion concentration at the interface to the hydration-dehydration phenomena which control membrane adhesion [2,3]. We showed in this connection that the interfacial concentration of monovalent cations ($Li^+$, $Na^+$, $K^+$) may influence the state of hydration of the polar head group region of small, unilamellar vesicles constituted of phosphatidylcholine (SUV-PC) [4]. In addition, a dynamic densitometry study

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Abbreviations: αT, α-tocopherol; *D*, diffusion coefficient; DPPE-PY, *N*-(1-pyrenesulfonyl)dipalmitoyl-L-α-phosphatidylethanolamine; ε, dielectric constant; $I_E$, fluorescence intensity of PY or DPPE-PY at 480 and 495 nm, respectively; $I_M$, fluorescence intensity of PY or DPPE-PY at 395 and 399 nm, respectively; I/III, ratio of PY emission maxima at 373 and 384 nm, respectively; LP, vesicles' concentration given as lipid phosphorus; PC, phosphatidylcholine; PY, pyrene; SUV-PC, small unilamellar vesicles constituted of PC; $\tau_D$, mean time to encounter of colliding vesicles; $\tau_f$, fluorescence lifetime of PY or DPPE-PY.

[5] provided evidence of a variation in size of the water-lipid interface as the salt concentration in the bulk solution increased. In brief, we measured the molecular specific volume ($\bar{v}_m$) of phosphatidylcholine (PC) molecules incorporated into SUV-PC as a function of the amount of NaCl present in the bulk aqueous solvent, and found that $\bar{v}_m$ diminished with increasing salt concentrations ranging from 0.05 to 0.20 M. It is worth noting, in this respect, that a study of PC bilayers by $^{23}$Na-NMR quadrupole splitting [1] showed that an increase in salt content induces conformational changes of the polar head groups. A possible explanation for these observations is the weakening of interfacial hydration forces upon association of monovalent cations and anions with the phosphate and choline moieties of PC molecules, respectively [6].

The fluorescent probe pyrene (PY) is well suited to the study of these questions on account of its versatility that makes possible the investigation of a variety of interfacial properties such as polarity, fluidity and diffusion (see, e.g., ref. 7). In a previous paper [8], we observed that the formation of PY excimer is enhanced by SUV-PC collisions. To explain this effect, we postulated the presence of free PY in the vicinity of the water-lipid interface, and the transient fusion, or merging, of the lipid polar head groups (see description of model in ref. 8). We believe that this mechanism facilitates PY-PY interactions at distances within the range determined for PY excimer formation which is between 3 and 10 Å [9–12].

In the present work, we studied the formation of excimers by free PY and a phospholipid-bound PY, namely, *N*-(1-pyrenesulfonyl)dipalmitoyl-L-α-phosphatidylethanolamine (DPPE-PY, see fig. 1), incorporated into SUV-PC solubilized in media containing various NaCl concentrations, and observed that the ionic strength has no marked effect on PY or DPPE-PY excimer formation. In addition, it does not affect the magnitude of the ratio of excimer to monomer fluorescence intensities which is generally used as a measure of the probe's mobility in the lipid membrane (see, e.g., ref. 13). Henceforth, we shall discuss the data with reference to the transient-fusion model of ref. 8 and the hypothesis of repulsive hydration forces proposed in ref. 2.

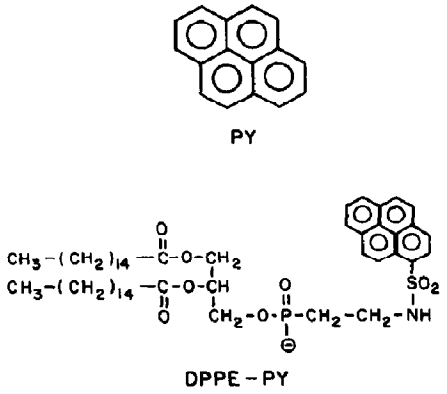


Fig. 1. Chemical structure of pyrene (PY) and *N*-(1-pyrenesulfonyl)dipalmitoyl-L-α-phosphatidylethanolamine (DPPE-PY).

## 2. Experimental

PY from Sigma (St. Louis, MO) was purified by column chromatography on silica gel G with cyclohexane as solvent. DPPE-PY was purchased from Molecular Probes and used without further purification. PC was extracted from egg yolk according to the method of Singleton et al. [14]. The purity of lipids was checked by thin-layer chromatography on silica gel.

The vesicles were prepared by using the method of Huang [15] as slightly modified. In brief, the dried mixture of PC and probe was dispersed in 0.01 M Tris-HCl buffer (pH 8.0), 0.1 M NaCl (~ 10 mg/ml). The solution was then sonicated for 15–20 min in a sonifier cell disrupter (Heat Systems-Ultrasonics) at an output setting of about 60 W, under a stream of $N_2$ gas. The dispersion was then centrifuged for 1 h at 100000 × *g*, followed by concentration in an Amicon cell to about 1–2 ml, and finally fractionated on a Sepharose-4B (Pharmacia) column. In order to control the NaCl concentration in the fractions, we first dialysed the homogeneous vesicles (class III vesicles; see fig. 1 of ref. 4) against the same Tris-HCl buffer (pH 8.0) without salt for a period of 20 h. Thereafter, we added an appropriate amount of salt to

the solution in order to obtain the desired final concentration.

The PC content of the vesicles is expressed in terms of lipid phosphorus (LP) as determined according to a modified method of Bartlett [16]. The pyrene content was evaluated as described elsewhere [8].

The fluorescence measurements were performed on a SPEX Fluorolog 2-spectrofluorometer (SPEX Industries, NJ) which was equipped with a 450 W xenon lamp, an R928 photomultiplier (Hamamatsu) and a photon-counting system. The emission spectra were corrected relative to a correction curve obtained with a standard of spectral irradiance (Optronic Labs, model 220 M, no. M-320). The excitation wavelength was set at 335 nm for PY and 337 nm for DPPE-PY. The excitation and emission bandwidths were 5 and 2 nm, respectively, throughout this work.

## 3. Results and discussion

### 3.1. Fluorescence characteristics of PY and DPPE-PY in NaCl solutions

The effects of NaCl on PY or DPPE-PY excimer formation were investigated by studying the ratio $I_E/I_M$ of the fluorescence intensities of the excimer ($I_E$) at 480 nm (PY) and 495 nm (DPPE-PY), and peak V of the monomer ($I_M$) at 395 nm (PY) and 399 nm (DPPE-PY) (see ref. 7 for a thorough description of band maxima). Fig. 2 displays the variations in $I_E/I_M$ of PY incorporated into SUV-PC as a function of NaCl concentration. The PY content of the lipid vesicles was 3.0 mol% which is well above the probe's critical concentration of excimer formation (cf. fig. 4a of ref. 8). Figure 2 shows that up to 0.4 M NaCl in the bulk aqueous solvent, no significant changes in $I_E/I_M$ occur (●, ▲ and ■ straight lines). In contrast, $I_E/I_M$ varies considerably when the vesicles' concentration (or lipid phosphorus, LP) is changed: average excimer/monomer emission intensity ratios were calculated to be 0.24, 0.32 and 0.38 (S.D. = 0.02) (average values of ●, ▲ and ■ straight lines, respectively) for LP concentrations of 23 (●), 47 (▲) and 70 μM (■),

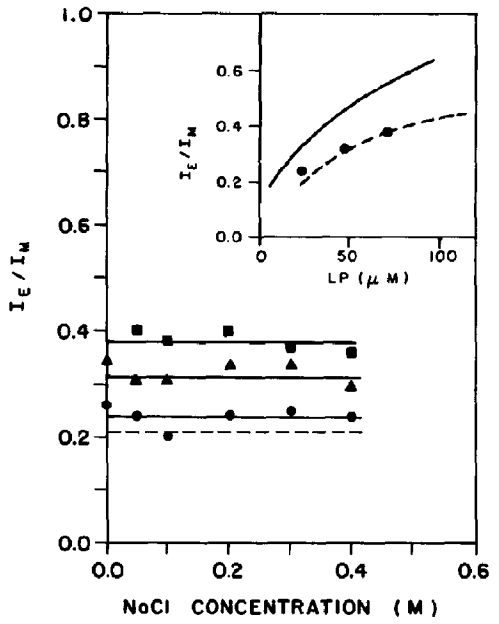


Fig. 2. Variation of the excimer/monomer ratio of fluorescence intensities ($I_E/I_M$) of PY and DPPE-PY with NaCl concentration in the bulk phase of SUV-PC solutions. PY and DPPE-PY concentrations were 3.0 (———) and 14.0 mol% (- — —, no data points shown), respectively. Vesicles' concentrations in solution, i.e., LP, were 23 (●), 47 (▲), 70 μM (■), and 42 μM (— — —). The straight lines represent the $I_E/I_M$ averages, i.e., 0.24 (●), 0.32 (▲) and 0.38 (■) (S.D. = 0.02). (Inset) Variation of $I_E/I_M$ of fluorescence intensities of PY with the vesicles' concentration in solution (LP). (———) 3.7 mol% PY (data from ref. 8). (— — —) 3.0 mol% PY (this curve was drawn with eight $I_E/I_M$ values obtained between 20 and 110 μM LP). The data points (●) represent the $I_E/I_M$ averages at 23, 47 and 70 μM LP (see above). LP, lipid phosphorus (see section 2).

respectively. This is consistent with data obtained previously with 3.7 mol% PY also incorporated into similar SUV-PC (cf. fig. 2, inset (———), and also fig. 5 of ref. 8). The higher $I_E/I_M$ values of ref. 8 are ascribed to the concentration of PY, i.e., 3.7 mol%, which is greater than that used in the present experiments, that is to say 3.0 mol%. We wish to remark that the results of fig. 2 differ from those of Ohyashiki and Mohri [17]. These authors studied the ratio $I_E/I_M$ of PY incorporated into intestinal brush-border membranes of rabbit. They observed an increase of $I_E/I_M$ between 0 and 0.1 M NaCl which was followed by a plateau up to

0.2 M NaCl. These discrepancies arise from the great differences between the two types of membranes. For instance, it is reasonable to assume that the presence of proteins in the brush-border membranes is at the origin of specific interactions with the ionic species in solution which may perturb the fluorescence of PY.

Fig. 2 (— — —) also presents the results of experiments performed with DPPE-PY. As with PY, no variation in $I_E/I_M$ was observed (average $I_E/I_M = 0.21 \pm 0.01$) when SUV-PC containing 14 mol% DPPE-PY were dispersed in NaCl solutions ranging from 0.05 to 0.5 M at 42 $\mu$M LP. We note, however, that the need for using such a high mol% DPPE-PY comes from the difficulty in obtaining a measurable $I_E/I_M$ ratio within reasonable limits of statistical certainty at less than 14 mol%. This is discussed further in section 3.2.

Finally, we also observed a rather steep increase in $I_E/I_M$ to $2.21 \pm 0.03$ and $2.26 \pm 0.03$ at LP concentrations of 39 and 78 $\mu$M, respectively, with raising of the DPPE-PY content of SUV-PC to 33 mol% (data not shown in fig. 2). This is indicative of a molecular rearrangement, or phase transition, in the lipid bilayer membrane. It is plausible to attribute these variations of $I_E/I_M$ to formation of DPPE-PY domains, or aggregates, which are most probably already present in the ground state (see, in this respect, refs. 12 and 18). We emphasize that even in this case the variation in the excimer/monomer ratio displayed no dependence on the NaCl concentration in the bulk phase.

### 3.2. PY and DPPE-PY diffusivities

To explain the differences between the relative concentrations of PY and DPPE-PY in SUV-PC which give rise to $I_E/I_M$ ratios of about 0.2, i.e., 3.0 and 14.0 mol%, respectively, we postulate slower diffusion of DPPE-PY in the bilayers as compared to PY which, if operative, will delay the onset of excimer formation. In fact, the coefficient of lateral diffusion, $D$, of PY in lipid membranes was evaluated to be nearly $10^{-7}$ cm$^2$ s$^{-1}$ [9,11,19,20], whereas $D$ of DPPE-PY is about 50% smaller. For instance, diffusion studies of pyrene bound to lecithin derivatives yielded $D = 5.3 \times 10^{-8}$ cm$^2$ s$^{-1}$ [20] and $5.8 \times 10^{-8}$ cm$^2$ s$^{-1}$ [21]. This is in good agreement with lipid diffusion studies by fluorescence recovery after photobleaching (FRAP) with the probe *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine incorporated into PC bilayers where $D$ is about $3.5–4.4 \times 10^{-8}$ cm$^2$ s$^{-1}$ [22–25]. We remark, in addition, that excimer formation of PY-containing membranes is favored by the long excitation lifetime ($\tau_f$) of the probe, i.e., 100–200 ns [12,21], as compared to 13 ns for DPPE-PY [26]. Thus, using an expression for the mean square displacement, $\langle r^2 \rangle$, such as (see, e.g., refs. 25 and 27)

$$\langle r^2 \rangle = 4D\tau_D,$$

where $\tau_D$ is the mean time to encounter, here equated to $\tau_f$, one estimates the relative diffusion distances of PY and DPPE-PY in lipid bilayers to be about 20–28 and 5 Å, respectively. Furthermore, taking into account that the overall displacements of PY occur laterally and in a direction normal to the plane of the membrane (see discussions in refs. 8 and 28), it is clear that the final trajectory has a path length greater than the above calculated 20–28 Å. In such a framework, it is obvious that the probability of excimer formation of DPPE-PY will be identical to that of PY only at much higher probe/host lipid molar ratios as shown in fig. 2.

The afore discussed corroborates the previously held views that the magnitude of $I_E/I_M$ provides a measure of the diffusion rate and collision frequency of the pyrenyl probes (see, e.g., ref. 13), and is thereby dependent on the excited-state lifetime of the monomer. Thus, the variation in $I_E/I_M$ reflects the dynamic state (or fluidity) of the membrane [29]. From this and the data displayed in fig. 2, we conclude that NaCl concentrations up to 0.5 M do not affect the mobility of PY and DPPE-PY in SUV-PC. A second major conclusion is that the fluidity of the water-lipid interface most probably remains unchanged under the influence of monovalent ions ($Na^+$ and/or $Cl^-$). It is important to mention at this point that a study of the lateral diffusion of the chloroplast protein plastocyanin at the surface of PC bilayers (see, in this connection, ref. 25), i.e., at least inside the water-lipid interface, revealed at most a very small

effect of the ionic strength on the interfacial viscosity (M. Fragata, unpublished data).

### 3.3. Ionic effects on interfacial hydration and SUV-PC interactions

Variations in the degree of hydration of the water-lipid interface of SUV-PC as a result of ionic strength changes have been inferred from studies on the interfacial polarity (dielectric constant, $\epsilon$). A description of this is given in ref. 4 for SUV-PC dispersed in solutions containing LiCl, NaCl or KCl. Briefly, $\epsilon$ was determined by following the polarity-dependent formation of an intermediate in the reaction of $\alpha$-tocopherol ($\alpha$T) incorporated into SUV-PC with the free radical 1,1-diphenyl-2-picrylhydrazyl. The region of the membrane which is probed is the interface where the chromanol ring of $\alpha$T is located, that is to say between the phosphorylcholine moiety and the ester carbonyl region of the PC molecules [4]. In the present work, we used PY as a polarity probe of SUV-PC on account of its ability to explore a region closer to the edge of the water-polar head group interface (see ref. 8). We measured the I/III ratio (see table 1), which is a function of $\epsilon$ [7], of PY emission maxima at 373 and 384 nm, respectively (see description of PY peaks I–V in ref. 7). DPPE-PY was not used in these experiments, since its pyrenyl moiety is covalently bound to an $SO_2$ group (cf. fig. 1), thus inducing a reduction in the molecular symmetry [30] which is accompanied by a substantial loss of the fine structure typical of pyrene monomer fluorescence (see, e.g., fig. 1 of ref. 8). This renders the evaluation of I/III a rather difficult task, if not impossible.

Table 1

Variation of I/III of PY incorporated into SUV-PC [a] with NaCl concentration

| [NaCl] (M) | I/III |
|---|---|
| 0 | 1.32 |
| 0.05 | 1.31 |
| 0.10 | 1.30 |
| 0.20 | 1.31 |
| 0.30 | 1.31 |
| 0.40 | 1.29 |

[a] In these experiments the incorporated PY/PC ratio was 3.0 mol% at 47 $\mu$M LP. LP (lipid phosphorus) gives a measure of the vesicles' concentration in the bulk aqueous solvent (see section 2). I/III denotes the ratio of peaks I and III of the emission spectrum of PY (cf. e.g., fig. 1 of ref. 8). PY, pyrene.

Table 1 lists the I/III ratio of fluorescence intensities of PY as a function of NaCl concentration in the bulk aqueous phase of SUV-PC dispersions. It is observed that the dielectric environment of PY is not affected by variations in the ionic strength. The insensitivity of I/III to ionic strength may have a two-fold meaning, i.e., either the water content or the molecular organization of $H_2O$ arrays in the vicinity of the probe is not altered by monovalent ions. The present experiments do not permit us to distinguish between the two hypotheses. Moreover, it is noted that the experiments of table 1 were performed at LP concentrations of 47 $\mu$M. We found, in addition, that similar results are obtained with lipid phosphorus contents of 23 and 70 $\mu$M. Taken together, these observations are a clearcut demonstration that the transient-fusion mechanism of vesicles' merging described in ref. 8 (see section 1) is neither enhanced nor hindered by the presence of monovalent ions in the bulk aqueous phase, or, more precisely, in the water-lipid interface. This conclusion is most interesting, since this phenomenon is directly related to the function of repulsive hydration forces in membrane-membrane interactions at separations less than about 25 Å [2].

The idea of hydration repulsion as a barrier against molecular contact between lipid bilayers is associated with the finding that $H_2O$ molecules in the proximity of the polar head groups form a special layer with a supramolecular organization which is dependent on distance (see, e.g., ref. 31). The hydration forces are apparently related to the work involved in removal of the $H_2O$ molecules from the special layer. Therefore, increasing electrolyte concentrations in the surrounding solution should lead to the progressive destruction of the hydration barrier. We would thus expect $I_E/I_M$ to rise with the NaCl content in the aqueous solvent as a consequence of the intensification of probes' collisions. This, however, was not verified (cf. fig. 2). Vesicles' concentration, i.e., LP, was the only

factor which induced a rise in $I_E/I_M$ (cf. fig. 2, inset). To explain these results we postulate a heterogeneous distribution of dehydrated domains, or contact areas, on the outer surfaces of colliding vesicles that would ensure the operation of an efficient transient-fusion mechanism against a gradient of repulsive hydration forces.

Another major conclusion in this work is that monovalent ions do not necessarily play a key role in counteracting repulsive hydration forces in interacting SUV-PC. This, therefore, indicates the need for improvement of the appropriate theory.

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