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Micropolarities of lipid bilayers and micelles

5. Localization of pyrene in small unilamellar phosphatidylcholine vesicles

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The excimer/monomer ratio of emission intensities (I_E/I_M) and the enhancement of the 0-0 vibronic transition in the fluorescence spectra of pyrene (PY) and 16-(1-pyrenyl)hexadecanoic acid (C_{16}PY) were used to investigate the localization of PY in the bilayers of small unilamellar vesicles constituted of phosphatidylcholine (SUV-PC). First, from comparison of the fluorescence characteristics of PY in water with those of PY incorporated into the SUV-PC membranes, we concluded that the probe is incorporated preferentially in the lipid phase of the vesicles and not in the bulk aqueous phase. In addition, we found that, contrary to what happens with the pyrenyl moiety of C_{16}PY, the location of PY varies with its relative concentration in the membrane space. The critical concentration was observed to be around 1.0 mol% of incorporated PY. At concentrations below this value, PY is located in the hydrocarbon core of the lipid bilayers. Above 1.0 mol%, the PY molecules reside preferentially in the neighbourhood of the glyceryl moiety region of the PC vesicles.

1. Introduction

The use of pyrene (PY) as an optical probe has become an important tool in various membrane and micelle studies such as permeability [1], diffusion [2–6], viscosity [7], osmotic pressure [8], phase transitions [4,9] and dielectric constant (ϵ) determinations [10–15]. In some particular cases, e.g., viscosity and dielectric constant, the necessity to ascertain the localization of the probe in the lipid bilayers comes from the observation of molecular gradients across the membranes (see, e.g., ref. 16). In this connection, it is believed that PY is incorporated in a region about 10–15 Å wide extending from the glyceryl moieties of the phospholipids into the inner core of the alkyl chains [2,3,8,12,17–25]. However, a recent work of Jones and Lee [26] suggests a significant exchange of PY between the membrane bilayer and the bulk aqueous phase of the lipid dispersion in water. Also, a study of the erythrocyte membrane by Dembo et al. [27] showed that the partition coefficient, PY_m/PY_s, of the PY distribution between the membrane (PY_m) and the bulk solvent (PY_s) is about 7×10^4. Note, however, that this value will only be generalized to other experimental systems with caution, since the partition of PY between the lipid and water phases may vary considerably with the type of membrane. Nevertheless, the possibility of PY displacements back and forth between the membrane and the solvent means that

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Abbreviations: C_{16}PY, 16-(1-pyrenyl)hexadecanoic acid; ϵ, dielectric constant; LP, lipid phosphorus; I_E, fluorescence intensity of PY or C_{16}PY excimer measured at 480 nm; I_M, fluorescence intensity of PY or C_{16}PY monomer measured at 394–395 nm; PC, phosphatidylcholine; PY, pyrene; SUV-PC, small unilamellar vesicles constituted of PC.

the probe tends to reside more frequently at the vicinity of the water/lipid interface than elsewhere. This view is obviously in contradiction with the assumption of the presence of PY in the hydrophobic core of the bilayers. Because of these uncertainties which hamper the interpretation of previous results (see, e.g., ref. 15), we undertook a study of the localization of PY and C_{16}PY in small unilamellar vesicles constituted of PC. Herein, we show that the location of PY in the lipid bilayers varies with its relative concentration, PY/PC, in the membranes. The results are consistent with a concentration-induced accumulation of PY monomers, or their aggregates (e.g., dimers), in the outer surface of the vesicles.

2. Experimental

2.1. Chemicals

Pyrene (from Sigma) was purified by chromatography in a silica column with cyclohexane as eluent. C_{16}PY was obtained from Molecular Probes and used without further purification. PC was extracted from egg yolks according to the method of Singleton et al. [28]. The purity of the final product was checked by thin-layer chromatography on silica gel G using a solvent mixture of chloroform/methanol/water (65 : 25 : 4).

2.2. Preparation of small unilamellar vesicles

The vesicles were prepared according to the method of Huang [29] slightly modified. Briefly, the dried mixture of PC (~ 150 mg) and probe (PY or C_{16}PY) was dispersed in 10 ml of 0.01 M Tris-HCl, 0.1 M NaCl (pH 8.0). The aqueous mixture was then subjected to 15–20 min sonication in a sonifier cell disrupter (Heat Systems-Ultrasonics, model W-225R) set at about 20 W output, having nitrogen bubbling into the mixture. This was followed by centrifugation at $100\,000 \times g$ for 1 h to eliminate the larger membranes and the titanium particles from the sonifier probe. The vesicle mixture (supernatant fraction) was then concentrated to 1–2 ml in an Amicon cell and fractionated in a Sepharose-4B (Pharmacia) column. Only the homogeneous fractions of the elution diagram (see details in ref. 15), henceforth designated as SUV-PC (i.e., small unilamellar vesicles constituted of phosphatidylcholine), were retained for the analyses described herein.

2.3. Determination of lipid phosphorus and PY contents

The PC content of the vesicles, expressed in terms of lipid phosphorus (LP), was determined by a modified method of Bartlett [30]. Upon color development the absorbance of the samples was read at 660 nm and compared to a KH_2PO_4 standardization curve. We wish to emphasize that LP is obviously a measure of the vesicles' concentration in the bulk aqueous solvent.

The PY content of the vesicles was determined as follows. First, a standardization curve of PY in a methanol/water mixture (95 : 5) was obtained. The curve was drawn by plotting the summation of the fluorescence intensities of PY between 360 and 400 nm (monomer emission) in 0.2-nm steps for each concentration used. Second, 1 vol. of vesicle solutions was diluted in 19 vols. of methanol to ensure complete solubilization of PY in the methanol/water mixture (95 : 5). The spectra of PY obtained under the two conditions described above were identical. Then, the summation of fluorescence intensities of PY from the vesicle preparations were compared to those of the standardization curve.

2.4. Fluorescence measurements

The fluorescence spectra were obtained with a SPEX Fluorolog 2 spectrofluorimeter (SPEX Industries, NJ), and corrected in relation to a correction curve obtained with a standard of spectral irradiance (Optronic Laboratories, model 220 M, no. M-320). In addition, the emission spectra of solvent and buffer were subtracted from the corresponding spectra with PY or C_{16}PY using the microcomputer facility (Spectroscopy Laboratory Coordinator, model DM1B) of the SPEX spectrofluorimeter. The instrument conditions were as follows. The pyrene excitation wavelength was set as 335 nm and that of C_{16}PY at 345 nm, the

excitation bandwidth being 5 nm in both cases. The emission spectra were taken between 360 and 500 nm with increments of 0.5 nm, and a 2 nm bandwidth. These conditions are sufficient for retaining the spectral fine structure of the PY monomer (see detailed discussion in ref. 15).

3. Results

3.1. Fluorescence spectra of PY and $C_{16}PY$

Fig. 1 represents the fluorescence characteristics of PY (fig. 1a) and $C_{16}PY$ (fig. 1b) incorporated into SUV-PC bilayers. Fig. 1a shows the five vibronic bands from the fine structure of the PY monomer fluorescence which are generally denoted I–V (see ref. 10 for a description of band maxima, vibrational modes and symmetry types, and also table 1 of ref. 13). Since the intensity of the 0-0 transition of PY (peak I) is very sensitive to the polarity of its environment contrary to peak III intensity [10–15], the I/III ratio of PY as a function of ϵ is widely used as a polarity scale. For example, the I/III ratios of solutions of PY in methanol ($\epsilon = 32.6$ at 25 °C) and *n*-hexane ($\epsilon = 1.88$ at 25 °C) are 1.40 and 0.60 [15], respectively. The spectra of PY in water ($\epsilon = 79$ at 25 °C) show, in addition, that I/III = 2.02 (cf. fig. 2).

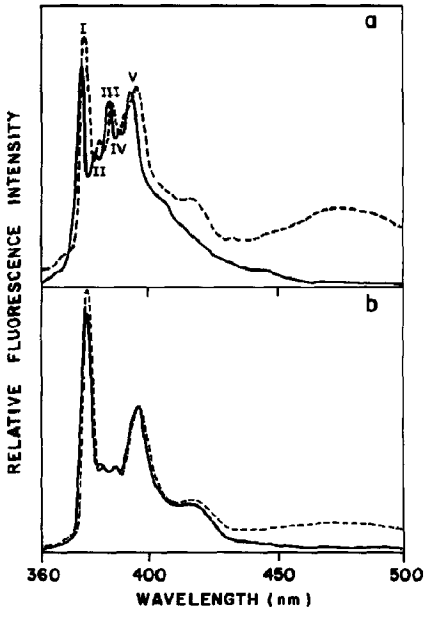


Fig. 1. Fluorescence spectra of pyrene (PY) and 16-(1-pyrenyl)hexadecanoic acid ($C_{16}PY$) incorporated into small unilamellar vesicles constituted of phosphatidylcholine (SUV-PC). (a) Ratios (mol%) of PY incorporated into SUV-PC: (———) 0.1, (- - - - -) 3.7. (b) Initial ratios (mol%) of $C_{16}PY$ in its mixture with PC: (———) 0.25; (- - - - -) 3.0. The spectra are normalized at the peak III wavelength (see text).

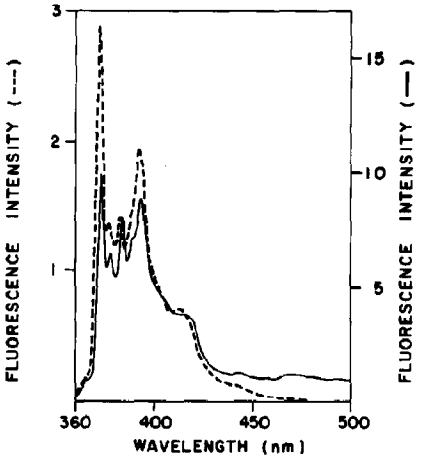


Fig. 2. Fluorescence spectra of 0.5 μM PY dissolved in water (- - - - -) and incorporated into SUV-PC (———). The vesicles' concentration (LP) in the bulk solution was 100 μM. The two fluorescence scales were adjusted at peak III in order to permit a better comparison of the I/III ratios. Note, however, that the absolute fluorescence intensity of PY in SUV-PC is 4-times greater than that of PY dissolved in water.

Fig. 1 also displays a broad band at 450–500 nm ($\lambda_{max} \sim 480$ nm) that appears in the emission spectra of vesicles prepared with high PY and $C_{16}PY$ concentrations, and which is attributed to excimer formation [7,8].

3.2. Evidence of PY incorporation into SUV-PC bilayers

Fig. 2 displays the fluorescence spectra of PY dissolved in water (0.5 mg PY/5 l H_2O), stirred for 24 h at 20 °C) and incorporated into SUV-PC membranes. Note that the PY concentration (0.5 μM) is identical in the two types of preparations. Also, their respective fluorescence scales were adjusted at peak III to permit a better comparison of

Table 1

Variation of I/III of PY incorporated into SUV-PC [a] with LP [b]

LP (μM) [c]	I/III
150	1.19
100	1.22
77	1.21
51	1.22
38	1.20
26	1.25
15	1.23
	Average 1.22 ± 0.02 [d]

[a] In these experiments the incorporated PY/PC ratio was 0.5 mol%.

[b] Lipid phosphorus (LP) is a measure of the vesicles' concentration in the bulk aqueous solvent (see section 2).

[c] The LP concentrations from 15 to 150 μM correspond to PY concentrations between 0.075 and 0.75 μM.

[d] Standard deviation.

the I/III ratios. The two spectra of fig. 2 differ markedly in several aspects. First, the absolute fluorescence intensity of PY in the membrane is about 4-times greater than that of PY in buffer (see legend to fig. 2 and also fig. 2C of ref. 26). Second, fig. 2 provides a measure of the sensitivity of PY to ϵ of its environment which in the case of the more polar medium (H_2O) brings about a greater enhancement of the 0-0 transition (see refs. 10–15): i.e., the measured I/III ratios of PY dissolved in water and incorporated in the lipid bilayers were 2.02 and 1.22, respectively.

An important observation (see table 1) is that the average I/III ratio of PY incorporated in the SUV-PC membranes at low mole ratio (< 1.0 mol%), i.e., 1.22 (S.D. = 0.02), does not vary significantly with the vesicles' concentration in the buffer solution expressed as LP (see section 2). It is clear from this that PY remains mostly, if not completely, in the lipid phase (bilayer membrane) during the dilution process, precisely because if PY had been displaced out of the membrane into the bulk aqueous solvent its I/III ratio would have increased to values much higher than 1.22, say close to 2.02 (cf. fig. 1). These conclusions, in addition, are consistent with the results obtained by plotting the intensity of PY monomer emission as a function of LP (fig. 3). The straight line of fig. 3 was drawn from a linear least-squares curve-fitting program and is represented by the expression

$$I_M = 0.198 + 8.65 \times 10^4 \mathrm{LP}$$

where I_M is the intensity of PY monomer emission at 394 nm, and LP is given in mol l^{-1}. The linear regression correlation coefficient is greater than 0.99. The linear relationship of fig. 3 indicates that the aqueous environment (bulk solvent) of PY is about the same irrespective of the LP concentration used between 15 and 150 μM in the bulk solvent. We wish to point out first that this LP range corresponds to PY concentrations between 0.075 and 0.75 μM which are close to reported solubility limits of the probe in water, i.e., 0.3 μM [31], 1.0 μM [17] or 2–3 μM [10]. However, the corresponding PY emission spectra between 0.075 and 0.75 μM LP are identical and always yield an average I/III value of about 1.22 but never close to 2.02, the observed I/III value of PY dissolved in water as was emphasized above. If one takes into account, furthermore, that the intensity of PY emission is much higher (about 4-times) in the membranes than in water (see above), then it is reasonable to conclude with good certainty that any PY molecules that could eventually be solubilized in the aqueous solvent are present at such low concentration that they cannot influence significantly the I/III data reported herein and consequently the interpretation

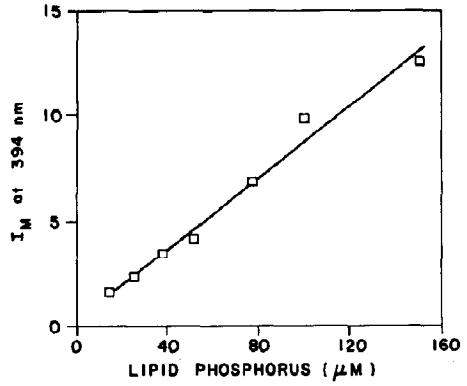


Fig. 3. Variation of the intensity of PY monomer emission at 394 nm (I_M) with the vesicles' concentration in solution, i.e., LP (see section 2). In these experiments the incorporated PY/PC ratio in SUV-PC was 0.5 mol%.

of the results. It is noteworthy, in this respect, that Blackwell et al. [25] reached similar conclusions in a recent work with PY incorporated into uni- and multilamellar vesicles obtained from aqueous dispersions of PC from membranes of chloroplast thylakoids.

3.3. Variation of I/III with concentration of incorporated probe

Fig. 4 depicts the variation of I/III with the mole ratio (mol%) of PY (fig. 4a) or C_{16}PY (fig. 4b) incorporated into the SUV-PC bilayers. Fig. 4a shows that up to 1.0 mol% of incorporated PY in the unilamellar vesicles the I/III ratio has an average value of about 1.21. However, at incorporated PY concentrations greater than 1.0 mol% the I/III ratio undergoes a transition to values higher

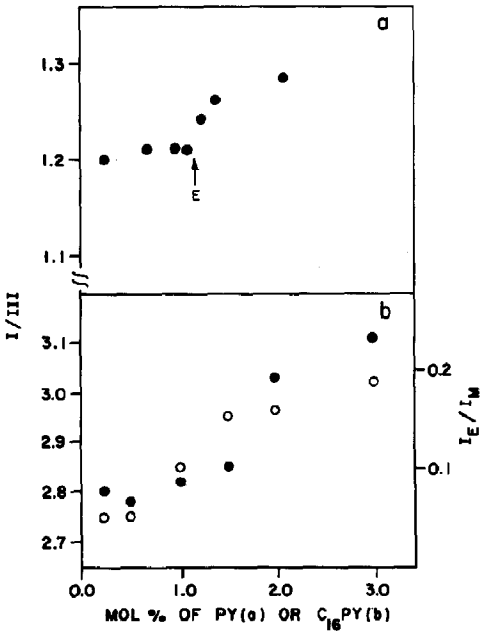


Fig. 4. (a) Variation of the I/III ratio of fluorescence intensities of pyrene with the ratio (mol%) of probe incorporated into SUV-PC. E (with an arrow) indicates the starting point of excimer formation; the variation of I_E/I_M (excimer/monomer ratio of fluorescence intensities) is not represented, since this ratio varies with the vesicles' concentration in solution (cf. fig. 5). (b) Variation of I/III (●) and I_E/I_M (○) ratios of C_{16}PY fluorescence intensities with the initial ratio (mol%) of probe in its mixture with PC.

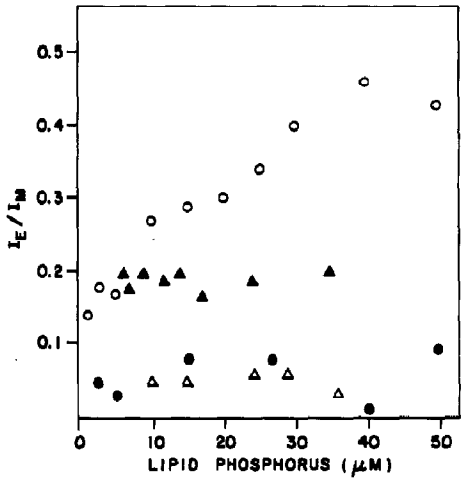


Fig. 5. Variation of the excimer/monomer ratios of fluorescence intensities (I_E/I_M) of PY and C_{16}PY with the vesicles' concentration in solution, i.e., LP. (○) 3.7 mol% of incorporated PY; (●) 0.1 mol% of incorporated PY; (▲), 3.0 mol% of initial C_{16}PY; (△) 0.25 mol% of initial C_{16}PY. Note that only the I_E/I_M of SUV-PC membranes prepared with mole fractions of free PY (○) above the critical point (cf. fig. 4) displays a significant variation with LP.

than 1.26 which is concomitant with the formation of excimer species emitting at 450–500 nm. This fluorescence behaviour of PY was also observed with C_{16}PY (fig. 4b), but I/III is approx. 2.80 and greater than 3.00 below and above 1.0 mol%, respectively *. Fig. 4b also shows that the I/III transition is accompanied by a similar variation of I_E/I_M, i.e., the ratio of fluorescence intensities of the excimer at 480 nm (I_E) and the peak V of the monomer around 394–395 nm (I_M) [7,8]. This is also in agreement with the work of Blackwell et al. (cf. fig. 5C of ref. 25 with our fig. 4b).

3.4. Effects of vesicles' concentration on I_E/I_M

Fig. 5 represents the variation of the ratio I_E/I_M with LP which is a measure of the PC content of

* We note, in this respect, that the higher values obtained for I/III of C_{16}PY compared with PY are caused by the covalent bond between the C_1 atom of PY and the C_{16} atom of hexadecanoic acid. This induces a reduction of molecular symmetry and, thereby, a more intense 0-0 vibronic transition (see, e.g., ref. 11).

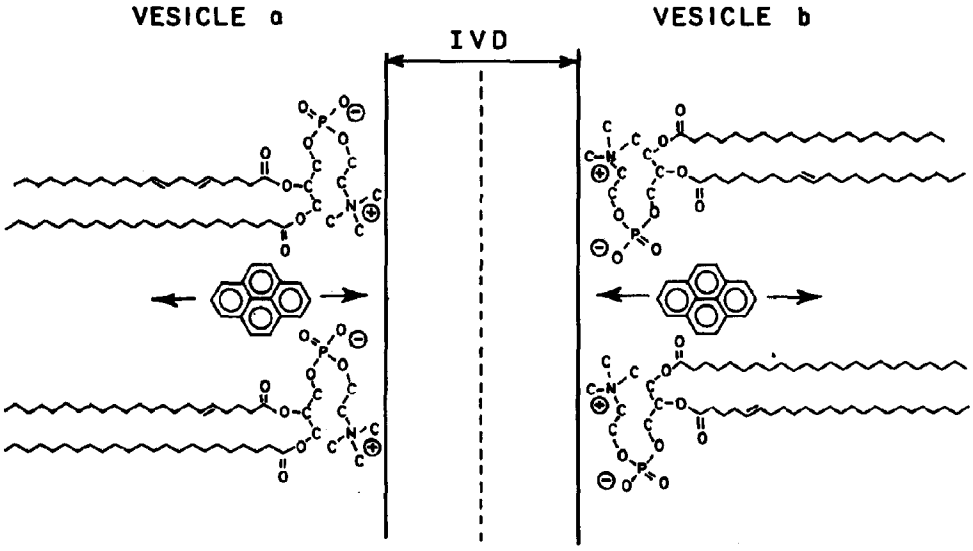


Fig. 6. Molecular diagram depicting the interaction between two PC vesicles with incorporated PY. The thickness of the outer hydration shells was set at 6 Å (see text and also ref. 34). On account of this, the intervesicular distance (IVD) is 12 Å. The long axis of the PY molecule is about 10 Å. The geometry of the PC polar head groups is the one suggested in ref. 39.

the vesicles and thereby of the vesicles' concentration in the bulk aqueous solvent (see section 2). It is seen that at 3.7 mol% of incorporated PY in SUV-PC bilayers (fig. 5, ○), thereby above the critical concentration of the probe (cf. fig. 4), I_E/I_M increases steadily with the vesicles' concentration. In contrast, I_E/I_M is practically insensitive to the increase of LP if the vesicles contain PY concentrations below the critical value, say 0.1% under the conditions of our experiments (cf. fig. 5, ●).

Another interesting observation is made from the study of the variation of I_E/I_M of $C_{16}PY$ as a function of the vesicles' concentration in solution (fig. 5). In contrast to free PY, fig. 5 shows that the I_E/I_M value of $C_{16}PY$ does not vary significantly with LP. The average I_E/I_M values are approx. 0.05 (fig. 5, △) and 0.2 (fig. 5, ▲) for 0.25 and 3.0 mol% of initial $C_{16}PY$ in the lipid mixtures, respectively. This is in the range of I_E/I_M values obtained, for instance, with 0.1 and 3.7 mol% of incorporated PY, i.e., approx. 0.04 and 0.15, respectively, at low LP in the vesicles' solutions (e.g., approx. 1.5 μM) but not at higher concentrations (cf. fig. 5).

Finally, it should be mentioned that the gap of approx. 0.10–0.15 observed between the I_E/I_M values of PY at low LP (~ 1.5 μM) as referred to above (fig. 5, ○ and ●) and $C_{16}PY$ at all LP concentrations (fig. 5, △ and ▲) stems from the transition from low to high excimer levels upon increasing the contents of PY and $C_{16}PY$ incorporated into the PC membranes above the probe/lipid critical ratio of 1.0 mol% as is clearly indicated in fig. 4.

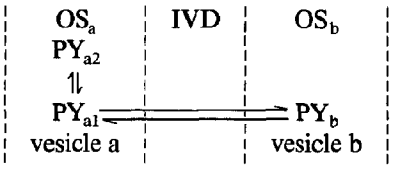
4. Discussion

4.1. Localization of PY and $C_{16}PY$ in the lipid bilayer

One of the possible explanations of the sudden changes of the I/III and I_E/I_M ratios displayed in fig. 4 is a concentration-induced generation of PY-PY and $C_{16}PY$–$C_{16}PY$ aggregates at critical molar ratios of probe to phospholipid greater than 1.0%. This view is supported by the work of Kaneda et al. [32] on excimer formation between the pyrene moieties of *N*-(1-pyrenesulfonyl)dipalmitoyl-L-α-phosphatidylethanolamine incorporated into fat cell plasma membranes. These authors showed on the one hand that about 50% of the excimer fluorescence originates from

ground-state dimers, and on the other that the excimer formation involves direct excitation of the dimers. These conclusions were corroborated by the work of Blackwell et al. [25] with aqueous lipid dispersions containing PY, and also by the studies of Yamazaki et al. [33] with Langmuir-Blodgett monolayer films prepared with $C_{16}PY$ and mixtures of $C_{16}PY$ and stearic acid. While the ground-state dimer hypothesis [32] is sufficient to explain the molecular mechanism of formation of $C_{16}PY$ excimers, it cannot elucidate reasonably our data obtained with PY.

A more plausible interpretation of the observed I/III (fig. 4a) or I_E/I_M transitions (fig. 5) of PY is a concentration-driven migration of PY monomers towards the outer surfaces of the lipid bilayers at mole ratios of incorporated PY above 1.0 which, if effective, will give rise to a two-step mechanism. First, the PY migration is accompanied by molecular aggregations, as suggested by Kaneda et al. [32], that occur most probably near the edge of the membranes. Second, and most importantly, the migration of PY brings about successful encounters between PY molecules originating from colliding vesicles. This is best visualized in the following scheme



which represents pyrene molecules PY_{a1} and PY_{a2} located at the outer surface of vesicle a (OS_a), and pyrene molecule PY_b located at the outer surface of vesicle b (OS_b) separated from vesicle a by the intervesicular distance, IVD. Here, the double arrows signify possible interactions between PY molecules located in the same or different vesicles. These interactions give rise to excimer formation by a mechanism which is represented, among other possibilities, by the expressions

$$PY_{a1} + h\nu \rightarrow PY_{a1}^*$$

$$PY_{a1}^* + PY_{a2} \rightarrow (PY_{a1}PY_{a2})^*$$

$$PY_{a1}^* + PY_b \rightarrow (PY_{a1}PY_b)^*$$

where the asterisk denotes an excited state molecular species.

At first, it is easily seen that interactions of PY_{a1} with PY_{a2} are responsible for the I_E/I_M differences discussed above of about 0.10–0.15 at approx. 1.5 μM LP (cf. fig. 5, ○ and ●). In contrast to this, increment of the vesicles' concentration in the bulk solvent (i.e., LP increase from ~ 1.5 to 50 μM, cf. fig. 5) cannot enhance $PY_{a1}PY_{a2}$ dimerizations but will favour instead the formation of transient molecular pairs of PY_{a1}, or PY_{a2}, with PY_b. In summary, the $C_{16}PY$ data discussed above can be explained in terms of formation of excimers in the deeper part of the hydrocarbon core of the SUV-PC bilayers as a result of constraints arising from the anchoring of the pyrenyl moiety to the hydrophobic end of hexadecanoic acid. In contrast, PY excimers will be formed preferentially (though probably not exclusively) in the neighbourhood of the water/lipid interface (cf. models depicted in fig. 4 of ref. 2 and in our fig. 6). This is a process which is most likely facilitated by the apparently high mobility of the PY molecule.

We also showed that the localization of PY, but not $C_{16}PY$, molecules in the membrane space of the SUV-PC bilayers is largely influenced by the relative concentration, or mol% PY/PC, of incorporated probe with a transition from inner core to outer surface location at approx. 1.0 mol% of incorporated PY. This molar ratio coincides with the start of excimer formation (cf. fig. 4).

4.2. *The transient-fusion model*

Taking into account that the interaction radius for excimer formation amounts to between 3 and 10 Å [2,17,25,27], then the data discussed above give strong support to the function of a mechanism of transient collision-enhanced merging of the vesicles' polar head groups. To make this conjecture plain, we will examine first the nature of collisions between PY-containing vesicles. The model displayed in fig. 6 depicts a scheme of two vesicles before collision. For practical reasons only one half of each vesicle's bilayer is represented in the figure. In addition, the intervesicular distance (IVD) is set at about 12 Å, i.e., twice the lower

limit thickness of the outer hydration shell (HS_0), i.e., 6 Å, which was determined by Huang and Mason [34]. Another possible HS_0 value is 10 Å [35], but its use renders the conditions for PY excimer formation, i.e., IVD = 20 Å, still more stringent (see discussion below) and, therefore, will not be applied here.

Close scrutiny of fig. 6 reveals that intervesicular excimer formation involving PY molecules situated at the nearness of the outer hydration shells is essentially under the control of conditions that are beyond the classical kinetic (collision frequency) and geometric constraints. The figure shows, in effect, that even under the most favourable conditions for colliding which bring about the contact between the hydration shells of two vesicles with an appropriate PY-PY geometry, the critical distance for excimer formation would never be attained. This implies that one of the first steps for effective collision of vesicles, i.e., a collision which brings two PY molecules into the region inside the critical excimer interaction radius, is one of fusion of the vesicles' hydration shells. We predict that during the process some interfacial H_2O molecules are excluded from the collision interface thus lowering its polarity (ϵ) and favouring at the same time the displacement of the more hydrophobic PY molecules toward the upper part of the phospholipid head groups. Since the PC vesicles are not rigid spheres but visco-elastic particles, it is reasonable to expect at least a brief interpenetration of the relatively fluid head group regions of the colliding vesicles *. What is more, on account of the fact that the lifetime of the excited monomer PY in SUV-PC membranes is quite long, ~ 350 ns [22], the probability of successful encounters, and thereby of excimer formation, should be considerably increased.

We note, finally, that a factor which may influence the vesicles' collisions is the surface charge of the phospholipids which is capable of creating repulsive forces between the outer surfaces of the bilayers. Some preliminary work from our laboratory shows that this is especially true in the case of charged lipids such as phosphatidic acid, phosphatidylglycerol and phosphatidylserine (F. Bellemare and M. Fragata, manuscript in preparation). However, since the lipid used in the present study, i.e., egg PC, is a mixture of zwitterionic PC molecules which differ only in the size and number of double bonds of their hydrocarbon chains, the short-range electrostatic forces which are developed give rise only to a weak repulsive effect even when the polar head group regions of colliding vesicles share the same membrane space at the impact area as was discussed above. Therefore, the perturbation effect was assumed to be negligible in the PY-PY interactions described here and was thereby neglected, as a first approximation, in the afore-mentioned model (cf. fig. 6).

* The most probable value of the surface viscosity of a PC bilayer was calculated to be 0.36 P (cf. ref. 36) in contrast with the viscosity of the lipid bilayer itself which ranges from 0.3 P [37] to 0.6–0.8 P [38]. We recall, for comparative reasons, that the viscosity of water at 293–295 K, the temperature at which our experiments were performed, is about 0.01 P.

Acknowledgments

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