Lateral Organization of Pyrene-labeled Lipids in Bilayers as Determined from the Deviation from Equilibrium between Pyrene Monomers and Excimers*

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In lipid bilayers, pyrene and pyrene-labeled lipids form excimers in a concentration-dependent manner. The aromatic amine N,N-diethylaniline (DEA), which has a high membrane-to-medium partition coefficient, quenches the monomers only, and therefore it is expected that under conditions in which the monomers are in equilibrium with the excimers due to the mass law, the Stern-Volmer coefficient \( K_{sv} \) for monomers (M), defined as \( K_{sv} = \frac{[M]}{[E]} \), should be identical to that of the excimer (E), defined as \( K_{E} \). This is indeed the case for pyrene and pyrene valerate in egg phosphatidylcholine small unilamellar vesicles. However, for pyrene decanoate and pyrene dodecanoate in these vesicles, and for N-[12-(1-pyrenyl)dodecanoyl]-sphingosylphosphocholine in a matrix of either N-stearoyl sphingosylphosphocholine or 1-palmitoyl-2-oleoyl phosphatidylcholine, \( K_{E} < K_{M} \). This can be explained either by the existence of (a) two subpopulations of excimers, one in fast equilibrium with the monomers and the other, related to ground-state protoaggregates of pyrene lipids; (b) two monomer subpopulations where part of M cannot be quenched by DEA; or (c) two monomer subpopulations, both quenched by DEA, but only one of which produces excimers. The good agreement between the photophysics processes determined by steady state and time-resolved measurements supports the third explanation for the bilayers containing pyrene phospholipids. It also suggests that the main factors determining the immiscibility of pyrene lipids in phospholipid bilayers are temperature, the difference in the gel-to-liquid-crystalline phase transition temperature \( \Delta T_m \) between the matrix and the pyrene lipid, and the structural differences between the matrix lipid and the pyrene-labeled lipid. These results indicate that the \( K_{E}/K_{M} \) ratio can serve as a very sensitive tool to quantify isothermal microscopic immiscibility in membranes. This novel approach has the following advantages: applicability to fluid phase immiscibility, requirement of a relatively low mol fraction of pyrene lipids, and conceivably, applicability to biological membranes.

Biological membranes are complex multicomponent assem-
blies. It is well established that the matrix of these membranes, the lipid bilayer, has two faces that are compositionally distinct (for review, see Refs. 1–3). At the level of resolution of the wavelength of visible light (0.5 μm), studies based on determination of the immobile fraction in a fluorescence recovery after photobleaching experiment indicate that biological membranes are laterally heterogeneous and have in-plane domains distributed in a homogeneous lipid continuum (4–6). More precise fluorescence recovery after photobleaching studies were recently performed in lipid bilayers of defined composition in which solid and fluid phases coexist, and the phase diagrams are well characterized (Refs. 7 and 8 and references therein). However, not much information is available on lateral organization at the submicron range. The functional significance of lateral heterogeneity must be related to creation of microenvironments that enable better control of functional assemblies in the membranes such as channels and signal transduction systems. It is expected that the kinetics of interaction between membrane components should be very different when the interaction occurs in a restricted domain or a continuum (9). Since most of these assemblies are relatively small (<100 nm), it is important to study how they are affected by both the micron and submicron scales of lateral heterogeneity. Our goal is to better understand the lateral organization of biological membranes. Since they are complex multicomponent systems, it is necessary first to study well-defined one-, two-, and three-component lipid bilayers in the liquid, liquid-ordered, and solid-ordered phases (10) as well as systems in which various combinations of these phases coexist. The model systems to be used to characterize lateral organization at the submicron range should be applicable to both lipid bilayers and biological membranes, which is the case for the fluorescence recovery after photobleaching approach used for the micron range organization (5, 7, 8, 11). Most of the approaches available for quantification of lateral heterogeneity at the submicron range (1, 12–14), although informative, suffer from either theoretical or practical drawbacks such as low sensitivity, inability to measure fluid-phase immiscibility, and inability to be used in parallel in both model systems and biological membranes (13, 15).

Here we evaluate a novel approach based on the ability to detect and quantify the lateral organization of pyrene-labeled lipids present in lipid bilayers of defined compositions. This method can be applied to biological membranes that can be labeled by introducing a pyrene fluorophore either metabolically (for review, see Ref. 16), or enzymatically by phospholipid exchange proteins (17) using liposomes as donors. A third approach of spontaneous diffusion from a labeled donor is rather limited due to the very low desorption rate \( K_{off} \), which is the
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rate-limiting step in the exchange process (for review, see Ref. 3).

Pyrene-labeled lipids were extensively used in membrane research to determine the dynamics and organization of lipid membranes and biological membranes (Ref. 18 and references therein). The most common use of pyrene and pyrene lipid is based on the ability of one excited pyrene moiety, together with a nonexcited pyrene moiety, to form an excited-state dimer (excimer). This reaction was studied extensively in organic solvents and proved to be a diffusion-controlled bimolecular reaction (19, 20). This assumption was used to study the dynamics of lipid bilayers (21). The approach of simple controlled diffusion was slightly modified by Galli et al. (22). However, the importance of the diffusion became more of a controversial issue as recent data suggested that pyrene excimer formation in membranes may not be diffusion controlled. The explanation for the rate-limiting step in this process is not clear and may be dependent on the exact pyrene lipid and method used (compare Blatt et al. (23) and Lemmetyinen et al. (24)). It was found that for binary systems in which the temperature-dependent determination of the ratio between the level of excimers and monomers (E/M ratio)1 was studied, most of the methods and systems have only limited use to determine the lateral organization of the pyrene lipid in bilayers due to their inability to be applied to cases in which the matrix lipid has a lower transition temperature than the pyrene-labeled lipid (15). An important contribution is the finding of Martins and Melo (25), which supports previous suggestions that the Birks three-dimensional formalism is not applicable for systems of reduced dimensionality such as lipid bilayers. Of special importance in the two-dimensional system is the time dependence of the fluorescence decay rate coefficient, which eliminates the need to assume the long term association of ground-state (“static”) pyrene lipids in the bilayer plane. The product of this association is referred to as the pyrene ground-state protoaggregate. Recently Chong and co-workers (26, 27), using pyrene lipids, showed that the fluorescent lipids were organized into hexagonal super-lattices in the bilayer plane, information that could not be obtained by other methods.

In most studies done so far, the ability to distinguish between two monomer populations in bilayers in which solid and fluid domains coexist based on the discrepancy between the decay of the excited monomer and excimer populations. This was used to show that a fraction of the pyrene probes is isolated and does not participate in the domain formation (28). In this study, we present another approach, which is based on using aromatic amines as pyrene (or pyrene moiety) quenchers. The aromatic amines selectively quench only the pyrene monomers, thereby enabling one to determine the lateral insufficiency of pyrene and pyrene-labeled lipids in lipid bilayers.

MATERIALS AND METHODS

Lipids—Egg phosphatidylcholine (PC) of high purity (>99%) was obtained either from Sigma or from Avanti Polar Lipids (Alabaster, AL). 1-Palmitoyl-2-oleoyl-sn-PC was purchased from Avanti Polar Lipids. N-Stearoyl sphingosylophosphocholine (C16-SPM) was prepared, purified, and characterized as described by Cohen et al. (29).

Fluorescent Lipids—Pyrene (99.9% pure) was purchased from Fluka (Buchs, Switzerland), and pyrene fatty acids (see legend to Fig. 2) were obtained from Molecular Probes (Eugene, OR). N-[12-(1-Pyrenylo)dodecanoyl]phosphoglycolylphosphocholine (Py-C12-SPM) was prepared, purified, and characterized as described by Frank et al. (30) using the procedure of Cohen et al. (29).

1 The abbreviations used are: E, excimer; M, monomer; PC, phosphatidylcholine; C16-SPM, N-stearoyl sphingosylphosphocholine; Py-C12-SPM, N-[12-(1-pyrenyl)dodecanoyl]phospho-sylophosphocholine; DEA, N, N-diethylaniline; SUV, small unilamellar vesicles; eq, equilibrium; neq, not in equilibrium.

Other Reagents—N,N-Diethylaniline (DEA) of analytical grade was obtained from BDH (Poole, United Kingdom). The DEA was distilled and stored as described by Barenholz et al. (18). All other reagents were of analytical grade or better.

Liposome Preparation—Phospholipids and either pyrene or pyrene- labeled lipids were mixed in spectral grade chloroform/methanol, 2:1 (v/v). The desired mol ratio small unilamellar vesicles (SUV) were prepared and fractionated in 50 mM Tris-HCl buffer, pH 8.0, containing 20 mM KCl, as described by Barenholz et al. (18). This procedure ensures good microscopic mixing (31). The SUV were fractionated by differential centrifugation. Egg PC concentration was determined by a modified Bartlett procedure (32). The pyrene, pyrene fatty acid, and Py-C12-SPM concentrations were determined spectrophotometrically using a Uvikon 810 double-beam spectrophotometer (Kontron, Switzerland). The quantification is based on the optical density at 345 nm after solubilizing the vesicles in ethanol and using a calibration curve of each of the individual pyrene-labeled molecules.

Fluorescence Measurements—All vesicle dispersions were placed in sealed cuvettes, and wet nitrogen was bubbled through them for 30 min before measurement followed by sealing to eliminate the presence of oxygen.

Steady State Fluorescence Emission Measurements—Fluorescence intensity was measured by Perkin Elmer MFP-44A or Perkin Elmer 50B spectrophotometers using excitation in the range of 340–350 nm adjusted to obtain absorption lower than 0.16 OD in order to ensure homogenous absorption of the excitation light. The intensity of the fluorescence emission was determined at the monomer peak (393 nm) and excimer peak (480 nm).

Measurements of Fluorescence Lifetime—The measurement of lifetime of monomer and excimer fluorescence emission is described in detail in the footnote to Table II and in Ref. 18.

RESULTS AND DISCUSSION

Assumptions and Modeling—Our approach is based on the following principles.

The unique property of pyrene (and pyrene-labeled molecules) to form excimers by two mechanisms: (a) as a result of a dynamic collision between a ground-state and an excited-state monomer (M and M*, respectively), producing a “dynamic” excimer, and (b) as a result of a static interaction between M and M* in close proximity, producing a “static” excimer (20, 23, 24, 26, 27, 33).

The ability to distinguish between, and separately quantify, quenching of monomers (M*) and excimers (E) from Stern-Volmer plots under conditions that the quencher directly interacts only with M* and not with E.

Therefore, based on the mass law, it is expected that the Stern-Volmer constant for M* and for E (Km and Ks, respectively) will be equal. As is demonstrated below, this is not the rule for all cases studied. Depending on miscibility of the matrix lipid with the pyrene-labeled lipid and the temperature of the measurement, it was found that for many systems Ks > Ks.

Therefore, this can be explained in three alternative ways. (a) There exists at least one excimer subpopulation that is not in equilibrium with quenchable monomers; namely, that this population is of ground-state protoaggregates referred to as static excimers (19, 20, 23). (b) There exists an equilibrated subpopulation, M* + M ↔ E, where part of M* is not accessible to the quencher. Or (c) there exists a monomer subpopulation that does not form excimers. The pattern of cases in which Ks > Ks is not random; it usually occurs under conditions of coexistence of two phases, such as gel and liquid-crystalline. Use of the ratio Ks/Ks is proposed to quantify the two populations, either of excimers or of monomers (see a–c above).

En = Eeq + Eneq and M* = M*eq + M*neq, where M*eq are monomers in equilibrium with excimers, Eeq are dynamic excimers in equilibrium with M*, Eneq are static excimers not in equilibrium with monomers, and M*neq are quenchable monomers that do not form excimers. In all these cases (a–c above), the ratio Ks/Ks is defined as X, where X = 1 – X give the fraction of M*eq and M*neq respectively. These definitions are
valid only if all quenchable monomers have the same $k_q$ (bimolecular quenching rate constant) and $\tau_{M^*}$ (lifetime of the excited state of the monomer in dilute solution), and only the monomers are quenched by DEA.

First, it was demonstrated that DEA quenches only the monomers. This was then applied to examples of lipid vesicles composed of well-characterized binary mixtures of a matrix phospholipid and a pyrene-labeled lipid at defined temperatures. The steady state results were also confirmed by dynamic time-resolved measurements.

Our previous study (18) indicates that the aromatic amine DEA, which quenches excited pyrene moieties through a charge transfer mechanism, is a more efficient quencher ($k_q$ in ethanol at 25°C is $1 \times 10^{10}$ M$^{-1}$ s$^{-1}$) than heavy atom derivatives such as bromobenzene and iodide, which quench the pyrene through a spin-orbit coupling mechanism. One of the advantages of DEA as a quencher is its high membrane-to-scattering, which was minimal for the SUV used in this study. Oxygen was removed, and fluorescence measurements were performed as described under “Materials and Methods.”

Steady State Measurements in Lipid Vesicles—The existence of M$^*$, monomer, in bilayers was first suggested by the deviation from linearity in the curves describing the E/M ratio as a function of the mol fraction of the pyrene-labeled lipids in the matrix of fluid lipid bilayer (egg PC, Fig. 2). Fig. 2 demonstrates the effect of pyrene on pyrene-derivative mol fraction on the E/M ratio for pyrene, pyrene valerate, pyrene decanoate, and pyrene hexadecanoate in egg PC SUV.

Fig. 2 shows that the effect of probe mol fraction on its capability to form excimers was in the order pyrene > pyrene hexadecanoate > pyrene decanoate > pyrene valerate. It seems that the attachment of a paraffinic chain to pyrene reduces its ability to form excimers, which may be related to reduced

$$F_0/F - 1 = K_{SV}[DEA]$$

(Eq. 1)

$K_{SV}$ is the Stern-Volmer coefficient, and [DEA] is the quencher concentration, describing $F_0/F - 1$ as a function of DEA concentration for the two concentrations of pyrene, for monomers and excimers (the latter for the 1.8 $\times$ 10$^{-2}$ M pyrene only). DEA in the range of 4 $\times$ 10$^{-5}$ to 1 $\times$ 10$^{-2}$ M was used. $K_M$ of 4.9 $\times$ 10$^{9}$ M$^{-1}$ and 92.6 M$^{-1}$ were obtained for 3.6 $\times$ 10$^{-6}$ M and 1.8 $\times$ 10$^{-2}$ M pyrene, respectively (Fig. 1). Also, within experimental error, the data show that $K_M = K_E$, suggesting that no real quenching of excimers takes place and that the $K_M$ obtained is related to the dissociation of E to the monomers. This was further tested as follows. The bimolecular quenching rate constant ($k_q$) was calculated as

$$k_q = K_M/\tau_{M^*}$$

(Eq. 2)

(where $\tau_{M^*}$ is the lifetime of the excited state of the monomer obtained in dilute solution). In the presence of high concentration of M*, the measured lifetime of the excited state of the monomer (M*) is reduced according to

$$\tau_{M^*} = \frac{1}{k_q + k_n + [Py]}$$

(Eq. 3)

where $k_q$ is the first-order rate constant of the fluorescence, $k_n$ is the first-order rate constant of all the nonradiative processes, $k_{ex}$ is the rate constant for excimerization in ethanol, and [Py] is the pyrene concentration. Using the values obtained by Birks of 6.0 $\times$ 10$^5$ M$^{-1}$ s$^{-1}$ for $k_{ex}$ (20) and $\tau_{M^*}$ (475 ns) (in the absence of oxygen and at low pyrene concentration), and for the lifetime of the excimers, $\tau_{ex}$ (in the absence of oxygen) = 53 ns permits us to calculate the sum of $k_q + k_n$. These are the intrinsic characteristics of pyrene (19); from these, the $\tau_{M^*}$ for the concentrated pyrene solution was determined. The bimolecular quenching rate constant, $k_n$, was obtained for the DEA in dilute pyrene solution using $\tau_{M^*}$ values, the Stern-Volmer rate constant, and Equation 2. $k_n = 1.04 \times 10^{-10}$ M$^{-1}$ s$^{-1}$. For the concentrated pyrene solution, $\tau_{M^*}$ of 9.1 ns was calculated using Equation 3 (a shorter lifetime is expected due to the large extent of excimerization). This $\tau_{M^*}$ was used in Equation 2 and a $k_q$ of 1.02 $\times$ 10$^{-10}$ M$^{-1}$ s$^{-1}$ was obtained for DEA-pyrene quenching at high pyrene concentration. This value is almost identical to the $k_q$ value of 1.04 $\times$ 10$^{-10}$ M$^{-1}$ s$^{-1}$ obtained for the low pyrene concentration. The identity in $k_q$ for the low and high pyrene concentrations indicates that the excimerization does not interfere with the quenching of monomers by DEA and that only monomers are quenched by DEA.
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**Fig. 3.** Stern-Volmer plots for DEA quenching of pyrene (A), pyrene valerate (B), pyrene decanoate (C), and pyrene hexadecanoate (D). Egg PC SUV containing the pyrene or pyrene fatty acid were prepared as described in Fig. 2. DEA in an oxygen-poor ethanol solution was added to the oxygen-poor egg PC SUV suspension (1 mg egg PC containing 10 mol % pyrene or the specified pyrene fatty acid). The final ethanol solution did not exceed 0.5%. The DEA is volatile and therefore has to be added in the final step (18). The high pH of the medium (pH 8.0, “Materials and Methods”) ensures that most of the DEA (pK<sub>a</sub> = 6.5) is in its neutral form in order to obtain its favorable partition into the lipid bilayer. Fluorescence measurements were performed and the Stern-Volmer plots for monomers (•) and excimers (□) were obtained (after correction for light scattering, Fig. 2) as described in the legend to Figs. 1 and 2.

Such phase separation is expected from phase diagrams for phospholipid and fatty acid mixtures (37, 38), in agreement with what was suggested before by Lemmetyinen et al. (24). Accordingly, a subpopulation of the excimers is derived from collisions between M<sup>*</sup> and M, which are in close proximity at the time of excitation. Therefore, the high local concentration of monomers exists, which shifts the local equilibrium to form a high E concentration even at relatively low total mol fraction of pyrene hexadecanoate. These excimers are not in equilibrium with the total monomers, and therefore they are referred to as E<sub>exc</sub>. This favors the existence of two excimer populations for the pyrene hexadecanoate in egg PC.

Previous studies using pyrene hexadecanoate in PC SUV did not show this behavior (33). However, these studies were done in bilayers having lower mol fraction of the pyrene hexadecanoate and no data of E/M versus mol fraction of the pyrene hexadecanoate were reported.

Fig. 3 describes the DEA Stern-Volmer plots for M<sup>*</sup> and E for the same pyrene lipids described in Fig. 2. All (except the highest) DEA concentrations used were below the DEA/lipid concentration, which may have a large effect on bilayer properties (18). For pyrene (a) and pyrene valerate (b) the slopes of the DEA Stern-Volmer plots for M<sup>*</sup> and E are identical (K<sub>E</sub> = K<sub>M</sub>), which is not the case for pyrene decanoate (c) and pyrene hexadecanoate (d), for which K<sub>M</sub> < K<sub>E</sub>. The ratio K<sub>E</sub>/K<sub>M</sub> was used to calculate the mol fraction not in M<sup>*</sup> = E equilibrium, which was 25 and 39%, respectively, for these two pyrene-labeled fatty acids. All plots in Fig. 3 were fitted using linear regression. However, it is evident that some of the curves may deviate from linearity and can better be fitted to a hyperbola. This was not studied in detail, although such nonlinearity is consistent with the presence of two populations of fluorophores, one of which is not accessible to the quencher. This may be an artifact, as the highest concentration of DEA used (DEA/lipid ratio > 0.4) may have a large effect on membrane structure (18). The two different criteria represented by Figs. 2 and 3 agree and both describe deviation from the behavior expected if the systems were governed solely by the diffusion-dependent collision model. However, the DEA-quenching criterion seems to be more sensitive since it monitors the deviation at a lower mol fraction of the pyrene-labeled lipid than the E/M approach. No DEA-pyrene exciplexes (18) were found in the spectrum at 6 mol % pyrene lipid; that is, no other interaction between DEA and the pyrene moiety could be detected. Vesicles composed of phospholipids and fatty acids may represent a special case since there are large differences in structure and molecular dimensions of the fatty acids and the matrix PC (38, 39). Therefore, the study was extended to other systems in which both the matrix lipid and the pyrene-labeled lipids are phospholipids of defined acyl chain composition and gel-to-liquid-crystalline phase transition temperature (T<sub>m</sub>). The quenching experiments were conducted using 1 mg total phospholipids. Stern-Volmer plots were performed over a DEA concentration range of 0–350 μM (below the DEA/lipid ratio that may cause pronounced perturbation to vesicle structure (18). At least 10 different DEA concentrations were used for each 2 T<sub>m</sub> is defined as the temperature of maximum change in the specific heat capacity during the main gel-to-liquid-crystalline phase transition.
curve. All curves were fitted best for linear plots throughout all the DEA concentration range, with a correlation coefficient >0.993 (which was not the case in Fig. 3). Table I clearly demonstrates that in all the systems there is a deviation from a complete M* = E equilibrium. However, the degree of deviation varied to a large extent, from 5% for Py-C12-SPM in a complete M* matrix. This demonstrates that in all the systems there is a deviation from equilibrium. The ratio between the two can be expressed as:

\[ \text{RMS} = \frac{\Delta T}{T_m} \]

At least 10 different concentrations of DEA (0–4 mol %) were used for each curve. All curves were linear, having a correlation coefficient R² = 0.993.

### Table I

**Organization of pyrene lipids in lipid bilayers**

| Vesicle system* | Pyrene lipid | Tm (°C) | Measurement temperature | % Deviation from M* E equilibrium* |
|-----------------|-------------|---------|------------------------|----------------------------------|
|                  | Compound    | Mol %   | Matrix lipid           | Pyrene derivative² | Matrix lipid³ | °C | °C | %     |
| Py-C12-SPM      | 4           | C18-SPM | 43.0                   | 43.6               | 20             | 75 |
| Py-C12-SPM      | 4           | C18-SPM | 43.0                   | 43.6               | 50             | 5  |
| Py-C12-SPM      | 2           | POPC    | 43.0                   | -5.0               | 20             | 100|
| Py-C12-SPM      | 2           | POPC    | 43.0                   | -5.0               | 50             | 45 |
| Py-C12-SPM      | 4           | POPC    | 43.0                   | -5.0               | 37             | 45 |
| Py-C12-SPM      | 4           | POPC    | 43.0                   | -5.0               | 50             | 40 |

* SUV (20–25 nm diameter of the specified composition) were prepared by ultrasonic irradiation as described in the legend of Fig. 2. The use of SUV minimizes light-scattering perturbation. Stern-Volmer plots for monomers and excimers were obtained as described in the legend of Fig. 3. At least 10 different concentrations of DEA (0–4 mol %) were used for each curve. All curves were linear, having a correlation coefficient >0.993.

**Table II**

### Monomer and excimer lifetime of pyrene and pyrene-labeled sphingomyelin in egg PC SUV at 25°C in the absence and presence of diethylaniline

| Pyrene lipid | Mol % DEA | E/M | Single | Double |
|--------------|-----------|-----|--------|--------|
| Pyrene       | 0.5       | 0.03 | 133.0  | 0.15   |
| Pyrene       | 10        | 1.15 | 51.3   | 0.13   |
| Pyrene       | 10        | N.D. | 22.1   | 0.06   |
| Py-C12-SPM   | 1.0       | 0.03 | 59.4   | 0.4    |
| Py-C12-SPM   | 1.0       | 0.03 | 10.7   | 0.02   |
| Py-C12-SPM   | 1.0       | 0.19 | 34.4   | 0.15   |
| Py-C12-SPM   | 10        | N.D. | 17.7   | 0.03   |
| Blank (no pyrene) | 0        | 0.0  | 3.5    | 0.38   |

**a RMS, root mean square.**

### Egg PC SUV containing the specified mol % of either pyrene (1 mM egg PC) or Py-C12-SPM (0.1 mM egg PC) were prepared in KCl-Tris, pH 8.0, solution as described in the legend to Figs. 2 and 3 (Table I). Excitation was done at 340–350 nm, using a narrow path of 3–10 nm (18). Monomer lifetime analysis Excimer lifetime analysis

- **Single**
  - $t_1 = 140.0 \pm 2.9$
  - $t_2 = 0.127 \pm 0.047$
  - $t_{\text{RMS}} = 0.0079$
  - No excimers were observed

- **Double**
  - $t_1 = 55.4 \pm 8.3$
  - $t_2 = 0.03 \pm 0.12$
  - $t_{\text{RMS}} = 0.0028$
  - No excimers were observed

- **Excimer lifetime analysis**
  - $t_1 = 69.5 \pm 3.13$
  - $t_2 = 0.11 \pm 0.054$
  - $t_{\text{RMS}} = 0.0066$
  - No excimers were observed

- **Blank (no pyrene)**
  - $t_1 = 23.3 \pm 4.5$
  - $t_2 = 0.127 \pm 0.104$
  - $t_{\text{RMS}} = 0.0060$
  - No excimers were observed

- **Pyrene**
  - $t_1 = 133.0 \pm 0.15$
  - $t_2 = 0.127 \pm 0.047$
  - $t_{\text{RMS}} = 0.0079$
  - No excimers were observed

- **Py-C12-SPM**
  - $t_1 = 55.4 \pm 8.3$
  - $t_2 = 0.03 \pm 0.12$
  - $t_{\text{RMS}} = 0.0028$
  - No excimers were observed

- **Pyrene lipid**
  - $t_1 = 69.5 \pm 3.13$
  - $t_2 = 0.11 \pm 0.054$
  - $t_{\text{RMS}} = 0.0066$
  - No excimers were observed

- **Pyrene**
  - $t_1 = 23.3 \pm 4.5$
  - $t_2 = 0.127 \pm 0.104$
  - $t_{\text{RMS}} = 0.0060$
  - No excimers were observed

- **Blank (no pyrene)**
  - $t_1 = 23.3 \pm 4.5$
  - $t_2 = 0.127 \pm 0.104$
  - $t_{\text{RMS}} = 0.0060$
  - No excimers were observed

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pyrene (Py-C_{12}-SPM) was drastically reduced by DEA. The lifetime of the fluorescence of the excimer was not affected for both pyrene and Py-C_{12}-SPM. This confirms the conclusions obtained from the steady state measurements and indicates that indeed only monomers are quenched by DEA in homogeneous systems such as ethanol (Fig. 1) and in lipid bilayers (Table II).

(c) The dynamic mechanism of the excimer formation was confirmed by the observation of the negative preexponent in the decay process of the excimer fluorescence. Both the free pyrene and the lipid pyrene derivative showed this characteristic of the excited state reaction. The fact that the two preexponents in the decay curve of the excimer emission were not of the same absolute value may be an indication of heterogeneity of the population of monomer molecules. A possible source can be a subpopulation of molecules that does not form excimers (see "Assumptions and Modeling," above).

(d) As was discussed by Martins and Melo (25), there is no need to assume the presence of ground-state aggregate, explanation under "Assumptions and Modeling," above. Therefore, our data favor explanation c, namely that there are at least two populations of excited monomers, one of which cannot excimerize.

CONCLUSIONS

The good agreement between the photophysical processes of pyrene and pyrene lipid in lipid bilayers monitored by steady state and time-resolved measurements described above led us to suggest that the main factors contributing to immiscibility of the pyrene lipid in the matrix lipid are (a) the temperatures at which the measurement was performed, (b) the difference in T_m (ΔT_m) between the matrix lipid and the pyrene lipid, and (c) structural factors such as the presence of pyrene fluorophore on the ω position of the acyl chain, which, due to its bulkiness, increases the volume of the hydrophobic region of the phospholipid molecule. Therefore c may be the overriding factor in determining lateral immiscibility. It may be that c is also related to the hexagonal super-lattices found in bilayers of binary mixtures composed of pyrene PC and dimyristoyl-PC, or in other PCs (Refs. 26 and 27 and references therein). Recently (39) it was demonstrated that the immiscibility of pyrene PC in a PC bilayer is increased by partial dehydration of the phospholipid headgroup, which further increases the packing parameter (39, 40). The relevancy of bilayers composed of phospholipids and pyrene lipids to understanding the miscibility of lipids in biological membranes is not yet clear. However, biologically relevant structural features that may contribute to fluid (or liquid-ordered) lateral immiscibility do exist, for example, in sphingolipids where the mismatch between the acyl chain and the sphingosine base chain may be expressed as interdigitation or as protrusion of the headgroup above the plane of the headgroup of the symmetric matrix lipid (41, 42). Indeed, it was demonstrated that partial dehydration increased immiscibility between PC and glucosylceramide in bilayers (45).

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