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Photophysics of anthracene–indole systems in unilamellar vesicles of DMPC and POPC: Exciplex formation and temperature effects

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Abstract

The quenching of anthracene fluorescence by indole (IN), 1,2-dimethylindole (DMI), tryptophan (Trp) and indole 3-acetic acid (IAA) in dimiristoylphophatidylcholine (DMPC) and palmitoyloleoylphosphatidylcholine (POPC) lipid bilayers was investigated. The studies were carried out at 25 °C in POPC vesicles and below (15 °C) and above (35 °C) the phase transition temperature (24 °C) of DMPC. A very efficient quenching of the anthracene fluorescence by IN and DMI in the lipid membrane is observed in all cases. It is less efficient in the case of Trp and IAA. Stern–Volmer plots are linear for DMI but present a downward curvature for the other quenchers. This was interpreted as an indication of the presence of an inaccessible fraction of anthracene molecules. By a modified Stern–Volmer analysis the fraction accessible to the quenchers and the quenching constant were determined. Partition constants of the quenchers were obtained from the changes in the fluorescence emission of the indole moiety caused by the presence of the phospholipid. Using the partition constants bimolecular quenching rate constants were determined in terms of the local concentration of quencher in the lipid bilayer. These corrected rate constants are lower than those in homogeneous solvents. In the case of DMPC values the gel phase are higher than in the liquid-crystalline phase. In the quenching by IN and DMI a new, red shifted, emission band appears which could be assigned to an exciplex emission. The exciplex band is absent in the quenching by IAA and Trp.
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1. Introduction

Fluorescence techniques are widely employed in the study of organized systems [1], such as micelles [2] reverse micelles [3,4] and vesicles [5–7]. In particular fluorescence quenching [8,9] has proved to be a powerful tool to investigate the partition and mobility of small molecules (usually used as quenchers) in the microphases of the organized systems and also on the properties (micropolarity and microviscosity) in the environment of the fluorescent probe.

The quenching of aromatic probes by indolic compounds has been widely investigated. In homogeneous solvents the quenching reaction takes place by electron transfer from the indole to the excited probe [10,11]. The quenching of several probes in normal [12,13] and reverse [14] micellar systems was the subject of several papers. More scarce are the studies of this process in liposomes. We have previously reported on the fluorescence quenching of anthracene by indolic compounds in large unilamellar vesicles (LUV) of palmitoyloleoylphosphatidylcholine (POPC) [15]. In this paper we wish to report on the quenching process in dimiristoylphophatidylcholine (DMPC) vesicles. The importance of this system resides in that DMPC liposomes are widely used as a model system of biomembranes because lecithins are the major component of most mammalian biomembranes. Moreover, these vesicles present a sharp phase transition that takes place in the bilayer near room temperature (24 °C [16]) in the absence of dissolved molecules. In this sense it is of interest to explore the effect of the phase transition on the kinetics of the quenching process. To this end the effect of temperature

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on the fluorescence of anthracene in the presence of the indoles in the lipid bilayer and on the associated exciplex emission was investigated. A comparison is made with POPC vesicles that in the temperature range investigated are in the fluid phase, since the phase transition temperature of POPC vesicles is −2 °C [16].

2. Materials and methods

2.1. Chemicals

Anthracene (ANT) from Aldrich (Milwaukee, WI, USA) was used without further purification. It was checked that their photophysical properties coincide with those reported in the literature. Indole (IN) and 1,2-dimethylindole (DMI) from Sigma Chem. Co. (St. Louis, MO, USA), were purified by recrystallization. Indole-3-acetic acid (IAA) from Aldrich and tryptophan (Trp) from Sigma Chem. Co. (St. Louis, MO, USA), were used as received. POPC and DMPC were from Avanti Polar Lipids (Alabaster, AL, USA). Aqueous solutions containing 0.01 M phosphate buffer pH 7.4 and 0.15 M NaCl were freshly prepared using Millipore Milli-Q (Millipore Corp., Bedford, MA, USA) purified water.

2.2. Vesicles preparation

The phospholipid was dissolved in chloroform–methanol 2:1 v/v at the desired concentration. The solution was evaporated to dryness under nitrogen, and kept under vacuum for 2 h to remove any residual solvent. The lipid film was hydrated by adding an appropriate volume of buffer and vigorous vortex stirring. The mixture was freeze–thawed five times employing liquid nitrogen and a 40 °C bath to ensure solute equilibration between trapped volume and bulk solutions [17]. The final phospholipid concentration was about 1 mM. Large unilamellar vesicles (LUV) were obtained by extrusion at room temperature of the multilamellar vesicles through 100 nm pore diameter polycarbonate filters in an extrusion apparatus from Avestin (Ottawa, Ont., Canada) [18]. Freshly prepared vesicles were used for all the experiments.

2.3. Incorporation of probes into vesicles

Anthracene, from a concentrated ethanolic solution, was co-dissolved with the phospholipid solution before evaporation under nitrogen. The final phospholipid:anthracene molar ratio was 100:1. Indole and DMI were added by injecting a few microliters of a concentrated ethanolic solution to 2–3 ml of the vesicles-containing solution. IAA and Trp were added from concentrated aqueous solutions.

2.4. Fluorescence measurements

Steady-state fluorescence spectra were measured with a Spex Fluoromax spectrofluorometer (Jobin Yvon-Spex, Edison, NJ, USA) in air-equilibrated solutions. The excitation wavelength was 280 nm for the indoles and 340 nm for anthracene. In all cases the fluorescence spectra were corrected by subtracting the residual fluorescence and light scattering of the medium. Lifetime measurements were performed by using the single photon counting technique with an Edinburgh OB900 apparatus (Edinburgh Instruments, Edinburg, UK). All experiments were performed by duplicate in air-equilibrated solutions. Temperature was controlled to ±0.1 °C by water circulation through the cell holder.

3. Results and discussion

3.1. Quenching of anthracene fluorescence in vesicles by indolic compounds

The emission spectrum of ANT suffers a slight blue shift with an increase of the polarity of the medium in homogeneous solvents. In the vesicular system the position of the maxima, the spectral bandwidth and the peak to valley ratio are very similar to those in an ethanol–water 1:2 mixture. The spectrum is practically the same for POPC and DMPC vesicles. The same spectrum is obtained when the sample is prepared at phospholipid:anthracene molar ratio 100:1 or 500:1, indicating that anthracene is fully incorporated into the membrane already at a molar ratio 100:1. In the absence of phospholipid, anthracene at the same analytical concentration as used in vesicular systems (10^{-5}– 10^{-6} M), yields unresolved absorption and emission spectra due to aggregation of the aromatic molecule. In DMPC vesicles the spectrum is practically the same in the gel (15 °C) and the liquid-crystalline (34 °C) phases. The fluorescence decay in air-equilibrated solutions is mono-exponential and insensitive to the phase transition of DMPC, with lifetime of 3.6 and 3.9 ns at 15 and 35 °C, respectively. In POPC LUV the fluorescence decay is also mono-exponential with a lifetime of 3.9 ns at 25 °C. The mono-exponential decay observed in all the systems investigated may be interpreted in the sense that anthracene molecules conform a nearly uniform spatial localization in the bilayer. Since ANT is a highly hydrophobic molecule this localization should be in the interior of the bilayer, however, due to the similar emission spectrum in the vesicles and in ethanol–water mixtures, the region where most of ANT resides is one of relatively high polarity, i.e. near the polar heads or in zone that can be reached by water molecules and polar quenchers. This is consistent with the quenching observed with Trp and IAA (vide infra).

Upon addition of the indolic compounds a quenching of the anthracene fluorescence is observed. Typical Stern–Volmer plots are shown in Figs. 1 and 2.

The SV plots for the quenching by DMI are linear in all cases and it may be concluded that all anthracene molecules are accessible to the quencher in the three systems. DMI is the most hydrophobic of the quencher investigated and probably solubilizes in the interior of the bilayer in the

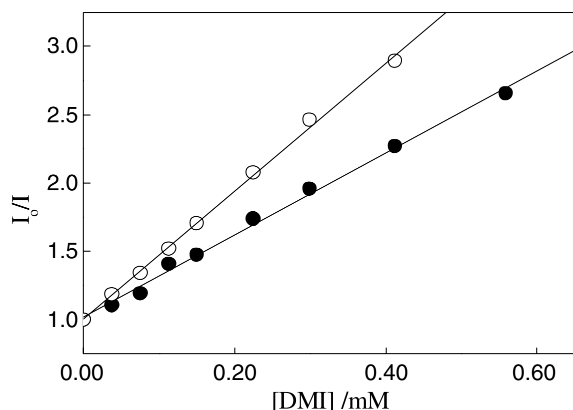

Fig. 1. Stern–Volmer plot for the quenching of ANT fluorescence by DMI in DMPC vesicles at 15 °C (●) and 35 °C (○).

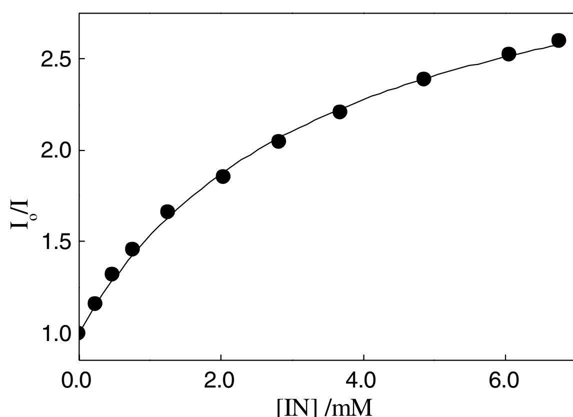

Fig. 2. Stern–Volmer plot for the quenching of ANT by IN in DMPC vesicles at 35 °C.

same region where the anthracene is located. In Fig. 2 it can be seen that IN is an order of magnitude less efficient than DMI and that the plot presents a negative deviation from linearity. Similarly, downward curvature was observed for the quenching by Trp and IAA. A possible interpretation of this downward curvature may be given in terms of a fraction of the anthracene molecules inaccessible to the quencher [19]. Therefore, in these cases the data may be analyzed in terms of a modified Stern–Volmer equation [15]:

$$\frac{I_0}{I} = \frac{1 + K_{SV}[\text{In}]}{1 + \chi K_{SV}[\text{In}]} \tag{1}$$

where [In] stands for the analytical concentration of indole. In Eq. (1) χ is the fraction of anthracene molecules inaccessible to the quencher and K_{SV} is the Stern–Volmer constant for the quenching of the accessible molecules. A non-linear least squares analysis of the data afforded the parameters collected in Table 1. For indole the fraction of ANT molecules inaccessible to the quencher is 0.25 in POPC and ca. 0.30 in DMPC. It is interesting that the last value is independent of the temperature. In part this may be due to changes in the phase transition temperature due to the presence of indole in the interior of the lipid bilayer.

Table 1
Stern–Volmer constants, K_{SV}, in POPC and DMPC vesicles and fraction of anthracene molecules, χ, inaccessible to the quencher

Quencher	POPC (20 °C)[a]		DMPC (15 °C)		DMPC (35 °C)	
	K_{SV} (M^{-1})	χ	K_{SV} (M^{-1})	χ	K_{SV} (M^{-1})	χ
DMI	3600	0	3000	0	4680	0
IN	505	0.25	896	0.35	974	0.30
Trp	87	0.47	190	0.35	160	0.38
IAA	80	0.51				

[a] From [15].

The existence of a fraction of ANT molecules inaccessible to the quenching by indole seems to be contradictory with the uniform spatial localization suggested by the fluorescence spectrum and lifetime. Nevertheless, a few nm in depth in the lipid bilayer may have dramatic effects on the quenching process, due to the quencher accessibility to the fluorophore and also to changes in the intrinsic quenching rate constant. These differences in localization do not necessarily reflect in the fluorescence parameters that are the result of an average over the whole population.

The K_{SV} is lower and χ is higher for tryptophan. This lower efficiency of the aminoacid as compared with the neutral quenchers is not due to a different intrinsic quenching ability, since in homogeneous media Trp is similar to DMI and IN for the quenching of anthracene derivatives. Therefore, the lower values of K_{SV} are featuring a different partition (i.e. lower local concentration) of tryptophan.

The indoles employed as quenchers may distribute between the lipid bilayer and the aqueous phase with partition coefficients that depend on the substituents on the indole moiety. In reverse micellar systems, DMI is totally dissolved in the organic phase [20], hence it is expected that in vesicles it will be localized preferentially in the hydrophobic region of the lipid bilayer.

The partition of indoles between the aqueous phase and the lipid bilayer was investigated by means of fluorescence emission spectroscopy. The fluorescence of the indole group is very sensitive to the polarity of the medium, while the absorption spectrum practically does not change [21]. The fluorescence spectrum of the quencher was recorded in the presence of varying concentrations of POPC at 25 °C and in DMPC at two temperatures, in the gel phase at 15 °C and in the liquid-crystalline phase at 34 °C. In Figs. 3 and 4 the emission spectra of IN and DMI, respectively, are shown as a function of DMPC concentration in the two phases. The emission maximum shifts to the blue in the presence of vesicles and it is between those observed in ethanol and in the buffer solution in the absence of vesicles. Besides, the width of the spectrum in the presence of the phopholipids is larger than in the pure solvents. From the spectra in Figs. 3 and 4 it is clear that the blue shift is more pronounced in the liquid-crystalline phase of DMPC.

The larger bandwidth in the emission spectra as compared with pure solvents points to a non-uniform distribution of IN and DMI molecules. Moreover, in homogeneous

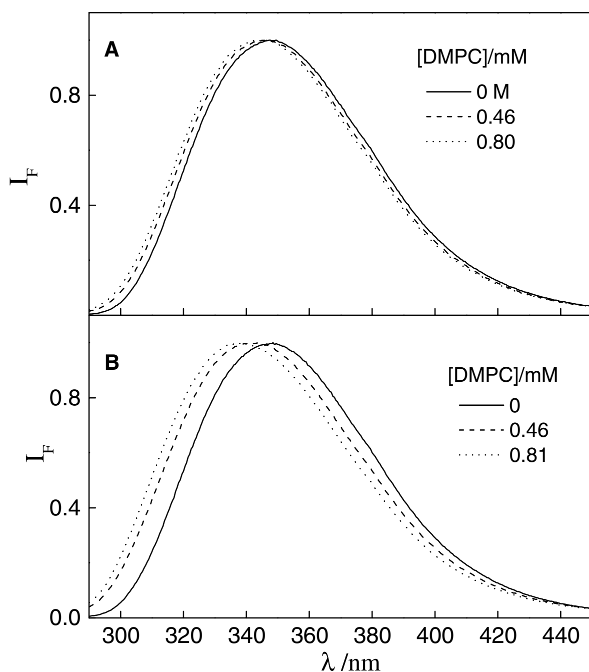

Fig. 3. Fluorescence emission spectra of IN in the presence of DMPC at 15.2 °C (A) and 34.1 °C (B). $[In] = 5.7 \times 10^{-5}$ M; $\lambda_{exc} = 280$ nm. Spectra were normalized at their respective maxima.

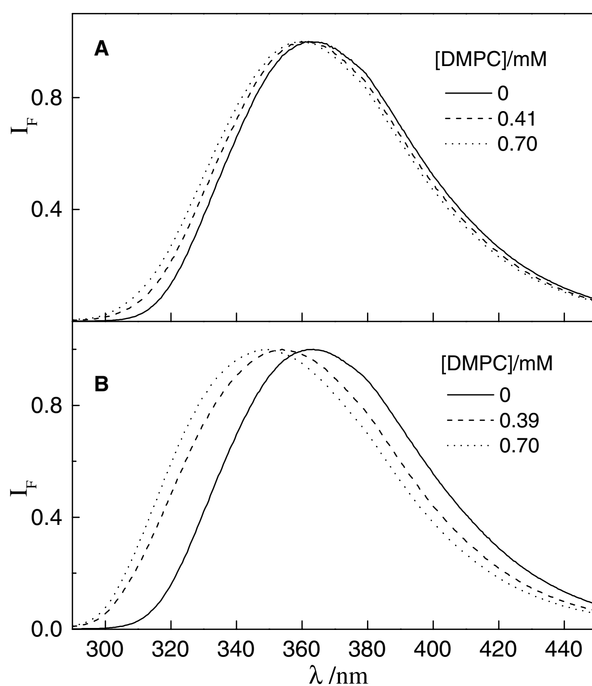

Fig. 4. Fluorescence emission spectra of DMI in the presence of DMPC at 15.1 °C (A) and 33.0 °C (B). $[DMI] = 9.4 \times 10^{-5}$ M; $\lambda_{exc} = 280$ nm. Spectra are normalized at their respective maxima.

solvents the fluorescence decay of the indolic compound may be fitted with a single exponential decay function. On the contrary, in the vesicles a lifetime distribution is required for the fitting. Indole itself presents a similar behavior to DMI in POPC and in DMPC in the liquid-crystalline phase, but in the gel phase the blue shift of the indole emission induced by the presence of DMPC is very minor. On the other hand, Trp and IAA practically do not interact with the membrane; their emission spectra remain unaltered in the presence of the vesicles.

From the changes in fluorescence intensity at a given wavelength as a function of phospholipid concentration, the partition constants of the indoles into lipid membranes can be determined as previously described [15,22]. These are collected in Table 2.

In order to discuss quantitatively the quenching by the different indolic compounds, rate constants must be evaluated in terms of the effective quencher concentration in the lipid bilayer, $[In]_b$. From the partition constants in Table 2 $[In]_b$ may be obtained by means of the following equation:

$$[In]_b = \frac{K_c[In]}{1 + K_c\gamma} \quad (2)$$

where [In] is the indole analytical concentration and γ is the volume fraction of the phospholipid bilayer, as given by the following equation:

$$\gamma = [P]\overline{V}_b \quad (3)$$

in terms of the phospholipid concentration $[P]$ and its molar volume $\overline{V}_b$. We used the same value of 0.7 L mol^{-1} for $\overline{V}_b$ of POPC and DMPC. The use of this value, which is typical for a phospholipid molecule, means that we are considering that the whole membrane volume is available for indole partitioning.

Bimolecular rate constants in the vesicles result from

$$k_q = K_{SV}^b \tau_0^{-1} \quad (4)$$

where K_{SV}^b is the Stern–Volmer constant recalculated with the effective concentrations for DMI and indole in the vesicle and τ_0 is the fluorescence lifetime of anthracene in the vesicles. For the quenching by Trp the rate constants were determined using the analytical concentration, since this quencher is assumed to remain in the aqueous phase, and its concentration at the bilayer interphase may be supposed to be the same as the bulk concentration. The corrected rate constants are collected in Table 3.

Table 2
Partition constants (K_c) of IN and DMI in LUVs of POPC and DMPC

	POPC 25 °C	DMPC	
		Gel phase	Liquid phase
IN	430 ± 70	–	500 ± 80
DMI	640 ± 80	270 ± 50	1360 ± 150

Table 3
Corrected bimolecular quenching rate constants in units of 10^9 M^{-1} s^{-1}

	POPC 25 °C	DMPC	
		Gel phase	Liquid phase
DMI	1.50	3.13	1.26
IN	0.50	1.0[a]	0.39

[a] Estimated value (see text).

For IN in DMPC at 15 °C (gel phase) the partition constant could not be determined with certainty, and the value of the rate constant was estimated assuming a factor with respect to DMI similar to that in the other two media. The values in the table show that the rate constant are lower than the value expected for a diffusion limited process in a medium of low viscosity, $5–20 \times 10^9\ M^{-1}\ s^{-1}$. This reflects the effect of a more rigid environment in the lipid bilayer. The rate constants have similar values in the three vesicular systems. Surprisingly, in the liquid-crystalline phase of DMPC the rate constants are lower than in the gel phase.

However, these values must be taken cautiously because in the presence of the quencher and the fluorophore the phase transition is not so clearly apparent as in the absence of them. The temperature dependence of the quenched fluorescence intensity does not show a clear inflection point in the temperature range 5–40 °C. Besides, the temperature dependence of the intrinsic quenching rate constant cannot be clearly disentangled from all the temperature dependent factors affecting the experimental value.

3.2. Anthracene–indole exciplex in vesicles

At the same time that the ANT emission is quenched a new band appears in the red, Figs. 5–7. This band is similar to that observed in the quenching of anthracene by indoles in homogenous solvents [11] and may be ascribed to an exciplex emission. Moreover, the excitation spectrum measured at 500 nm is the same as measured at 380 nm, confirming the exciplex nature of the red band. The exciplexes formed by anthracene derivatives and indolic compounds in low polarity solvents present a very large dipole moment, as evidenced by the dependence of the emission yield and position of the maximum with the solvent polarity [11]. In the presence of the phospholipid the exciplex emission, although weak, can clearly be seen with a concentration of the indolic compound very much lower than that required in homogeneous solvents. The exciplex

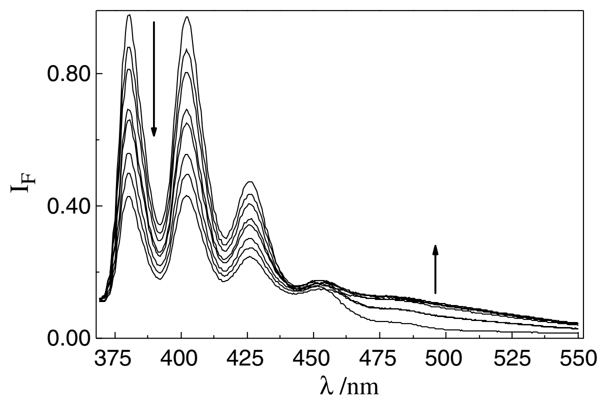

Fig. 5. Fluorescence quenching of anthracene by DMI in DMPC vesicles at 15.9 °C (gel phase). [DMPC] = 5×10^{-4} M; [POPC]/[Anthracene] = 100:1. [DMI] (analytical concentrations) = 0; 0.038; 0.075; 0.11; 0.15; 0.23; 0.30; 0.41 mM.

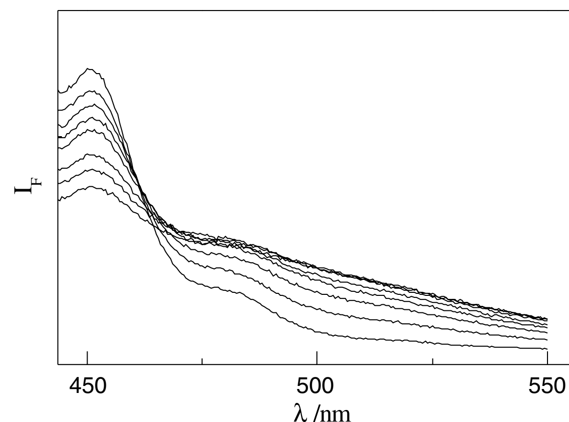

Fig. 6. Fluorescence quenching of anthracene by DMI in DMPC vesicles at 35 °C (liquid-crystalline phase). [DMPC] = 5×10^{-4} M; [POPC]/[Anthracene] = 100:1. [DMI] (analytical concentrations) = 0; 0.038; 0.075; 0.11; 0.15; 0.23; 0.30; 0.41 mM.

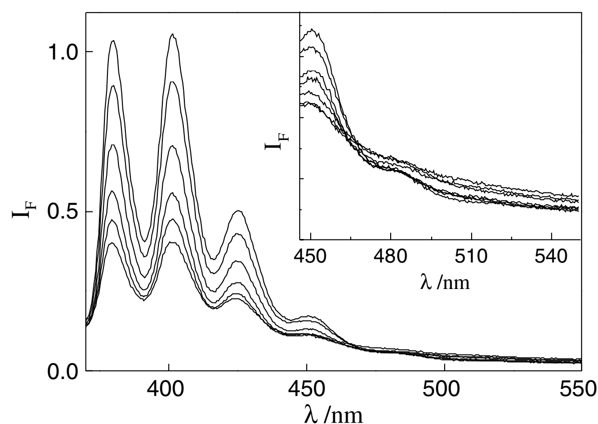

Fig. 7. Fluorescence quenching of anthracene by indole in DMPC vesicles (liquid-crystalline phase 35 °C) [DMPC] = 1.9×10^{-4} M, [IN] (analytical concentration) = 0; 0.241 0.768; 2.04; 3.68; 6.75 mM. λ_{exc} = 340 nm. Inset: enlargement of the exciplex emission region.

emission is clearly noticeable with DMI but it is very feeble when IN is used as quencher and it is absent with IAA or Trp.

It can be seen from Figs. 5 and 6 that the exciplex emission for the system ANT–DMI is more intense in the gel phase, in spite of the local concentration of DMI being lower. The exciplex emission is very weak in the case of IN, Fig. 7. This is most likely due to the fact that IN is a less efficient quencher of ANT.

In Fig. 8, the emission of the anthracene–DMI exciplex in the DMPC vesicles in the liquid-crystalline phase is shown. This spectrum was obtained by subtracting the local excited state emission from the total emission in the region of interest. The spectrum is practically indistinguishable from that taken at the gel phase. Moreover, it closely resembles the exciplex spectrum in POPC [15].

The exciplex emission for the system ANT–DMI in heptane was studied varying the quencher concentrations in a range similar to the local concentrations in the vesicular

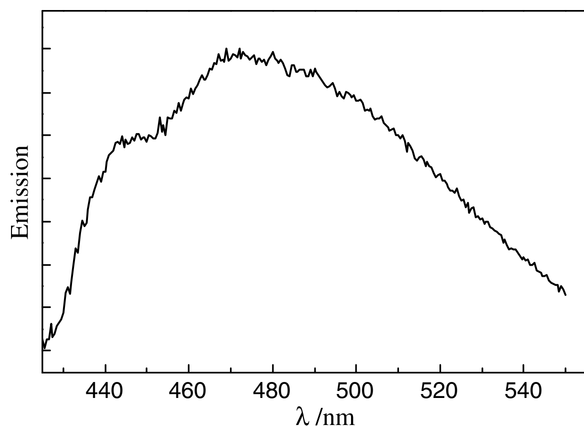

Fig. 8. Emission spectrum of the anthracene–DMI exciplex in DMPC vesicles, liquid-crystalline phase.

system. The maximum of the emission is now at 431 nm [15]. In Fig. 9A the Stern–Volmer plot I_0/I, where I_0 and I are the emission intensities of ANT at 374 nm in the absence and the presence of DMI, respectively, is presented. In Fig. 9B the plot of the exciplex emission relative to the quenched monomer I_{exc}/I_{mon} is shown, where I_{exc} and I_{mon} are the emission intensities at 431 and 374 nm respectively. The I_{exc} values were corrected for the change in the emission residual monomer emission at 431 nm. It can be seen that while the Stern–Volmer plot presents an upward curvature, a typical behavior at high quencher concentration, the plot of I_{exc}/I_{mon} vs. quencher concentration follows a linear behavior as expected for the normal exciplex mechanism for the quenching [23]. A similar plot for the ANT–DMI in DMPC presents a downward curvature, Fig. 10. In this case I_{exc} is measured 472 nm and I_{mon} is 380 nm, this plot is also corrected for the change in the

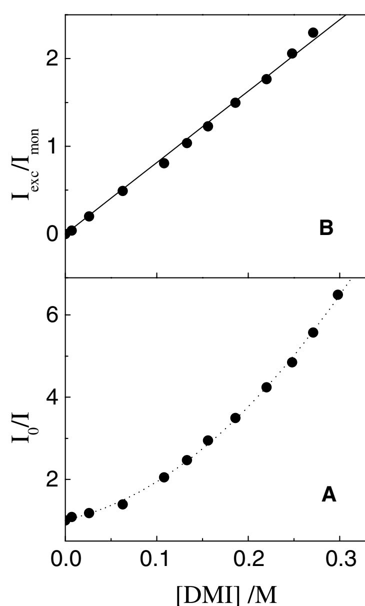

Fig. 9. Fluorescence quenching (A) and relative exciplex emission (B) for the system anthracene–DMI in *n*-heptane.

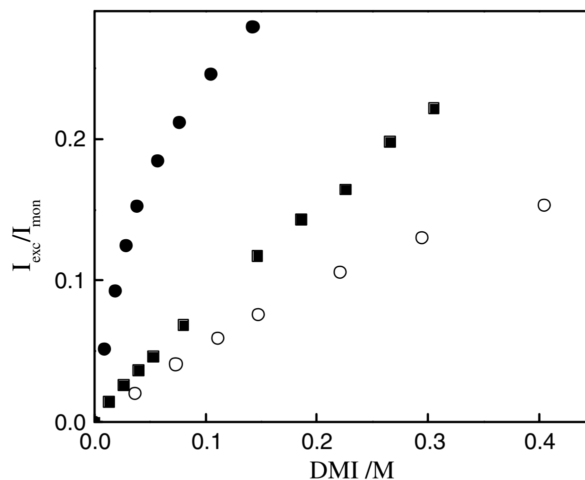

Fig. 10. Relative exciplex emission for the system anthracene–DMI in vesicles. (●) DMPC gel phase, (■) POPC, (○) DMPC liquid-crystalline phase. The DMI concentration is the effective concentration in the phospholipid bilayer calculated with the partition constant.

emission of I_{mon} with [DMI] and the concentration scale is expressed in terms of the local concentration of DMI in the lipid bilayer.

From a comparison of Figs. 9A and 10 it can be seen that for a DMI concentration in the bilayer similar to that in *n*-heptane, the exciplex emission is very much higher in the latter case. This is probably related to the polarity of the medium. In the vesicles the quenching and the exciplex formation is taking place in a relatively polar region, as may be inferred from the position of the emission [15]. The saturation type curves in Fig. 10 may be due to several factors: an exciplex quenching by DMI at high local concentrations, a saturation of the membrane with the quencher or a change in the micro-polarity and micro-viscosity of the bilayer at high quencher concentration. The first one is rather unlikely, since in a homogeneous solvent there is no evidence for the quenching of the exciplex emission by DMI at high concentrations as can be seen in Fig. 9. The local concentration of DMI in DMPC is similar to that in Fig. 9; therefore, it is unlikely that the downward curvature in Fig. 10 should be due to a quenching of the exciplex emission by DMI. Although the local concentration is relatively high, the saturation of the membrane might also be disregarded since the Stern–Volmer plots are linear, Fig. 1, up to a similar local concentration of the quencher as that employed in Fig. 10. Therefore, changes in the microenvironment of the exciplex at high DMI concentration remains as a possible explanation for the observed downward curvature. The more intense emission in the gel phase as compare with the liquid-crystalline phase in DMPC and POPC vesicles is another interesting point of the exciplex behavior in these systems. The interpretation of this effect is complicated by the simultaneous operation of changes in the fluidity of the membrane and the temperature effect on the exciplex emission intensity, as manifested in homogeneous media [24].

Acknowledgments

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