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The interaction of DAPI with phospholipid vesicles and micelles

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Abstract

The interactions of the dye 4′,6-diamidino-2-phenylindole (DAPI) with phospholipids ordered in single bilayer vesicles of dioleylphosphatidylserine (DOPS) or dimyristoylphosphatidylcholine (DMPC) or micelles of monomyristoylphosphatidylcholine (MPC) have been investigated. Somewhat unexpectedly, the binding of this dye to such ordered structures is not affected by the ionic strength of the external medium, which suggests an embedding of DAPI into the hydrocarbon phase. The fluorescence enhancement of DAPI bound can be accomodated within a model previously proposed for the behaviour of DAPI bound to proteins (Mazzini et al., *Biophys. Chem.* 42 (1992) 101). From both static and dynamic anisotropy measurements, bound DAPI results severely restricted in its rotational freedom but insensitive to the temperature dependent phase transition of the saturated DMPC vesicles. The considerable tightness and specificity of the interactions between DAPI and ordered phospholipids are also deduced from preliminary fluorescence quenching studies (reduced accessibility of iodide ions towards DAPI bound and quenching effects by the chaotrop Nonidet P-40).

Keywords: 4′,6-Diamidino-2-phenylindole (DAPI); Vesicles; Micelles; Fluorescence; Phospholipid–dye binding

1. Introduction

The fluorescent dye 4′,6-diamidino-2-phenylindole (DAPI, see Fig. 1) has almost exclusively been used as a probe for double stranded nucleic acids, and its interactions with DNAs of different base pair composition and sequence has been throroughly investigated [1]. In particular it has been shown that DAPI binds strongly to AT rich sequences in the minor groove of the double helix and with much lower affinity to GC rich regions by intercalation [2]. No interaction of DAPI apparently occurs with single or double stranded RNA, except with synthetic poly(AU) [3], poly(rI)

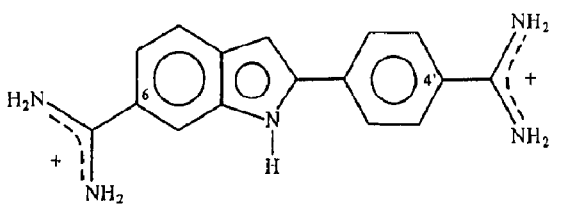

Fig. 1. Chemical structure of 4′,6-diamidino-2-phenyl-indole (DAPI) cation.

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[4] and tRNAs [5]. The dye has also been used in cytometry for quantitative determination of cellular DNA content [6] and as a probe in systems other than nucleic acids only in two specific cases, namely to follow microtubule assembly [7] and sarcoplasmic reticulum Ca^{2+} pump function [8].

The claimed specificity of DAPI towards nucleic acids has been recently questioned by the experimental finding that the dye is able to interact not only with proteins [9] but also with phospholipids (this paper).

These observations, if on one side may question the complete validity of studies performed on cellular DNA in the presence of other components, on the other may give rise to novel investigations on structural and functional aspects of proteins as well as membrane assemblies.

Whereas the investigations we have performed on other protein systems will be the subject of a next paper, here we describe the main results obtained from both static and dynamic fluorescence measurements of DAPI interacting with single bilayer vesicles of either dimyristoylphosphatidylcholine (DMPC) or dioleylphosphatidylserine (DOPS) as well as micelles of monomyristoylphosphatidylcholine (MPC). This choice has been dictated by the intention to see how the interaction of DAPI is modulated by the different chemical structure, electric charge and assembly (vesicles and micelles) of these phospholipids.

2. Materials and methods

4′,6-Diamidino-2-phenylindole (DAPI · 2HCl) has been purchased from Serva and used without further purification. DMPC, DOPS and MPC were obtained from Avanti Polar Lipids and chaotrop Nonidet P40 from Sigma. The quenchers used were acrylamide from Bio-Rad and KI from Merck. Mono- and bi-sodium phosphate salts were from Merck or C. Erba. All solutions were prepared using water from a Milli-RO plus Milli-Q purification system.

2.1. Preparation of vesicles and micelles

Vesicles of DOPS and DMPC were prepared according to the extrusion procedure [10]: briefly, a vortexed suspension of the phospholipid (1–2 mg/ml), in 10 mM pH 7 phosphate buffer, was filtered once through a 400 nm Nucleopore polycarbonate membrane (PCM) and three times through two superimposed 100 nm PCM filters. Vesicles were prepared at 33°C with DMPC and at room temperature with DOPS, well above the corresponding phase transition temperature of these phospholipids (23°C for DMPC, −11°C for DOPS [11]).

Micelles were prepared by simple mixing of MPC and phosphate buffer, above the phospholipid critical micellar concentration (150 μM).

2.2. Fluorescence titrations

Titrations have been performed by monitoring the fluorescence of DAPI, according to the progressive dilution method [12] using a thermostated Jasco model FP770 spectrofluorimeter, at several temperatures as follows:

1. at constant dye (4 μM) and variable vesicles (from 500 μM downwards) or micelles (from 4.8 mM downwards) concentrations ($\lambda_{ex} = 380$ nm, $\lambda_{em} = 400$–550 nm). The corresponding double reciprocal and Scatchard-like plots allow one to determine the ΔF_{max}, hence the quantum yield of ligand bound and k_d for the highest affinity site [13].

2. at constant vesicle (200 μM) or micelle (600 μM) and variable DAPI (25–200 μM) concentration ($\lambda_{ex} = 400$ nm, $\lambda_{em} = 400$–550 nm). In this case a longer excitation wavelength was chosen in order to minimize the inner filter effect due to the higher concentration of DAPI. The number of binding sites n of DAPI per phospholipid molecule was obtained from double reciprocal plots by extrapolation at infinite DAPI concentration, and accounting for the different absorbance at 400 nm with respect at 380 nm [13]. A more convenient parameter is $m = 1/n$, because it gives the minimum number of phospholipid molecules involved with the binding of each molecule of DAPI.

2.3. Fluorescence measurements

Steady state fluorescence measurements were carried out on a LS-50 Perkin-Elmer lumines-

cence spectrometer, equipped with a four position motor driven water thermostatted stirred cell holder.

Single photon counting measurements were obtained with an apparatus previously described [9]. Briefly the experimental set up was composed by a nitrogen flash lamp (199F Edimburg Instruments) pulsed at 20 kHz, a stop photomultiplier (Philips XP2020Q) and fast NIM electronics (Ortec, Silena, Tennelec). Data analysis was performed according to the global procedure [14].

The anisotropy of DAPI bound to the phospholipid structures has been determined exciting at 350 nm with emission at 450 nm, according to the following expression [15]:

$$A = \frac{I_{vv} - I_{vh}G}{I_{vv} + 2I_{vh}G} \tag{1}$$

where I is the fluorescence intensity, "v" and "h" refer to the vertical and horizontal orientation of the excitation and emission polarizers and the correction factor $G = I_{hv}/I_{hh}$.

The anisotropy $A(t)$ and total intensity $I(t)$ decays were obtained from the best fitting of the experimental $I_{vv}(t)$ and $I_{vh}(t)$ decays according to:

$$I_{vv}(t) = \tfrac{1}{3}I(t)[1 + 2A(t)] \tag{2}$$

$$I_{vh}(t) = \tfrac{1}{3}I(t)[1 - A(t)] \tag{3}$$

using the global procedure and assuming $I(t)$ and $A(t)$ to be each a sum of exponentials:

$$I(t) = \sum \alpha_i \, e^{-t/\tau_i} \tag{4}$$

$$A(t) = \sum A_i \, e^{-t/\phi_i} + A_\infty \tag{5}$$

where A_∞ is the anisotropy at infinite time, ϕ_i are the correlation times, τ_i are the fluorescence lifetimes and α_i and A_i are the pre-exponential factors. The anisotropy at zero time can be defined as:

$$A_0 = \sum A_i + A_\infty \tag{6}$$

The decay associated spectra, which represent the fluorescence spectra of each lifetime component, were obtained by combining time resolved and steady-state data as follows:

$$I_i(\lambda) = I(\lambda)\frac{\alpha_i(\lambda)\tau_i}{\sum \alpha_i(\lambda)\tau_i} \tag{7}$$

where $I_i(\lambda)$ and $I(\lambda)$ are the intensity of component i and the total corrected steady-state intensity at wavelength λ, respectively.

3. Results and discussion

3.1. DAPI-phospholipids interactions

The binding of DAPI to DOPS or DMPC vesicles and MPC micelles is characterized by a small bathochromic (red) shift (from 5 to 10 nm, depending on the phospholipid) in the absorbance spectrum of the dye. However, the fluorescence quantum yield of DAPI increases dramatically, from 0.035 up to nearly 1 with DOPS and MPC and up to 0.2 with DMPC, with a small hypsochromic (blue) shift, from 5 to 10 nm, again depending on phospholipid. Representative absorbance and fluorescence spectra are shown in Figs. 2a and 2b, respectively.

Titrations at constant DAPI and variable phospholipid concentrations have been performed at several temperatures between 15°C and 35°C (representative fluorescence spectra and Scatchard-like plot are shown in Figs. 3a and 3b). The pattern of the binding constants as a function of temperature are shown in Fig. 4, whereas the corresponding standard thermodynamic parameters, calculated at 20°C, are summarised in Table 1.

One can notice that in the case of vesicles the entropic term determines the negative sign of ΔG^0, ΔH^0 being positive, whereas the opposite is true with micelles. To rationalize these results appears difficult because both hydrophobic and electrostatic interactions may come into play in the formation of these complex systems.

From titrations at variable DAPI and constant phospholipid concentrations, allowing for inner filter effects due to DAPI and for scattering due to phospholipids, the lowest number m of or-

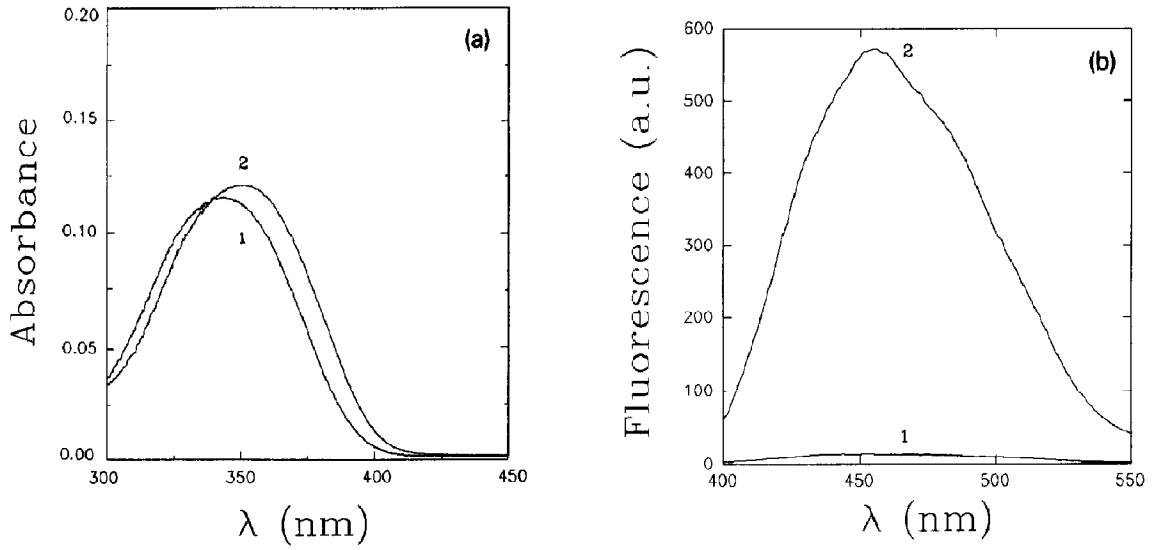

Fig. 2. Absorbance and fluorescense spectra of DAPI free and bound to MPC micelles. (a) Absorbance and (b) fluorescence spectra of 5.4 μM DAPI alone (1) and with 4.8 mM MPC (2), 10 mM phosphate buffer pH 7.0, 25°C. In (b) λ_{ex} = 380 nm.

dered phospholipid molecules involved in the binding of one molecule of DAPI was obtained: with DMPC vesicles m is close to 130, suggesting that about 130 molecules of DMPC structured in a vesicle appear to be involved with the binding of one molecule of DAPI. With DOPS vesicles m could not be determined due to precipitation of DAPI above 50 μM, whereas with MPC micelles m is close to 100. Since the estimated error on m is no more than 10%, as deduced from repeated measurements, one can relate the different values of this parameter with structural (compacteness) and dynamic (mobility) differences between vesicles and micelles, as seen by DAPI.

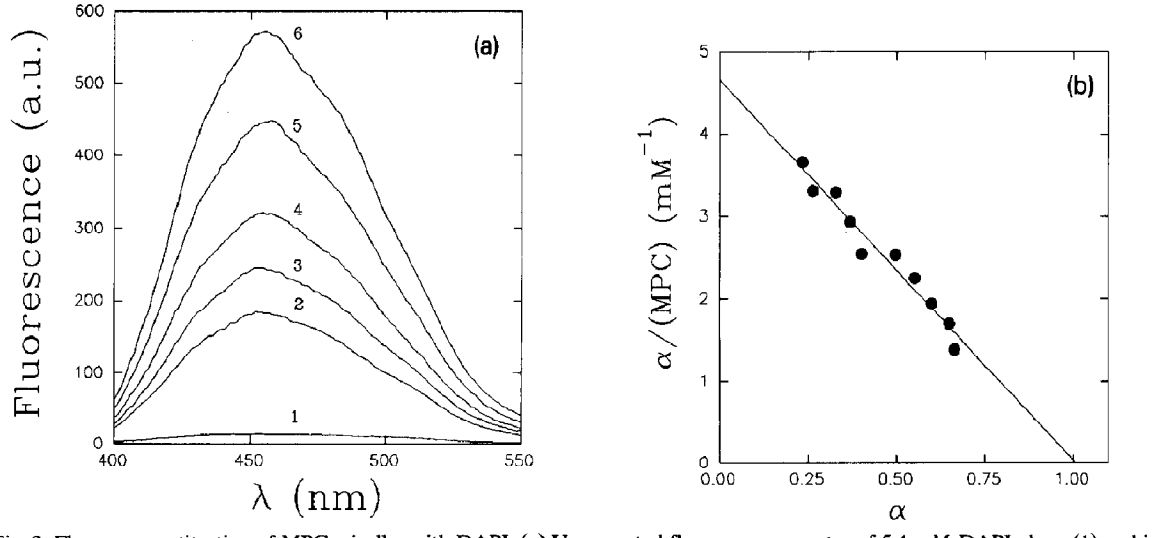

Fig. 3. Fluorescence titration of MPC micelles with DAPI. (a) Uncorrected fluorescence spectra of 5.4 μM DAPI alone (1) and in the presence of decreasing concentrations of micelles: (6) 4.8; (5) 3.08; (4) 1.96; (3) 1.58; (2) 1 mM. (b) Scatchard-like plot, as derived from the complete set of experimental points investigated in the titration, from 4.8 to 0.64 mM MPC at constant DAPI. Other conditions as in Fig. 2.

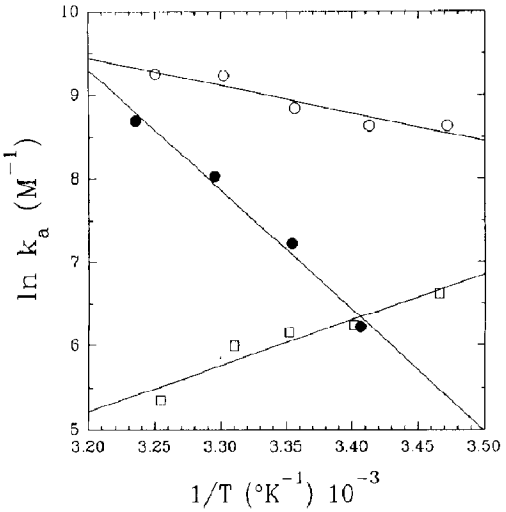

Fig. 4. Van 't Hoff plots of DAPI binding to phospholipid structures. The concentration of DAPI was kept constant at 4 μM whereas that of phospholipid was made variable during the titration. (○———○) DOPS vesicles, (●———●) DMPC vesicles, (□———□) MPC micelles). For other conditions see Section 2.

More specifically, considering DAPI as a planar molecule 15 Å long [16], the found values of m appear to be much larger than expected on purely sterical considerations. In fact, the average dimensions of a micelle (180 MPC molecules on a 20,000 Å^2 surface area [17]) are such that no more than two molecules of DAPI should bind on average to each micelle.

Considering a diameter of 80 Å, the micellar surface area per molecule of DAPI bound appears to be about 10,000 Å^2, or 50 times that for a freely rotating DAPI molecule. On a vesicle of 100 nm diameter the estimated number of phospholipid molecules is about 80,000 [18]. Considering that the ratio of lipid in the inner monolayer compared with lipid in the outer monolayer is 0.8 [18], and assuming that DAPI binds to the outer monolayer only, the estimated number of lipid molecules on the outer layer per molecule of DAPI bound is 70. Therefore, in the case of DMPC vesicles, considering an average area of 70 Å^2 per DMPC molecule, the lipid surface area involved with the binding of one molecule of DAPI appears to be 5,000 Å^2 or 25 times that of DAPI. The difference of a factor two between the surface area of micelles compared with that of vesicles, involved with the binding of one DAPI molecule, is difficult to explain, but a possibility is that it may be due to the higher mobility of the phospholipids in micelles with respect to that in vesicles. However, one should also consider that DAPI is a charged molecule bearing two positive charges and therefore that repulsive electrostatic effects between DAPI molecules on the surface should come into play, thus increasing the value of m. More speculatively, the binding of DAPI could also perturb the surrounding lipid environment so profoundly to hinder the binding of other dye molecules in the neighbourhood.

Table 1

Values of ΔG^0, ΔH^0 and ΔS^0 for the binding of DAPI to vesicles of DOPS or DMPC, and to micelles of MPC, calculated at 25°C from the data shown in Fig. 4

DAPI with	ΔG^0 (kJ/mol)	ΔH^0 (kJ/mol)	ΔS^0 (kJ/mol K)
DOPS	−22.1	+27.4	+0.17
DMPC	−17.5	+120	+0.46
MPC	−15.1	−45	−0.10

3.2. *Ionic strength effects*

In order to evaluate the effect of the electrostatic interactions on the binding between DAPI and ordered phospholipids structures, titrations at three different salt concentrations (0, 0.1 and 1 M NaCl) have been performed with each phospholipids in 10 mM phosphate at pH 7. In all cases investigated almost no appreciable variation but a small increase of the binding constant k_a has been detected. This result is somewhat amazing considering that DAPI is a charged molecule which presumably binds on or near the outer surface of the phospholipid structures. One possible explanation can be found considering a two step binding process: first DAPI is attracted electrostatically on the charged surface, then it rearranges its rings within the hydrophobic phospholipid tails, while retaining only its two amidino groups on the surface. This binding mode recalls that proposed for DAPI binding to AT clusters in DNA [19], according to which the dye is first electrostatically actracted by the phosphate groups and then rearranges into the minor groove

with formation of hydrogen bonds with A and T acceptor groups, the binding, however, remaining sensitive to the ionic strength [2]. A similar result has also been observed with the protein BSA [9], suggesting that electrostatic effects are predominant in that case too. In contrast here no effect or even a small increase of affinity of DAPI towards phospholipid structures in the presence of salt has been observed, which strongly suggests a large hydrophobic contribution that overwhelms the electrostatic one, presumably arising from the embedding of the aromatic moiety of DAPI into the hydrocarbon layer. It is interesting to note that a similar behaviour has been reported for the interaction of the hydrophobic ion tetraphenylphosphonium with egg phosphatidylcholine vesicles (namely one ionic molecule adsorbed per 100 lipids and no screening effect by salts) [20].

3.3. *Fluorescence decay associated spectra (DAS) of DAPI*

The DAS of DAPI bound to phospholipid vesicles (or micelles) have been determined in the 400–500 nm emission range exciting DAPI at 340 nm. The lifetime values are shown in Table 2, and the corresponding decay associated spectra are represented in Fig. 5.

A global analysis has been performed assuming either 2 or 3 lifetimes linked components plus a very short component to allow for scattering. As it appears from the χ^2 values in Table 2, two lifetime components are sufficient to account for the spectra, except with DMPC, where a third longer component (3.66 ns) appears to be necessary. In all cases, however, τ_2 is only marginally reduced in comparison with that of DAPI alone ($\tau_2 = 2.78$ ns), whereas τ_1 is increased twice with MPC, three times with DMPC and up to five times with DOPS ($\tau_1 = 0.16$ ns for DAPI alone). The presence of a third component with DMPC is difficult to explain, since it is not due to any impurity in the lipid sample.

When one compares the DAS of bound and free DAPI [1] the two spectral components appear to be inverted, namely the long lifetime component is shifted towards longer wavelength, whereas the short lifetime component towards

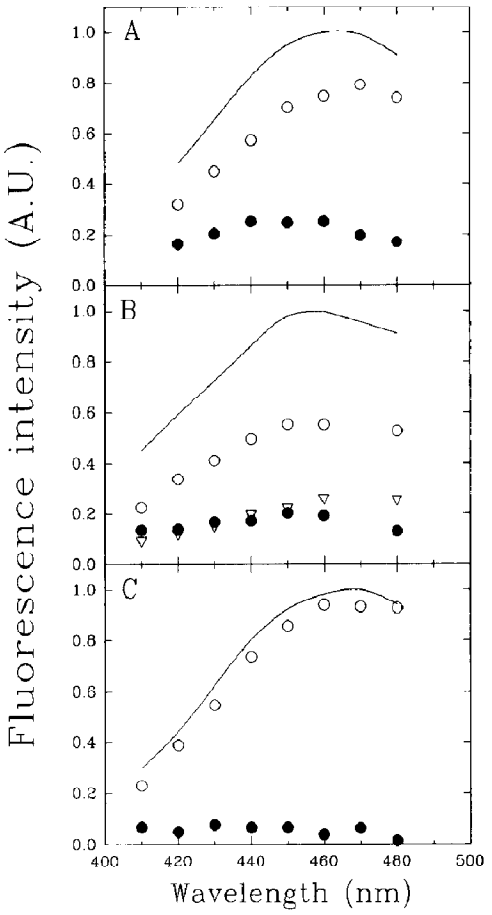

Fig. 5. Fluorescence decay associated spectra of DAPI bound to phospholipid structures. DAPI (4 μM) plus (A) DOPS (500 μM) vesicles, (B) DMPC (500 μM) vesicles and (C) MPC (3 mM) micelles. Excitation at 340 nm, $T = 20$°C.

Table 2

Fluorescence lifetime values of DAPI bound to vesicles (DOPS and DMPC) and micelles (MPC), as obtained from 2 or 3 exponential decay global analysis. For measurement conditions see Fig. 5

DAPI with	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	Global χ^2
DOPS	0.83	2.31	–	1.23
	0.83	2.56	2.21	1.27
DMPC	0.59	2.75	–	2.18
	0.45	2.25	3.66	1.16
MPC	0.34	2.74	–	1.25
	0.08	1.44	3.55	1.58

shorter wavelength in bound DAPI. This result should imply the involvement of both rotamers of DAPI free into the binding with phospholipids. As already proposed for the binding of DAPI to bovine serum albumin [9], the large increase of τ_1 with DOPS vesicles may be explained by a reduced probability of an intramolecular proton transfer from the 6-amidino group in the excited singlet state of bound DAPI, consequent to the breaking of the planar conformation of the 6-amidine group with respect to the indole ring.

3.4. Static anisotropy measurements

The static anisotropy value of DAPI in the presence of DMPC vesicles, obtained under stationary irradiation conditions, is well above that of DAPI alone; however in the range between 15 and 30°C the anisotropy decreases monotonically in both cases and with no change of slope in correspondence with the phospholipid phase transition (Fig. 6). This result implies that DAPI, though bound to vesicles, is not influenced by the phase transition of the fatty acids and therefore cannot be used as a probe of fluidity of the bilayer. With micelles of MPC the situation is similar except that the slope is slightly higher than for DAPI alone. This suggests a progressive displacement of the dye from the micelles as the temperature is increased, in agreement with a decrease of affinity resulting from titrations.

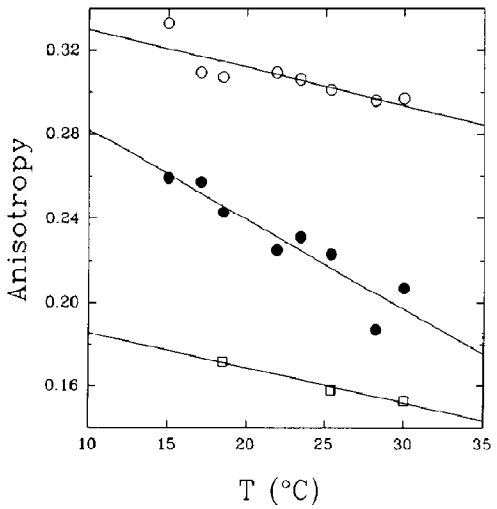

Fig. 6. Static anisotropy of DAPI as a function of temperature. (□——□) DAPI free, (●——●) DAPI bound to MPC micelles, (○——○) DAPI bound to DMPC vesicles. For conditions see Fig. 5 and Section 2.

Table 3

Time resolved anisotropy parameters of DAPI alone and in the presence of (DOPS or DMPC) vesicles or (MPC) micelles. For measurement conditions see Fig. 6

DAPI with	ϕ_1 (ns)	A_0	A_∞
–	0.14	0.12	–
DOPS	3.66	0.35	0.17
DMPC	1.55	0.39	0.18
MPC	3.18	0.34	0.03

3.5. Dynamic anisotropy measurements

The best fit values of the anisotropy parameters ϕ_1, A_0 and A_∞ are given in Table 3. They have been calculated with the global analysis by fitting the experimental data for the parallel and perpendicular decays to a single exponential decay:

$$A(t) = A_1 \, e^{-t/\phi_1} + A_\infty \qquad (8)$$

where $A_1 = A_0 - A_\infty$. A limiting value of anisotropy $A_\infty > 0$ is expected whenever restrictions to movement are present in a fluorophore [15] bound to a rigid or very slowly revolving matrix. In the case of our vesicles, given their very large dimensions, the corresponding rotational correlation times are far larger than the average lifetime of DAPI bound, therefore the found A_∞ value can be almost entirely ascribed to the restricted motion of DAPI bound. This result is not observed with micelles because of their small size and in fact $A_\infty \to 0$. The values of the rotational correlation time ϕ_1 clearly indicate that DAPI is bound to the phospholipid structures. However, the lower value of ϕ_1 obtained with DMPC is suggestive of a higher rotational freedom than in the other two cases, even if the allowed solid angle of rotation is apparently restricted. With DOPS vesicles both ϕ_1 and A_∞ values result to be relatively high, suggesting both tightness and hindrance to rotation of DAPI bound to these negatively charged phospholipid vesicles are important.

Table 4

Stern–Volmer (K_{SV}) and dynamic quenching (k_q) constants for DAPI (either alone or bound to phospholipids) in the presence of iodide ions. K_{SV} is in M^{-1} and k_q in $M^{-1}\ s^{-1}$ ($\times 10^{-9}$) ($k_q = K_{SV}/\bar{\tau}$, where $\bar{\tau} = \Sigma \alpha_i \tau_i$ is the average lifetime of DAPI measured in all cases at 460 nm). For measurement conditions see Fig. 7

DAPI with	K_{SV}	k_q
–	6.7	23.0
DOPS	0.9	0.56
DMPC	≈ 0	–
MPC	8.8	4.11

3.6. Quenching

In order to gain further insight into the binding of DAPI to phospholipids the accessibility of DAPI by the negative iodide ions and by the neutral acrylamide molecules has been preliminarly investigated. The corresponding Stern–Volmer plots are shown in Fig. 7. As it can be seen, non-linear behaviour has been observed in some cases, indicating that both dynamic and static quenching effects may be present. Stern–Volmer and dynamic quenching constants of DAPI alone and bound to phospholipids, referred to the quenching by iodide ions, are summarised in Table 4.

The effects of the two different quenchers are briefly commented as follows:

- Iodide ions (Fig. 7a): with DAPI free a considerable quenching is observed; the downwards curvature can be tentatively attributed to a conformational heterogeneity of this fluorophore. This interpretation, which agrees with a model with two different conformers previously proposed from fluorescence lifetime measurements [1], suggests a different accessibility of the two conformers by iodide ions. In the presence of micelles the ratio F_0/F increases linearly with the iodide concentration, indicating that the quenching process is essentially dynamic. Nevertheless the collisional quenching constant is reduced by at least a factor five in the presence of micelles. In contrast only a small quenching effect is observed with either vesicles, suggesting that DAPI bound to them is poorly accessible to iodide ions.
- Acrylamide (Fig. 7b): unexpectedly DAPI free is not quenched by acylamide, but rather a consistent enhancement of its fluorescence has been observed. This result can presumably be interpreted as due to the formation of a complex between free DAPI and acrylamide, which prevents the proton transfer process responsible for the low quantum yield of DAPI in aqueous solution. The situation is different in the presence of phospholipids: no appreciable quenching of DAPI is observed with either vesicles, whereas a small quenching is observed

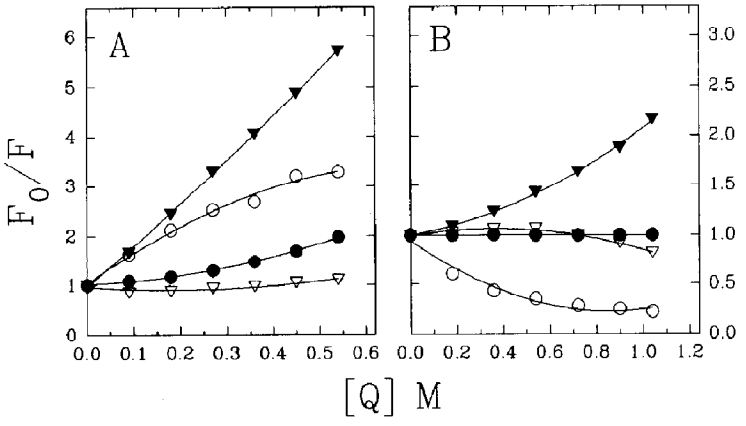

Fig. 7. Stern–Volmer plots for the quenching of DAPI. A: KI; B: Acrylamide. (○——○) DAPI alone, (●——●) with DOPS vesicles, (▽——▽) with DMPC vesicles, (▼——▼) with MPC micelles. Excitation at 380 nm, emission at 460 nm. Other conditions as in Fig. 5.

with MPC micelles. One can speculate that DAPI, when bound to phospholipids, is no more available to form a complex with acrylamide, and therefore no further enhancement, but also no quenching, of fluorescence is observed. However, it must be said that this case is difficult to rationalize, since acrylamide does not quench free DAPI.

The general conclusion, which can be drawn from these preliminary studies with these quenchers, is that the accessibility of DAPI is negligible when bound to vesicles and strongly reduced when bound to micelles with respect to DAPI free, this effect being clear when referred to iodide ions.

3.7. Effects of chaotropes

In order to assess whether or not the interaction of DAPI with DOPS, DMPC and MPC is dependent upon the ordered disposition of phospholipids in vesicles or micelles, the chaotropic agent Nonidet P-40 (polar but electrically neutral), has been added to these structures, and the fluorescence intensity of DAPI measured (Fig. 8). As the concentration of Nonidet P-40 increases the fluorescence intensity of the solution with DAPI alone also increases, whereas that of DAPI in the presence of vesicles or micelles decreases towards that of DAPI alone. In the case of DOPS vesicles this can be inferred by extrapolation to higher concentrations of chaotrope.

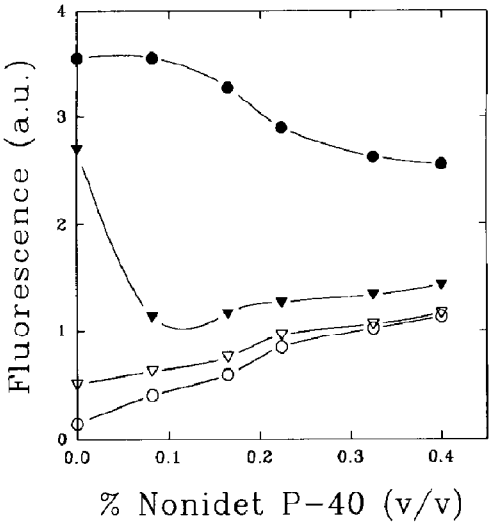

Fig. 8. Effect of chaotropes on the binding of DAPI to phospholipid structures. (○——○) DAPI alone, (●——●) with DOPS vesicles, (▽——▽) with DMPC vesicles, (▼——▼) with MPC micelles. Excitation at 380 nm and emission at 460 nm. Other conditions as in Fig. 5.

The increasing fluorescence observed with DAPI alone suggests that the dye interacts with Nonidet P-40, probably because this substance also forms micellar structures, whereas the progressive breakdown of the ordered phospholipid structures brought about by the increasing concentration of chaotrope, when vesicles or MPC micelles are originally present in solution, is responsible for the observed trend of fluorescence observed with DAPI alone.

The main conclusion that can be drawn from the use of this chaotrope seems to be that DAPI increases its fluorescence quantum yield very considerably by interaction with phospholipids only when these are organized in ordered layered structures.

4. Conclusions

The fluorescent dye 4′,6-diamidino-2-phenylindole (DAPI) has been and still is mainly used as a structural probe for assemblies containing double stranded nucleic acids. Recently, however, we have reported on specific binding of DAPI to a protein [9].

Further information on the apparently large specificity of DAPI is given in this paper, where we have shown that DAPI can also bind specifically to ordered phospholipid structures. The thermodynamic equilibrium parameters of the interaction between DAPI and unilamellar vesicles of either DOPS or DMPC as well as micelles of MPC have been measured by taking advantage of the large fluorescent enhancement of DAPI bound to these structures.

A surface area, equivalent to 70 phospholipids in a vesicle and to 130 in a micelle is apparently required for the binding of DAPI. Although DAPI cannot in principle be considered a probe for the interior of the bilayer, owing to its polarity and positive net charge, no appreciable effect of the ionic strength on the binding has been found, this fact implying a largely hydrophobic driving force. Support to this hypothesis also comes from the

reduced accessibility of DAPI bound by iodide ions. Results obtained with acrylamide are not easy to rationalize, considering that an increase, rather then a quenching, of fluorescence intensity has been observed with DAPI alone.

The mechanism of binding of DAPI to ordered phospholipids can be rationalised according to a model originally proposed to explain the photophysical behaviour of DAPI by itself, as deduced from fluorescence lifetime measurements [21], and also useful to explain the binding of DAPI to DNA [1] and proteins [9]. This model is based on the presence of two rotamers of DAPI with different lifetimes. The lifetime and percentage of the short component increases upon binding to macromolecular assemblies, whereas the longer lifetime remains approximately constant, thus accounting for the observed quantum yield increase of DAPI bound.

The importance of the ordered phospholipid structures for the specific interaction with DAPI has been evidentiated by the use of the neutral chaotropic agent Nonidet P-40, which progressively reduces the fluorescence of DAPI bound to that of DAPI free.

As a final remark, we would like to point out that DAPI can no more be considered a probe specific for dsDNA, since we have previously shown that it is able to bind to proteins [9] and here also to phospholipid ordered structures. Therefore our observations, while recommending a careful use of DAPI with complex biological samples, allow one to expand the field of investigation with this dye.

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