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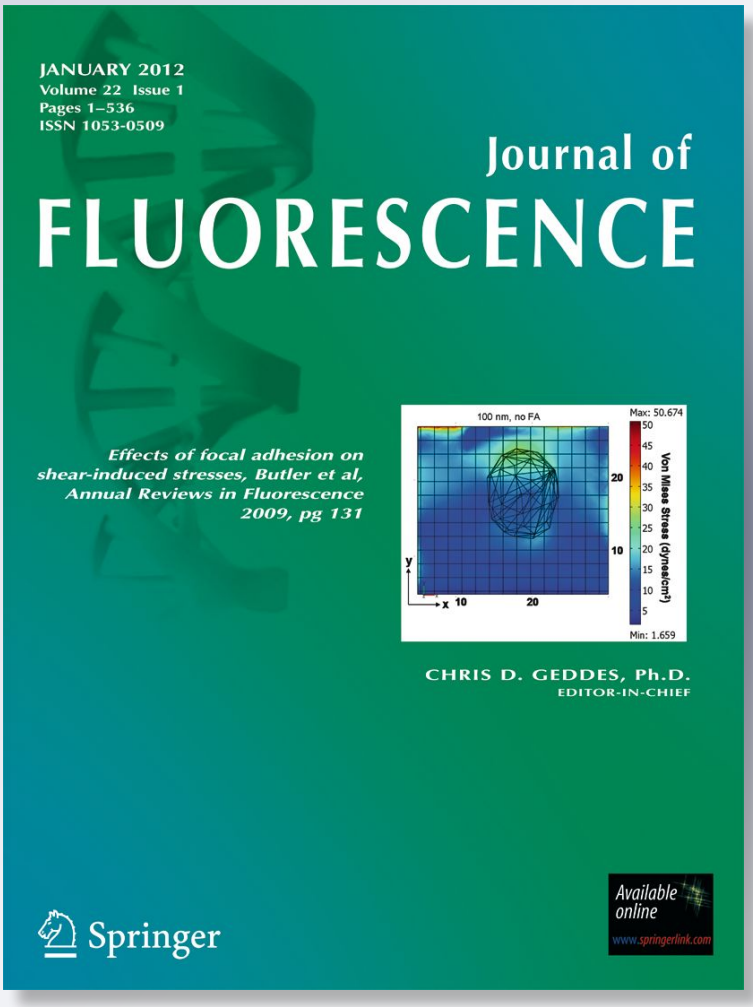

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ORIGINAL PAPER

Novel Benzanthrone Aminoderivatives for Membrane Studies

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Abstract The potential of novel benzanthrone aminoderivatives to trace the changes in physicochemical properties of lipid bilayer has been evaluated. Binding of the dyes to the lipid bilayers composed of zwitterionic phospholipid phosphatidylcholine (PC) and its mixtures with anionic phospholipid cardiolipin (CL) and cholesterol (Chol) was followed by significant quantum yield increase with small blue shift of emission maximum. Analysis of partition coefficients of the dyes under study showed that all aminobenzanthrones possess high lipid-associating ability. The dyes A8 and AM2 proved to be sensitive to the variations in membrane chemical composition responding to the changes in bilayer hydration induced by CL and Chol.

Keywords Aminobenzanthrone dyes · Lipid membranes · Bilayer hydration · REES

Introduction

The unique photophysical properties of aminobenzanthrones have resulted in their extensive use as disperse dyes for textiles, polymers, daylight fluorescent pigments and laser dyes [1, 2]. Despite technological utilization of these compounds is continuously growing, their applicability as fluorescent probes in biological assays so far remains scantily evaluated. Meanwhile, spectral characteristics of aminobenzanthrones satisfy all the requirements for an ideal fluorescent tracer. Bright fluorescence, high extinction coefficient, photo-, thermo- and chemical stability, and reduced background signal make benzanthrone dyes particularly attractive as bioimaging agents. The main advantageous photophysical characteristic of aminobenzanthrones is their ability to form intramolecular charge transfer (ICT) state [3]. The appearance of ICT state is controlled by electron-donating and -accepting properties of the fluorophore functional groups. Accordingly, charge transfer from amine substituent to carbonyl group results in significant increase of the dye dipole moment after excitation. This, in turn, induces the reorientation of solvent dipoles around the excited-state dipole of a probe and energy loss which manifests itself in a red shift of emission band upon increasing microenvironmental polarity [4].

It has been shown previously that one representative of aminobenzanthrones, ABM, exhibits high affinity for model and biological membranes [5–7]. Specifically, it was found that spectral characteristics of this probe correlate with a number of important parameters of artificial and cellular membranes such as physicochemical state, microviscosity, proliferating and lipid metabolic activities of cells, distribution of lymphoid subsets, etc. Furthermore, our recent studies revealed that ABM is highly sensitive to the conformational and aggregation state of proteins [8]. Cumulative data from the binding, resonance energy transfer and red-edge excitation shift (REES) studies allowed us not only to distinguish between native monomeric lysozyme and its fibrillar aggregates, but also to characterize the protein oligomeric species in terms of their fractal-like dimensions and polarity of the dye microenvironment. All the above findings inspired us to extend the

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evaluation of aminobenzanthrone performance in biological media and concentrate our efforts on evaluating the ability of five novel dyes (referred to here as A4, A8, AM2, AM3 and AM4) to monitor the properties of lipid bilayers. Model lipid membranes were composed of zwitterionic lipid phosphatidylcholine (PC) and its mixtures with cholesterol (Chol) and anionic lipid cardiolipin (CL) in different molar ratios.

Experimental

Materials

Egg yolk phosphatidylcholine and beef heart cardiolipin were purchased from Biolek (Kharkov, Ukraine). Both phospholipids gave single spots by thin layer chromatography in the solvent system chloroform:methanol:acetic acid:water, 25:15:4:2, v/v. Cholesterol was from Sigma. Aminobenzanthrone dyes A4, A8, AM2, AM3 and AM4 were synthesized at the Faculty of Natural Sciences and Mathematics of Daugavpils University as described previously [9, 10]. All other chemicals were of analytical grade and used without further purification.

Preparation of Lipid Vesicles

Unilamellar lipid vesicles composed of neat PC and PC mixtures with a) 5 or 10 mol% of CL and b) 30 mol% of Chol were prepared by the extrusion method [11]. The thin lipid films were obtained by evaporation of lipids' ethanol solutions and then hydrated with 1.2 ml of 5 mM Na-phosphate buffer (pH 7.4). Lipid suspension was extruded through a 100 nm pore size polycarbonate filter. The phospholipid concentration was determined according to the procedure of Bartlett [12]. Hereafter, liposomes containing 30 mol% Chol or 5 or 10 mol% CL will be referred to as Chol30, CL5 and CL10, respectively.

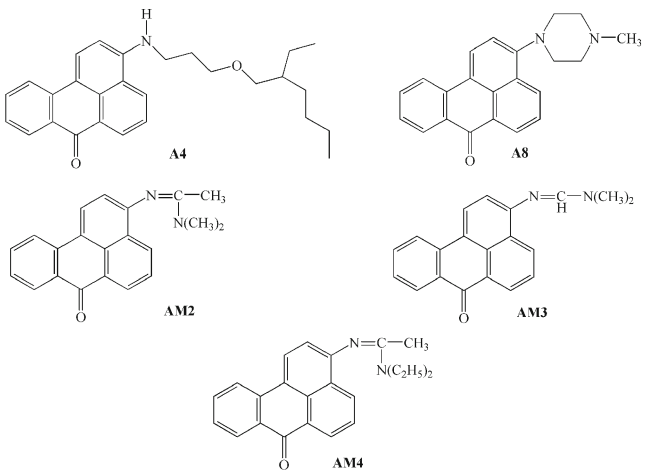

Fig. 1 Chemical structures of examined aminobenzanthrone dyes

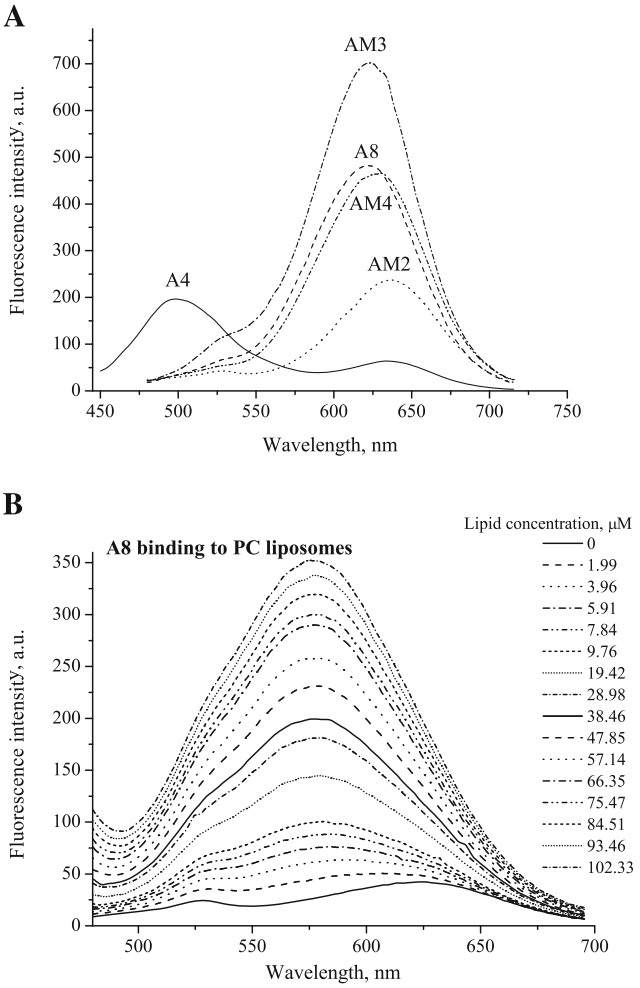

Fig. 2 Fluorescence spectra of aminobenzanthrones in ethanol (**a**). Typical emission spectra of benzanthrone aminoderivatives in lipid bilayer (**b**). Dye concentration was 1.6 μM

Fluorescence Measurements

Fluorescence measurements were performed at 20 °C with LS-55 spectrofluorimeter equipped with magnetically stirred, thermostated cuvette holder (Perkin-Elmer Ltd., Beaconsfield, UK) using quartz cuvettes with a path length of 10 mm. Excitation wavelengths were 450 nm for A4 and A8, and 460 nm for AM2, AM3 and AM4. Excitation and emission

Table 1 Quantum yield of aminobenzanthrones in different media

System Dye	Buffer	Ethanol	PC	CL5	CL10	Chol30
A4	0.02	0.1	0.1	0.092	0.089	0.23
A8	0.05	0.25	0.6	0.5	0.48	0.49
AM2	0.007	0.13	0.07	0.045	0.07	0.055
AM3	0.016	0.25	0.14	0.11	0.15	0.164
AM4	0.021	0.2	0.078	0.12	0.108	0.128

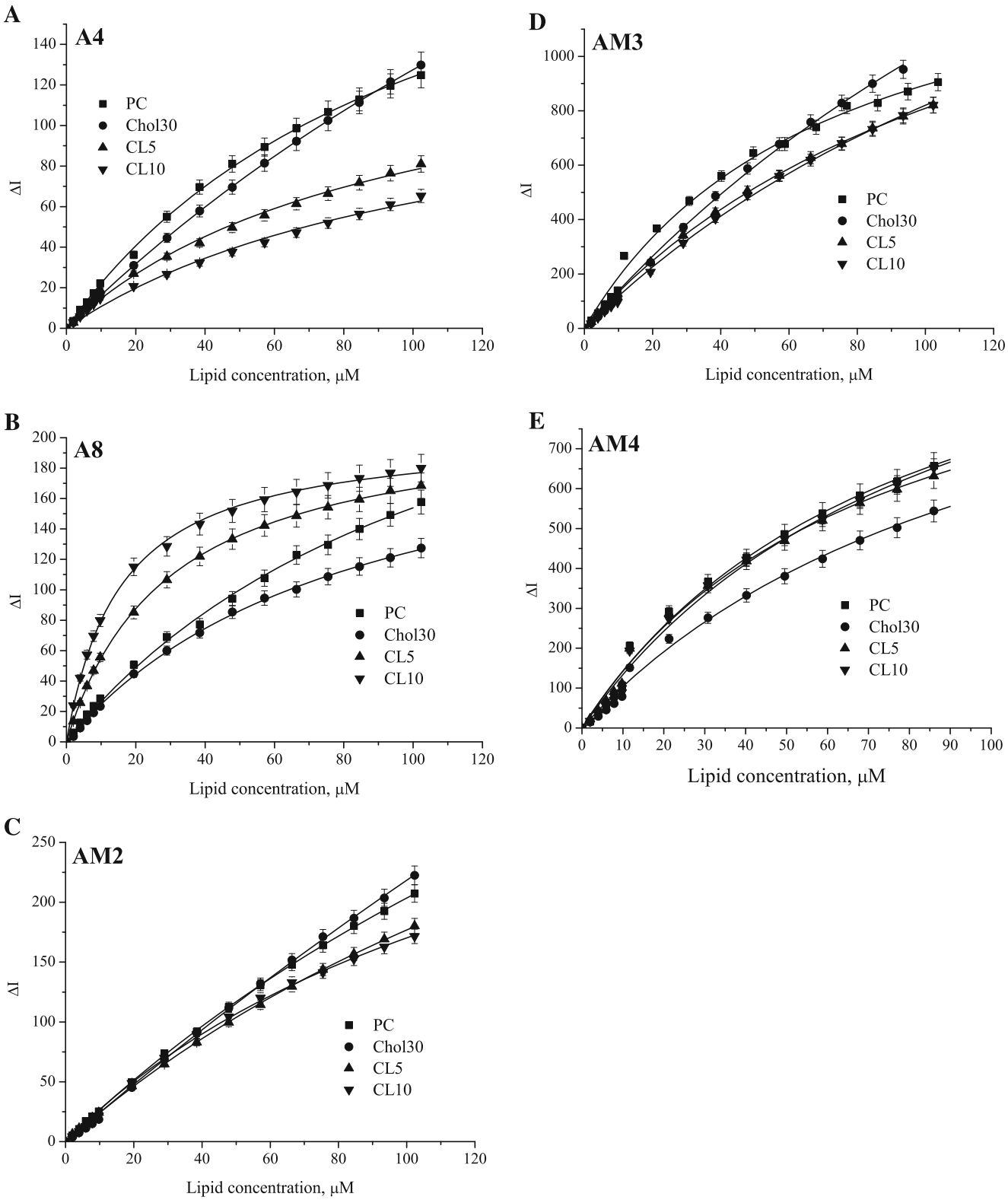

Fig. 3 Isotherms of aminobenzanthrone binding to model lipid membranes. Dye concentration was 1.0 μM (**a**), 1.6 μM (**b**), 2.0 μM (c), 3.5 μM (**d**), 1.2 μM (**e**)

slit widths were set at 10 nm. The concentrations of the dyes were determined spectrophotometrically, using extinction coefficients 1.5×10^4, 8.5×10^3, 1.2×10^4, 1.3×10^4 and $1.4\times10^4\ M^{-1}cm^{-1}$ for A4, A8, AM2, AM3 and AM4, respectively.

Quantum Yield Determination

The quantum yields of benzanthrone dyes (Q_{dye}) were estimated using Rhodamine 101 as standard. The integral fluorescence intensity (I_F) is known to depend on the sample absorbance at excitation wavelength (A) in the following manner:

$$I_F = KQ(I_0 - I_{tr}) = I_0KQ(1 - T) = I_0KQ(1 - 10^{-A}) \quad (1)$$

where I_0 is the intensity of excitation light, I_{tr} is the intensity of the light that has passed through the sample, Q is the fluorophore quantum yield, T is the transmittance, K is device-dependent coefficient of proportionality. Based on the relationship (1), the equation for quantum yield determination has been written in its most strict form:

$$Q_{dye} = \frac{Q_{R101}\left(1 - 10^{-A_{R101}}\right)S_{dye}}{\left(1 - 10^{-A_{dye}}\right)S_{R101}} \quad (2)$$

where A_{R101} and A_{dye} stand for the absorbance at the excitation wavelength of Rhodamine 101 and aminobenzanthrone dye, respectively, S_{R101} and S_{dye} are the integrated areas of fluorescence spectra of Rhodamine 101 and aminobenzanthrones, respectively.

Partition Model

When the probe binds to lipid vesicles its total concentration in the sample (Z_{tot}) can be represented as:

$$Z_{tot} = Z_f + Z_L \quad (3)$$

where Z_f stands for the probe concentration free in bulk solution, Z_L is the concentration of the dye incorporated into lipid bilayer. The coefficients of dye partitioning into lipid phase (K_p) can be written as [13]:

$$K_P = \frac{Z_L V_w}{Z_f V_L} \quad (4)$$

here V_w, V_L are the volumes of aqueous and lipid phases, respectively. Given that under the employed experimental conditions the volumes of lipid phase is much less than the total volume of the system V_t, we assume that $V_w \approx V_t = 1$ dm^3. The volume of lipid phase was calculated as:

$$V_L = N_A C_L \sum v_i f_i \quad (5)$$

where C_L is the molar lipid concentration, f_i is mole fraction of the i-th bilayer constituent, v_i is its molecular volume taken as 1.58 nm^3, 3 nm^3 and 0.74 nm^3 for PC, CL and Chol, respectively [14]. For cholesterol-containing systems condensing effect of this lipid was taken into account, so that the above v value for PC was reduced by the factor 1.3 [14]. The relationship between K_p and fluorescence intensity increase (ΔI) can be written as [13]:

$$\Delta I = I_L - I_W = \frac{K_p V_L (I_{\max} - I_W)}{1 + K_p V_L} \quad (6)$$

where I_L is the fluorescence intensity observed in the liposome suspension at a certain lipid concentration C_L, I_W is the probe fluorescence intensity in buffer, $I_{\max}$ is the limit fluorescence in the lipidic environment.

Results

Photophysical Properties of Aminobenzanthrones

As seen in Fig. 1, the dyes under study are asymmetrical aminobenzanthrone derivatives. The dyes proved to be nearly non-emissive in buffer solution but exhibited strong fluorescence in ethanol with emission maxima around 500 nm for A4 and 625–635 nm for other dyes (Fig. 2a). The corresponding quantum yields of the dyes are presented in Table 1.

Binding of Aminobenzanthrone Dyes to Model Lipid Membranes

Binding of the examined dyes to the model lipid membranes was followed by the increase in fluorescence intensity (Fig. 2b) and quantum yield (Table 1) with small blue shift (~5 nm) of the emission maximum. The enhancement of the probe fluorescence in liposomal suspension can be explained by two main factors: (i) the dye transfer to a membrane environment of reduced polarity, and (ii) immobilization of the probe molecules within the lipid bilayer resulting in strongly hindered fluorophore rotation. To derive the dye partition coefficients for different lipid systems, experimental dependencies $\Delta I(C_L)$ presented in Fig. 3, were approximated by Eq. (6). The values of K_p were found to fall in the range

Table 2 Parametrs of aminobenzanthrone partitioning into lipid systems

Dye	Parameter	PC	Chol30	CL5	CL10
A4	K_p,×10^4	0.99±0.04	0.91±0.01	1.08±0.11	0.89±0.13
	$I_{\max}$	255±7	519±12	150±9	132±13
A8	K_p,×10^4	0.9±0.07	1.7±0.07	3.4±0.05	6.2±1.6
	$I_{\max}$	333±16	229±5	214±1	204±1.5
AM2	K_p,×10^4	0.36±0.12	0.47±0.04	0.53±0.11	0.66±0.05
	$I_{\max}$	784±21	212±4	578±17	419±22
AM3	K_p,×10^4	0.81±0.15	0.84±0.07	0.78±0.01	0.75±0.04
	$I_{\max}$	1536±81	3492±354	1858±125	2397±138
AM4	K_p,×10^4	1.3±0.174	0.97±0.12	1.4±0.15	1.1±0.17
	$I_{\max}$	1264±91	1219±100	1158±63	1329±112

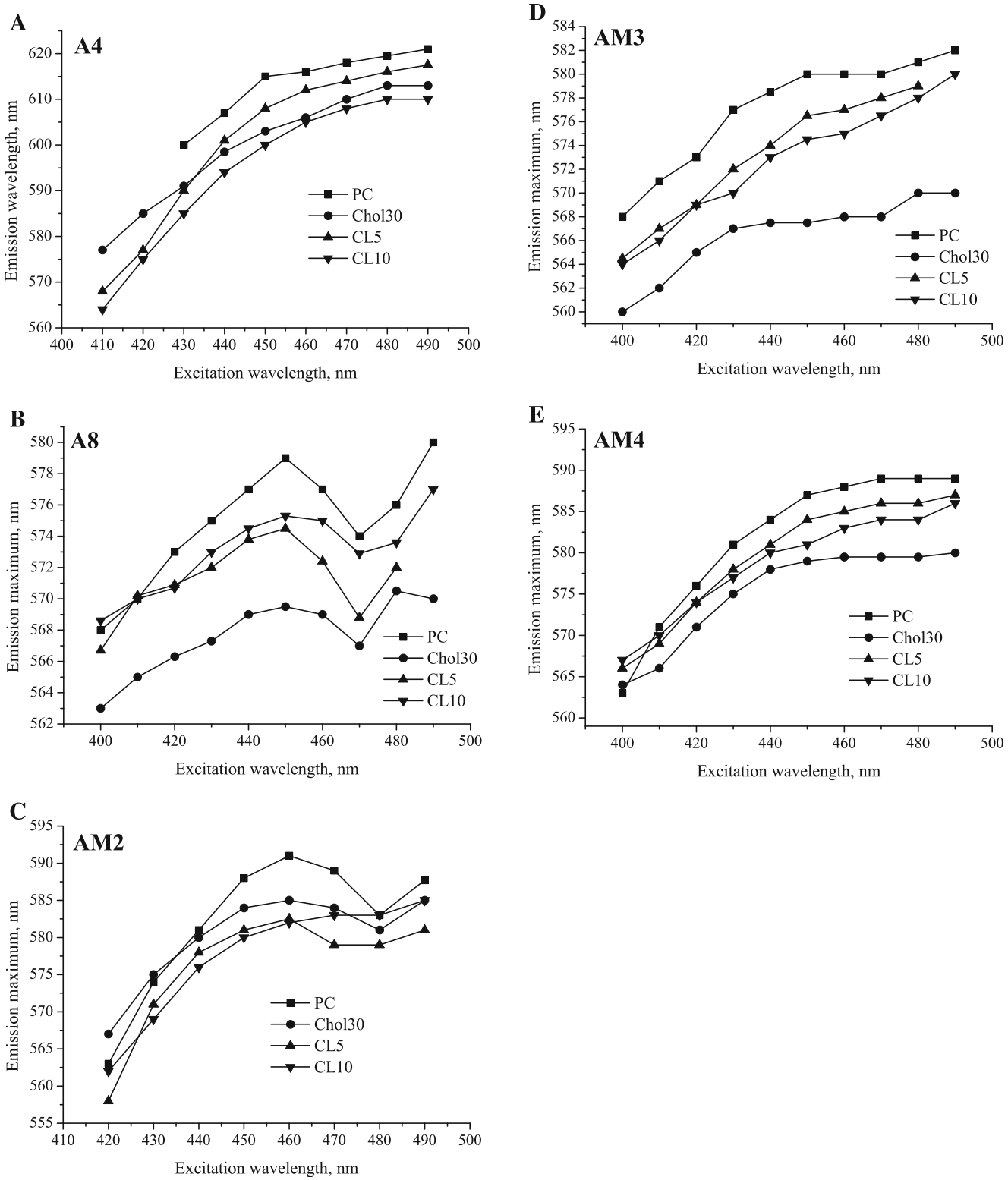

Fig. 4 REES dependencies for benzanthrone aminoderivatives in different lipid systems. Dye concentration was 0.9 μM (**a**), 1.5 μM (**b**), 1.8 μM (**c**), 3.2 μM (**d**), 1.1 μM (**e**). Lipid concentration was 102 μM

$(0.4–6.2)\times10^4$ indicating that the examined dyes possess high lipid-associating ability. As seen from Table 2, inclusion of Chol or CL into PC bilayer didn't influence the partition coefficients of A4, AM3 and AM4. On the contrary, increase of K_p value compared to neat PC bilayers was revealed for A8 and AM2 in Chol- and CL-containing membranes.

At the next step of the study we address ourselves to the solvent relaxation phenomenon which in steady-state fluorescence experiments can be traced by measuring the red shift of emission maximum with increasing excitation wavelength (REES effect) [15]. As shown in Fig. 4, all dyes under examination exhibited REES, with the magnitude of emission shift being dependent on the probe structure. The most pronounced REES was observed for A4, while the smallest emission shift was revealed for A8. The estimation of REES values allowed us to range the dyes according to the depth of bilayer location. As follows from the "dipstick" rule, the magnitude of REES is directly correlated with the depth of probe penetration in membrane interior – the less the REES, the deeper probe locates. Examination of REES in our systems showed that A8 exhibits the deepest bilayer penetration, while A4 prefers the shallowest membrane location.

The last step of the work was directed towards the estimation of fluorescence anisotropy of aminobenzanthrones in different membrane systems. Anisotropy of membrane-bound probe is determined by the rate of its rotational diffusion, which, in turn, depends on the free volume of the dye microenvironment [16]. As seen from Table 3, anisotropy values of aminobenzanthrones are virtually independent of the membrane composition. Allowing for condensing effect of Chol and CL on the structure of PC bilayer [14, 17], we concluded that aminobenzanthrone dyes are insensitive to the changes in lipid chain order.

Discussion

The photophysics of aminobenzanthrone dyes is based mainly on the intramolecular charge transfer (ICT) phenomenon consisting in electron transfer from electron-donating substituents at 3-position to electron-withdrawing carbonyl group of chromophore system upon excitation. The concomitant electronic polarization accounts for the dye absorption in visible region. In the excited state the proton-acceptor ability and the energy of H-bonds of aminobenzanthrone dyes are greater than in the ground state. As a result, the energy of $\pi\pi^*$-level becomes the lowest one, and aminobenzanthrones begin to fluoresce [18]. The major advantageous property of these dyes is their high sensitivity to microenvironmental polarity. In low-polar solvents aminobenzanthrones are characterized by so-called locally excited (LE) state in which dipole-dipole interactions between the dye and solvent molecules are absent [19]. When polarity increases, solvent dipoles tend to reorganize their configuration, thereby promoting the switch between LE and ICT states and long-wavelength shift of emission band. Extremely high sensitivity of this kind of fluorophores to milieu conditions provoked extensive utilization of ICT dyes as 'polarity probes' in biophysical and biochemical research, especially in membrane studies. In the present paper, the potential of novel benzanthrone aminoderivatives (A4, A8, AM2, AM3 and AM4) to trace the physicochemical properties of lipid bilayers has been evaluated. These dyes exhibited weak structureless emission in aqueous solution, but their association with the model membranes composed of PC and its mixtures with Chol or CL resulted in significant increase of fluorescence intensity and small blue shift of the spectral maximum (Fig. 2b). Quantitative analysis of the dye partitioning into different lipid systems showed that A4, AM3 and AM4 are insensitive to the variations in membrane composition as can be judged from the invariance of K_p values (Table 2). On the contrary, the binding of A8 and AM2 to Chol- and CL-containing membranes resulted in K_p increase compared to neat PC bilayers. This effect cannot be explained by electrostatic dye-lipid interactions because molecules of aminobenzanthrones are uncharged. In an attempt to explain the observed K_p changes, it seemed reasonable to consider possible bilayer location of the above probes. According to the fundamental works of Chattopadhyay et al. [20, 21], the anisotropy of membrane properties along the normal to its plane permits a rough division of lipid bilayer into three regions: i) bulk aqueous phase characterized by fast solvent relaxation, ii) highly anisotropic polar region of lipid bilayer where solvent relaxation is restricted and slow, and iii) isotropic hydrophobic part of membrane with fast solvent relaxation. Among these, membrane hydrophilic part is recognized as possessing typical features of environment favorable for manifestation of red edge effects. Since all aminobenzanthrones under study exhibited REES (Fig. 4), we supposed that these dyes reside in the polar region of lipid bilayer. This assumption is also corroborated by the finding that inclusion of CL or Chol into PC bilayer did not cause any statistically significant changes of the dye anisotropy. Allowing for the reported ability of CL and Chol to affect the ordering of lipid acyl chains [14, 17], it seems probable that insensitivity of the anisotropy of aminobenzanthrones to bilayer composition is associated with the location of these probes in the membrane polar region. Therefore, while attempting to interpret the increase of A8 and AM2 partition coefficients we should search for the mechanism that would underlie the effect of CL and Chol on the physicochemical characteristics of lipid-water interface. Among the possible modifying impacts of

Table 3 Anisotropy of aminobenzanthrones fluorescence in lipid bilayers

Dye	PC	Chol30	CL5	CL10
A4	0.38±0.01	0.378±0.013	0.349±0.012	0.396±0.01
A8	0.19±0.006	0.17±0.005	0.17±0.005	0.19±0.006
AM2	0.244±0.008	0.235±0.008	0.205±0.007	0.208±0.007
AM3	0.134±0.009	0.145±0.01	0.134±0.008	0.15±0.009
AM4	0.167±0.004	0.174±0.005	0.155±0.005	0.159±0.006

these lipids, one of the most well studied concerns the bilayer hydration. A wealth of evidence indicates that CL and Chol are capable of inducing the elevation of water content in polar region of lipid bilayer [22–25]. Specifically, it was shown that the amount of interfacial bound water considerably increases in the presence of CL. This effect was explained by the fact, that CL headgroup exerts less sterical hindrance for water binding than PC headgroup [24]. Furthermore, CL negative charge tends to move the N^+-end of PC P-N dipole parallel to the membrane surface, thereby causing the rearrangement of water bridges at lipid bilayer surface and stabilization of the intramolecular hydrogen bonds including the water molecules of hydration layer [26]. This effect eventually leads to the increase of membrane polarity. In turn, in the case of Chol, it is suggested that Chol inclusion into PC bilayer alters lipid packing density allowing a greater number of water molecules to penetrate in the membrane headgroup region [22]. Apparently, OH-group of sterol, which protrudes into bilayer carbonyl region, increases intermolecular separation, thereby enhancing water penetration into the membrane interior. Increased polarity of lipid bilayer induced by CL or Chol may favor the partitioning of highly polar A8 and AM2 into CL- and Chol-containing model membranes.

In conclusion, the present study has been undertaken to evaluate the potential of aminobenzanthrone dyes as novel probes for monitoring the physicochemical properties of lipid membranes. All dyes were found to possess high lipid-associating ability. Furthermore, spectral responses of A8 and AM2 in different lipid media proved to correlate with increased bilayer hydration brought about by CL and Chol. These findings allowed us to conclude that benzanthrone aminoderivatives may be effective fluorescent probes for examining membrane-related processes, especially those coupled with the change in the degree of bilayer hydration.

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