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SPECTRAL BEHAVIOR OF NOVEL BENZANTHRONE PROBE IN MODEL MEMBRANES

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The present study was undertaken to evaluate the sensitivity of a newly synthesized benzanthrone dye to the changes in physicochemical properties of lipid bilayer. It was shown that the dye under study is non-emissive in buffer but exhibites strong fluorescence in lipid phase. Partitioning of AM15 into model membranes composed of zwitterionic lipid phosphatidylcholine (PC) and its mixtures with anionic lipid cardiolipin and cholesterol was followed by significant increase of fluorescence quantum yield. Analysis of the partition coefficients showed that inclusion of cardiolipin and choleterol into phosphatidylcholine bilayer gives rise to the decrease of AM15 incorporation into lipid phase compared to the neat phosphatidylcholine membrane. It is assumed that AM15 resides in the hydrophobic bilater region, being oriented parallel to the lipid acyl chains.
KEY WORDS: benzanthrone dye, liposomes, phosphatidylcholine, cardiolipin, cholesterol, lysozyme.

СПЕКТРАЛЬНА ПОВЕДІНКА НОВОГО БЕНЗАНТРОНОВОГО ЗОНДУ В МОДЕЛЬНИХ МЕМБРАНАХ

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В даній роботі була проведена оцінка чутливості нового бензантронового барвника до зміни фізико-хімічних властивостей ліпідного бішару. Показано, що зонд АМ15 проявляє яскраво виражену флуоресценцію в ліпідній фазі у порівнянні з буфером, а його зв'язування з модельними мембранами, що складались із цвіттеріонного ліпіду фосфатидилхоліну і його сумішей із аніонним ліпідом кардіоліпіном та холестерином супроводжувався зростанням квантового виходу флуоресценції. Включення кардіоліпіну та холестерину в фосфатидилхоліновий бішар викликало зменшення ефективності вбудовування АМ15 в ліпосомальні мембрани у порівнянні з фосфатидилхоліновими ліпосомами. Припускається, що АМ15 локалізується в гідрофобній області бішару, орієнтуючись паралельно до ацильних ланцюгів ліпідів.

КЛЮЧОВІ СЛОВА: бензантронові барвники, ліпосоми, кардіоліпін, фосфатидилхолін, холестерин, лізоцим.

СПЕКТРАЛЬНОЕ ПОВЕДЕНИЕ НОВОГО БЕНЗАНТРОНОВОГО ЗОНДА В МОДЕЛЬНЫХ МЕМБРАНАХ

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В данной работе была проведена оценка чувствительности нового бензантронового красителя к изменению физико-химических свойств липидного бислоя. Показано, что исследуемый краситель проявляет ярко выраженную флуоресценцию в липидной фазе по сравнению с буфером, а его связывание с модельными мембранами состоящими из цвиттерионного липида фосфатидилхолина и его смесей с анионным липидом кардиолипином и холестерином сопровождалось увеличением квантового выхода флуоресценции зонда. Включение кардиолипина и холестерина в фосфатидилхолиновый бислой уменьшало эффективность встраивания красителя в липосомальные мембраны по сравнению с фосфатидилхолиновыми липосомами.

Предполагается, что AM15 локализуется в гидрофобной области липидного бислоя, ориентируясь параллельно ацильным цепям липидов.
КЛЮЧЕВЫЕ СЛОВА: бензантроновые красители, липосомы, фосфатидилхолин, кардиолипин, холестерин, лизоцим.

Membrane physical properties are known to control a variety of biological processes such as partitioning of proteins and peptides into lipid bilayer, membrane fusion, modulating the enzyme activity, just to name a few [1]. One powerful physical technique for detecting physical and chemical properties of model and biological membranes is based on the use of a wide variety of fluorescent probes [2-4]. The newly synthesized probes must meet several requirements: (i) several orders of magnitude higher quantum yield in the media under investigation compared to the dye fluorescence on buffer; (ii) small perturbing influence on biological systems, (iii) high sensitivity to the physicochemical changes of membrane environment.

The structure and dynamics of the membrane-bound probe molecule are of great interest because of the usefulness of their fluorescence properties in a wide variety of biological applications [5, 6]. A number of fluorescence properties (emission maximum, quantum yield, anisotropy, etc.) are sensitive to the variation in the membrane structure and/or dynamics Moreover, the exact molecular mechanisms behind the change in fluorescence property in many applications are not fully understood [7]. Identification of the dye location within the membrane is essential for understanding the molecular mechanisms underlying the changes in fluorescence properties.

In many complex biological systems fluorescence probe may be situated both in aqueous and membrane phases where in turn the probe tends to distribute between various locations, such as surface and interior regions [8]. In the lipid membrane an organic molecule can be located in three distinct regions: (i) The surface region where the dye is exposed to the external aqueous phase, (ii) the interfacial region where the dye exposure to the aqueous phase is limited, and (iii) the core region where the dye is entirely in a hydrophobic environment.

The present study was undertaken to evaluate the spectral properties of a newly synthesized benzanthrone dye (referred here as AM15). The structure of this compound is given in Fig.1. The spectral characteristics of AM15 have been examined using model membranes composed of egg yolk phosphatidylcholine (PC) and its mixtures with cholesterol (Chol) and anionic phospholipid cardiolipin (CL).

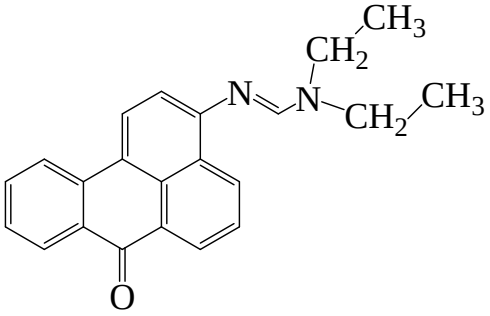

Fig. 1. Structure of the benzanthrone dye AM15.

MATERIALS AND METHODS

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. Chol esterol was from Sigma. Benzanthrone dye AM15 was synthesized at the Faculty of Natural Sciences and

Mathematics of Daugavpils University. All other chemicals were of analytical grade and used without further purification.

Unilamellar lipid vesicles composed of pure PC and PC mixtures with a) 5 or 10 mol% of CL; b) 30 mol% of Chol; were prepared by the extrusion method [9]. 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 [10]. The dye-liposome mixtures were prepared by adding the proper amounts of the probe stock solutions in buffer to liposome suspension.

Steady-state fluorescence spectra were recorded with LS-55 spectrofluorimeter (Perkin Elmer, Great Britain) equipped with magnetically stirred, thermostated cuvettes. Fluorescence measurements were performed at 20°C using 10 mm path-length quartz cuvettes. Excitation wavelengths for benzanthrone dye AM15 was 480 nm. Excitation and emission slit widths were set at 10 nm.

Quantum yields of AM15 was calculated according to the formula (1):

$$Q = \frac{Q_s(1 - 10^{-A_s})S_p}{(1 - 10^{-A_p})S_s} \quad (1)$$

Where Q_s is the dye quantum yield of standard, S_p and S_s are the areas under fluorescence spectra of the dye and standard, respectively, A_p and A_s are absorbances of the dye and standard at excitation wavelength, respectively. For correct quantum yield evaluation it was mandatory for the dye and standard to have close absorbance values, not exceeding 0.1. Absorption measurements were performed with SF-46 spectrophotometer using 2 mm path-length cuvettes.

RESULTS AND DISCUSSION

At the first step of the study we compared the sensitivity of AM15 to the membrane environment. Fluorescence spectra of this dye were recorded in buffer solution (5 mM Na-phosphate, pH 7.4) and liposomal suspensions.

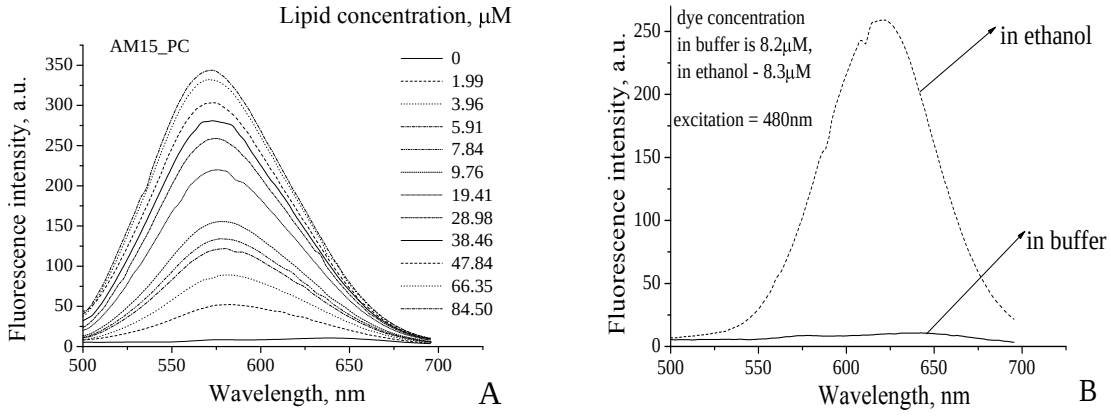

Fig. 2. Emission spectra of AM15 in PC liposomes (A) and ethanol (B). Extinction coefficient is $\varepsilon_{480} = 14491\ M^{-1}cm^{-1}$

The typical fluorescence spectra of probe AM15 in liposomal suspension are presented in Fig. 2, A. The dye under study is an asymmetrical benzanthrone derivative, which was

found to be nearly non-emissive in buffer but exhibited strong fluorescence in ethanol solution (Fig. 2, B).

The observed enhancement of AM15 fluorescence in lipid phase can be explained by the fluorophore transfer into membrane environment with reduced polarity and higher viscosity, decreased rate of non-radiative relaxation processes involving excited state dissipation via vibrations, hydrogen bonding to the solvent cage and the probe rotation [11, 12].

In the study of interaction of any compound with model membranes, the determination of the partition coefficient should be the basic step. To characterize AM15-lipid binding quantitatively, we determined the dye partition coefficient (K_p) for different lipid systems. The fluorescence spectroscopy methodology was employed to quantify the dye partitioning into a lipid phase.

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

$$Z_{tot} = Z_f + Z_l \tag{2}$$

where Z_f stands for the probe concentration free in bulk solution, Z_L is concentration of the dye, incorporated into the model membranes.

The coefficient of dye partitioning into the lipid phase (K_P) can be written as [13]:

$$K_P = \frac{Z_L V_W}{Z_f V_L} \tag{3}$$

or

$$Z_L = Z_f K_P \frac{V_L}{V_W} \tag{4}$$

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

It is easy to show that:

$$Z_f = \frac{Z_{tot}}{1 + K_P V_L} \tag{5}$$

The dye fluorescence intensity measured at a certain lipid concentration can be derived from the following expression:

$$I = a_f Z_f + a_L Z_L = Z_f (a_f + a_L K_P V_L) \tag{6}$$

where a_f, a_L represent molar fluorescence of the dye free in solution, and in the lipid environment, respectively.

From Eqs. (5) and (6) we have:

$$I = \frac{Z_{tot}(a_f + a_L K_P V_L)}{1 + K_P V_L} \tag{7}$$

The volume of lipid phase can be determined from:

$$V_L = N_A C_L \sum v_i f_i \tag{8}$$

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 and 3 nm^3 for PC and CL, respectively [14].

The relationship between K_p and fluorescence intensity increase (ΔI) can be written as [14]:

$$\Delta I = I_L - I_W = \frac{K_p V_L \ I_{max} - I_W}{1 + K_p V_L} \tag{9}$$

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.

To derive the dye partition coefficients for different lipid systems the experimental dependencies $\Delta I(C_L)$ presented in Fig. 3 were approximated by eq. (9). Analysis of the recovered partition coefficients (Table 1) shows that inclusion of anionic CL or sterol lipid Chol into PC bilayer gives rise to the decrease of partition coefficient relative to the neat PC membrane.

Table.1. Quantum yields and partition coefficients of AM15 in various systems

System	Fluorescence quantum yield	Partition coefficient
buffer	0.018	
ethanol	0.19	
PC	0.6	$(6.1\pm0.2)*10^4$
PC/CL (5%)	0.47	$(2.7\pm0.2)*10^4$
PC/CL (10%)	0.36	$(1.6\pm0.5)*10^4$
PC/Chol (30%)	0.3	$(4.9\pm0.4)*10^4$

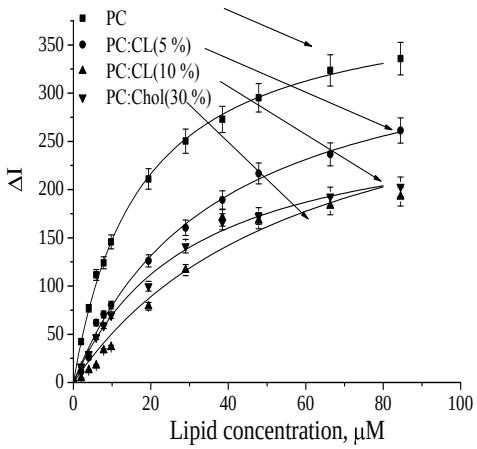

Fig.3. Fluorescence intensity increase as a function of lipid concentration.

To interpret the decrease of AM15 partition coefficients we should consider the mechanisms that may underlie the effect of CL and Chol on the physicochemical properties of lipid bilayer.

The charged backbone of cardiolipin, as well as the ester carbonyl group, contribute to the stability of an intra-and intermolecular hydrogen bonded network that includes water molecules of the hydration layer [15, 16]. It was shown that CL is capable to reduce liposome water permeability due to bilayer stabilization [17]. CL incorporation into lipid bilayer can lead to the changes in the lipid head group conformation [17]. Perturbation of the antisymmetric stretching vibrations of PC polar groups caused by CL confirms the above mechanism of CL influence on the molecular organization of a lipid bilayer [17].

Among the factors that may be responsible for the observed decrease of AM15 partitioning in Chol-containing membranes, the most essential seems to be cholesterol-induced structural reorganization of lipid–water interface. To date, the consequences of cholesterol inclusion in phospholipid bilayer are rather well characterized. For the liquid-crystalline lipid phase, the main chol effects include: (i) an increase in the separation of the

phospholipid headgroups [18, 19]; (ii) increased freedom of motion of the phosphocholine moiety [19]; (iii) enhanced headgroup hydration [19-24]; (iv) reduced content of the acyl chain gauche conformations [25, 26]; and (v) tighter lateral packing of the lipid molecules (condensing effect) [27]. In the lipid bilayer the amphiphilic cholesterol molecule is thought to adopt a quasiperpendicular orientation to the membrane surface, with the 3b-hydroxy group being located in the interfacial region and an apolar moiety composed of the tetracyclic ring and the isooctyl side chain embedded in the hydrophobic core. Modification of the physical properties of the lipid bilayer is considered as a predominant mechanism underlying cholesterol influence on the dye–lipid interactions. The change in the lipid packing density caused by Chol inclusion allows a greater number of water molecules to penetrate in the headgroup bilayer region. Probably Chol OH-group, which protrudes into carbonyl region of the bilayer, moves the neighbouring lipid molecules apart thereby increasing membrane hydration.

Taking into account the above effects, we can supposed that newly synthesized dye AM 15 is most probably located in the hydrophobic bilayer region being oriented parallel to the lipid acyl chains.

The next step of the work was directed towards the estimation of fluorescence anisotropy of AM15 in lysozyme-lipid systems (Table 2). It is known that fluorescence anisotropy of membrane-bound probe is determinedby an average angular shift of fluorophore occurring between absorption and subsequent emission of a photon, which depends on the lifetime of the probe excited state and the rate of its rotational diffusion. As seen from Table 2, anisotropy value increases in CL- and Chol-containing liposomes, compared to PC bilayer.

Table 2. Fluorescence anisotropy of AM15 in different lysozyme-lipid systems. Native lysozyme concentration was 4.5 μM.

System	anisotropy
PC	0.197
PC/CL(5%)	0.214
PC/CL(10%)	0.232
PC/Chol(30%)	0.215

Since the diffusive motions depend on free volume of the dye microenvironment, the angular shift reflects the ordering of lipid acyl chains. As a result, any variations in membrane free volume, available for probe motions, will lead to the changes in fluorophore anisotropy. What factors may cause the membrane free volume change? The free volume of lipid bilayer depends on its composition, degree of acyl chain saturation, extent of hydration, temperature, etc. [28]. The free volume model considers diffusion of membrane constituents or guest molecules as a three-step process*:* 1) opening of a gap in a lipid monolayer due to formation of kinks in the hydrocarbon chains; 2) jump of the diffusing molecule into a gap leading to the creation of a void; 3) filling the void by another solvent molecule.

According to the data of Shibata *et al.*, indicating that CL is capable to reduce liposome water permeability because of the bilayer stabilization, which in turn brings about reduction of the lipid bilayer free volume [17]. Bilayer free volume can be influenced by lysozyme inclusion into dye-lipid system. These changes most probably result from the restrictions of the probe rotation induced by the reduction of the membrane free volume upon the protein penetration into bilayer hydrophobic region. Likewise, protein binding may give rise to bilayer dehydration. Removal of water enhances the interactions between lipid molecules and increases ordering of lipid headgroups [29]. Restrictions imposed by the increased packing

density of lipid headgroups inhibit the probe photoisomerization and also may hinder its rotation [30]. Importantly, AM15 anisotropy increases with protein concentration and CL content. This finding implies that enhancement of electrostatic component of protein–lipid binding strengthens the hydrophobic interactions of the proteins with the acyl chains of lipid molecules.

CONCLUSIONS

In conclusion, the present study represents the first report about the behavior of a newly synthesized benzantrone dye AM15 in lipid membranes. The dye was found to be nearly non-emissive in buffer but exhibited strong fluorescence in liposomal suspensions. The results obtained indicate that benzanthrone probe readily binds to the liposomes and displays high sensitivity to the changes in physical and chemical properties of model membranes. AM15 partitioning into the lipid phases is followed by the increase of its fluorescence intensity without any shift of emission maximum. Based on partition coefficients and anisotropy values we supposed that newly synthesized dye AM15 is located in the hydrophobic bilayer region, being oriented parallel to the lipid acyl chains. The recovered properties of AM15 allowed us to recommend this dye for further use in membrane studies.

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