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INTERACTION OF BENZANTHRONE DYE ABM WITH MODEL MEMBRANES: THE EFFECTS OF IONIC STRENGTH AND CHOLESTEROL

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The influence of cholesterol and ionic strength on the partition and spectral properties of the novel benzanthrone probe ABM in the model lipid membranes has been examined using fluorescence-based approaches. The probe spectral characteristics, such as fluorescence emission maximum, intensity, anisotropy and lifetime were found to be dependent on cholesterol content and sodium chloride concentration, indicating high sensitivity of this dye to variations in the membrane physicochemical properties.

Keywords: benzanthrone dye, cholesterol, partition coefficient, fluorescence.

Among a wide variety of fluorescent dyes currently used in biomedical research, particular attention is given to the so-called environmentally-sensitive dyes. Of special interest in this regard are benzanthrone derivatives, whose fluorescence properties are modulated by the polarity of its surroundings due to a large increase of the dipole moment upon excitation [7]. This change in dipole moment has been attributed to charge separation between electron-donating part of the benzanthrone molecule in C-3 position and carbonyl group which serves as the electron acceptor [3]. The ability of benzanthrone dyes to form internal charge transfer (ICT) states makes them especially relevant for membrane studies. It has been shown previously that one representative of aminobenzanthrones, ABM, exhibits high affinity for the model and biological membranes [4, 5]. 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 alterations in conformational and aggregation state of protein molecules [2]. Despite the growing use of ABM in biomedical sciences, membrane properties of this probe are not yet fully ascertained. In view of this, in the present work we extended the evaluation of ABM performance in membrane studies and concentrated our efforts on assessing the ability of this probe to monitor the structural changes of phospholipid membranes produced by addition of cholesterol and sodium ions.

Materials and methods

Cholesterol (Chol) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were from Avanti Polar Lipids (Alabaster, AL). Benzanthrone dye ABM was synthesized at the Faculty of Natural Sciences and Mathematics of Daugavpils University as described in detail elsewhere [3]. All other chemicals were of analytical grade and used without further purification.

Unilamellar lipid vesicles composed of the neat POPC and POPC mixtures with 10, 20, 30 and 40 mol% of Chol were prepared by the extrusion method. The thin lipid films were first formed from the lipid mixtures in chloroform by removing the solvent under a stream of nitrogen. The dry lipid residues were subsequently hydrated with 1.7 ml of 5 mM Na-phosphate buffer (pH 7.4). Thereafter, lipid suspension was extruded through a 100 nm pore size polycarbonate filter (Millipore, Bedford, USA). The dye-liposome mixtures were prepared by adding the proper amounts of the probe stock solutions in ethanol to liposome suspension. Steady-state fluorescence spectra were recorded with Varian Cary Eclipse equipped with magnetically stirred, thermostated cuvette holder. Fluorescence measurements were carried out at 20°C using 10 mm path-length quartz cuvettes. Excitation wavelength for ABM was 460 nm.

Time-resolved fluorescence measurements were performed with a commercially available system (PTI, Ontario, Canada). A train of 500-ps excitation pulses at 337 nm at a repetition rate of 10 Hz was produced by a nitrogen laser. The solution of laser dye Coumarin 460 (Exciton, USA) in ethanol was used to excite ABM at 460 nm. The resulted data were fitted by biexponential functions and analyzed by the nonlinear least squares method:

$$I(t) = \sum_{i=1}^{n} \alpha_i \exp(-t/\tau_i), \qquad (1)$$

where τ_i and α_i are decay times and pre-exponential coefficients, n is the number of fluorescence lifetime components. The goodness of each fit was characterized by the value of reduced chi-square (χ^2), weighted residuals and autocorrelation function of the weighted residuals. A fit was considered good when χ^2 fell in the range of 0.8 to 1.3, and the plots of weighted residuals and autocorrelation function were randomly distributed around zero.

Results and discussion

Fig 1 represents the fluorescence spectra of ABM in POPC liposomes containing different amounts of cholesterol and 0.15 mM of NaCl at the last titration point. Increasing the concentration of cholesterol up to 20 mol% leads to the increase in ABM fluorescence intensity and shift of emission maximum from 598 nm in the neat POPC membrane to 600 nm in the vesicles containing 20 mol% of cholesterol. These effects reflect the changes in physicochemical properties of the membrane interfacial region, where ABM is supposed to be located [6]. The rise in cholesterol content up to 30 mol % results in the decrease of ABM fluorescence intensity compared to the case of 20 mol% Chol, but it remains higher than that in POPC liposomes.

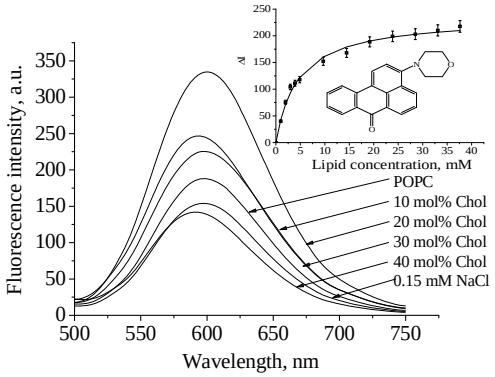

Fig. 1. Effect of cholesterol and ionic strength on fluorescence emission spectra of ABM in POPC vesicles. Concentration of ABM was 2.4 μM. Lipid concentration was 33 μM. Shown in inset are fluorescence intensity increase as a function of lipid concentration in POPC liposomes containing 10 mol% of cholesterol and chemical structure of ABM.

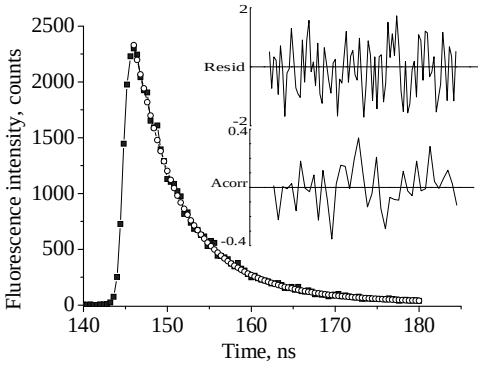

Fig. 2. Time-resolved fluorescence intensity decay of ABM in POPC liposomes (■), together with its fit (○). Concentration of ABM was 2.4 μM. The excitation wavelength was 460 nm and emission was monitored at 600 nm. Lipid concentration was 33 μM. Shown in insets are the weighted residuals and autocorrelation function of weighted residuals.

However, further increase in Chol concentration to 40 mol% was followed by the reduction of ABM fluorescence intensity relative to POPC and shift of the emission maximum to 596 nm. This effect can be attributed to the induction of liquid-ordered-like phase caused by high cholesterol content [9]. Moreover, fluorescence intensity drops by ~ 60% in the presence of 0.15 mM NaCl. Likewise, increase of ionic strength resulted in progressive blue shift of ABM emission maximum from 598 nm in POPC vesicles to 592 nm in the presence of 0.15 mM of NaCl. This observation can be considered as an additional argument in favor of ABM localization in the interfacial membrane region, since, as follows from MD simulations, sodium ions are capable of forming tight complexes with carbonyl oxygens of lipid molecules, thereby producing bilayer ordering [1]. Next,

ABM fluorescence intensity increase observed at elevating lipid concentration was used to quantify the probe partition coefficient K_p, according to the following equation:

$$\Delta I = \frac{\Delta I_{\max} K_p V_L}{1 + K_p V_L}, \quad (2)$$

where ΔI stands for the difference between ABM fluorescence intensity measured in the presence and absence of liposomes, $\Delta I_{\max}$ is the maximal value of this difference once the limiting intensity value is reached upon increasing lipid concentration and V_L is the volume of lipid phase. The recovered K_p estimates characterizing ABM partitioning into POPC model membranes containing different amounts of Chol and 0.15 mM NaCl are presented in Table. It can be seen that addition of cholesterol to POPC bilayer modifies partition ability of ABM. The rise in cholesterol content up to 20 mol% leads to the increase in AMB partition coefficients, but there were no statistically significant differences between K_p values found for 30 or 40 mol% Chol and the neat POPC bilayer. To interpret the observed differences in ABM partition behavior, the influence of Chol on lipid bilayer structure should be analyzed. In the liquid-chrystalline phase cholesterol can produce tighter lateral packing of lipid molecules (condensing effect), which enhances with Chol concentration [9]. However, condensing effect has significant impact on ABM partitioning only at 40 mol% of Chol, as follows from the increased anisotropy values (Table). At this concentration Chol can induce liquid-order-like phase, thereby preventing ABM bilayer partitioning. On the other hand, enhancement of ABM bilayer incorporation at lower Chol content can be attributed to Chol ability to affect lipid packing and bilayer polarity at hydrophobic-hydrophilic interface [8]. It should also be noted that due to the presence of carbonyl group in ABM structure, this probe seems to be capable of hydrogen bonding with cholesterol hydroxyl group. This capability may underlie the increase of K_p values in Chol-containing membranes. Notably, substantial decrease of partition coefficient was observed in the presence of sodium ions, most probably because of the aforementioned ordering effect of NaCl on POPC bilayer.

Partition coefficients, fluorescence lifetime and anisotropy of ABM in POPC liposomes with increasing cholesterol concentration

Chol (mol%)	$K_p \times 10^4$	α_1	τ_1	α_2	τ_2	χ^2	Anisotropy
0	6.4±0.7	0.8	6.49	0.2	1.98	0.9	0.278
10	7.9 ±0.2	0.8	6.75	0.2	1.57	0.96	0.274
20	8.1±0.4	0.76	7.62	0.24	1.39	1.01	0.276
30	6.9±0.3	0.75	8.07	0.25	2.96	0.93	0.283
40	5.9±0.1	0.6	9.62	0.4	2.31	1.06	0.304
0.15 mM NaCl	3.5±0.3	ND	ND	ND	ND	ND	0.296

The following experiments were directed towards the estimation of fluorescence anisotropy of ABM in different membrane systems. Anisotropy of membrane-bound probe is determined by the rate of its rotational diffusion, which, in turn, depends on free volume of the dye microenvironment. As seen from Table, increase of ionic strength and addition of up to 30 mol% cholesterol do not exert substantial influence on ABM fluorescence anisotropy, while statistically significant anisotropy increase was observed only at 40 mol% Chol.

At the last step of the study we performed fluorescence lifetime measurements of ABM in different model systems. Fluorescence lifetime serves as a faithful indicator of the local environment in which a given fluorophore is placed. A typical intensity decay profile of ABM in POPC vesicles with its biexponential fit and various statistical parameters used to check the goodness of fit are shown in Fig. 2. The calculated lifetime values together with their preexponential factors (see Eq. 1.) and chi-square values are presented in Table. It can be assumed that the longer lifetime component (τ_1) corresponds to the lifetime of membrane-bound dye (lower environmental polarity compared to buffer solution), while the shorter component accounts for the free dye in buffer (polar environment). Addition of Chol to POPC bilayer gives rise to pronounced increase in fluorescence lifetime of the lipid-associated dye. It is noteworthy that the fractional contribution of membrane-bound dye α_1 to the time-resolved decay appeared to attain the lowest value in the presence of 40 mol% of Chol, indicating the decrease of ABM membrane partitioning in liquid-order-like phase. According to MD simulation results, Chol may cause more water molecules to penetrate in the lipid bilayer interior [8]. In such a case the increase of ABM fluorescence lifetime in Chol-containing model membranes can be brought about by Chol-induced packing defects. Moreover, taken into account the red-shift of emission maximum in the presence of cholesterol we can hypothesize that ABM is a polarity sensitive probe.

Taken together, our results show that the novel benzanthrone fluorescent probe ABM is localized at lipid-water interface. In was demonstrated that spectral and partition properties of ABM depend on cholesterol content of the model membranes and ionic strength of the medium pointing to high sensitivity of this dye to the changes in its environment. These findings may prove of value in future studies involving ABM for probing composite biological membranes. This work was supported by the grant from Fundamental Research State Fund (project number F.54.4/015) and CIMO Fellowship (OZ)

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ВЗАЄМОДІЯ БЕНЗАНТРОНОВОГО ЗОНДА ABM З МОДЕЛЬНИМИ МЕМБРАНАМИ: ВПЛИВ ІОННОЇ СИЛИ І ХОЛЕСТЕРИНУ

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За допомогою флуоресцентних методів досліджено вплив холестерину та іонної сили на спектральні властивості нового бензантронового зонда ABM та його здатність до

зв'язування з модельними ліпідними мембранами. Показано, що спектральні характеристики АВМ, включаючи максимум, інтенсивність, анізотропію і час життя флуоресценції, залежать від вмісту холестерину та концентрації натрію в середовищі, що вказує на високу чутливість цього зонду до змін фізико-хімічних властивостей мембран.

Ключові слова: бензантронові зонди, холестерин, коефіцієнт розподілу, флуоресценція.

ВЗАИМОДЕЙСТВИЕ БЕНЗАНТРОНОВОГО ЗОНДА АВМ С МОДЕЛЬНЫМИ МЕМБРАНАМИ: ВЛИЯНИЕ ИОННОЙ СИЛЫ И ХОЛЕСТЕРИНА

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С помощью флуоресцентных методов исследовано влияние холестерина и ионной силы на спектральные свойства нового бензантронового зонда АВМ и его способность к связыванию с модельными липидными мембранами. Показано, что спектральные характеристики АВМ, включая максимум, интенсивность, анизотропию и время жизни флуоресценции, зависят от содержания холестерина и концентрации натрия в среде, что указывает на высокую чувствительность этого зонда к изменениям физико-химических свойств мембран.

Ключевые слова: бензантроновые зонды, холестерин, коэффициент распределения, флуоресценция.