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Lipid bilayer interactions of Eu(III) tris-β-diketonato coordination complex

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ABSTRACT

Membrane-associating and bilayer-modifying properties of Eu(III) tris-β-diketonato coordination compound (LC) displaying anti-tumor activity were evaluated using absorption and fluorescence spectroscopy techniques. Quantifying the lipid-induced changes in LC absorbance in terms of partitioning formalism yielded the partition coefficient of $(6.7 \pm 1.4) \times 10^3$. Examination of LC effect on the structural state of PC bilayer with fluorescent probe pyrene revealed the ability of europium complex to increase membrane free volume, supposedly associated with bilayer disordering and increased rate of *trans-gauche* isomerization of acyl chains.

1. Introduction

Lanthanide chelates, a wide class of metal coordination complexes with unique photophysical properties have found numerous applications in different areas, as components of light-converting optical devices, thermoluminescence dosimeters, indicator molecules in immunological and other nonisotopic assays [1–3], to name only a few. These compounds are of particular interest for biomedical research and diagnostics, because their spectral characteristics, such as long-lived fluorescence, large Stokes shift and long-wavelength emission are optimal to minimize interfering background from autofluorescence and light scattering of biological samples.

Recently it has been demonstrated that a series of Eu(III) tris-β-diketonates with common formula $Eu(L)_3Int$, where L is acetyl acetone, thenoyltrifluoroacetone, benzoylacetone, dibenzoylmethane and Int is 1,10-phenantroline or 2,2′-bipyridine are capable of exerting significant antineoplastic effect, with the abundance of DNA-intercalating motif (Int) being the major determinant of cytotoxic activity [4]. These findings open up new important area of the investigation implicating determination of the structure activity relationships and mechanisms of biological action of europium coordination complexes as a new class of potential antineoplastic drugs with alternative mode of cytotoxicity and nonoverlapping mechanisms of drug resistance. One aspect of this problem is concerned with elucidating the nature of lanthanide interactions with biological macromolecules and their assemblies, particularly, with lipid bilayer, a major structural element of cellular membranes. Such studies are of great significance at least in two respects: first, membrane damage may contribute to cytotoxic potential of europium complexes, and second, knowledge about lipid-associating abilities of a potential drug is important for the development of its liposomal formulations. Among a wide variety of the existing drug nanocarriers, liposome-based delivery systems are particularly attractive due to a number of advantages, such as biocompatibility, complete biodegradability, low toxicity, ability to carry both hydrophilic and lipophilic payloads and protect them from chemical degradation and transformation, increased therapeutic index of a drug, etc. [5,6]. The development of liposomal carriers is heavily based on evaluation of the membrane-partitioning and bilayer-modifying properties of the drug. This is important not only for achieving maximum payload without compromising liposome stability, but also for the prediction of therapeutic and toxic effects of a certain compound, because membrane interactions may prove critical for drug absorption, distribution, metabolism and elimination in an organism.

The present study was undertaken to gain insight into lipid bilayer interactions of one representative of the above class of cytotoxic europium complexes, referred to here as LC. Our goal was two fold: (i) to characterize the membrane partition properties of LC, and (ii) to clarify the effects of this compound on structural state of model lipid membranes. Phosphatidylcholine (PC), zwitterionic lipid, abundant in most cellular membranes was used to prepare monolamellar lipid vesicles.

2. Materials and methods

2.1. Chemicals

Egg yolk PC was purchased from Biolek (Kharkov, Ukraine). Phospholipid purity assessed by thin layer chromatography in

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the solvent system chloroform:methanol:acetic acid:water, 25:15:4:2, v/v) was found to exceed 90%. Eu(III) coordination complex (Fig. 1) was synthesized as described previously [4]. Pyrene was from Sigma (Germany).

2.2. Preparation of lipid vesicles

Lipid vesicles were prepared from PC using extrusion technique. The thin lipid film was obtained by evaporation of PC ethanol solutions and then hydrated with 1.2 ml of 5 mM Na-phosphate buffer (pH 7.4) at room temperature. Thereafter, lipid suspension was extruded 15 times through a 100 nm pore size polycarbonate filter (Nucleopore, Pleasanton, CA).

2.3. Spectrophotometric measurements

Absorption spectra were recorded with SF-46 spectrophotometer (LOMO, Russia) in 5 mM or 2 M sodium-phosphate buffer, pH 7.4, at room temperature. Stock solution of LC was prepared in dimethylsulfoxide. While measuring the absorbance of europium complex at varying lipid concentration two series of experiments have been conducted. In the first series, 10 μl of LC stock solution was added to a 1-cm-path-length quartz cuvette containing 1.5 ml of the buffer, and then titrated with liposomes. In the second series of experiments LC mixtures with liposomes were incubated for 60 min. Thereafter, LC absorbance was measured using 0.2-cm-path-length cuvettes. Drug-to-lipid molar ratios were varied from 0.04 to 0.44. Absorption spectra of lanthanide-liposome mixtures were recorded against liposomal suspensions of the same concentration, to allow for light scattering effect.

2.4. Fluorescence measurements

Fluorescence measurements were performed with CM 2203 spectrofluorimeter (Solar, Belarus) equipped with the magnetically stirred, thermostated cuvette holder (SOLAR, Belarus). Pyrene emission spectra were recorded with excitation wavelength 337 nm and excitation and emission slit widths set at 2 nm. The excimer-to-monomer pyrene fluorescence intensity ratio (E/M) was determined by measuring fluorescence intensity at the monomer (389 nm) and excimer (490 nm) peaks.

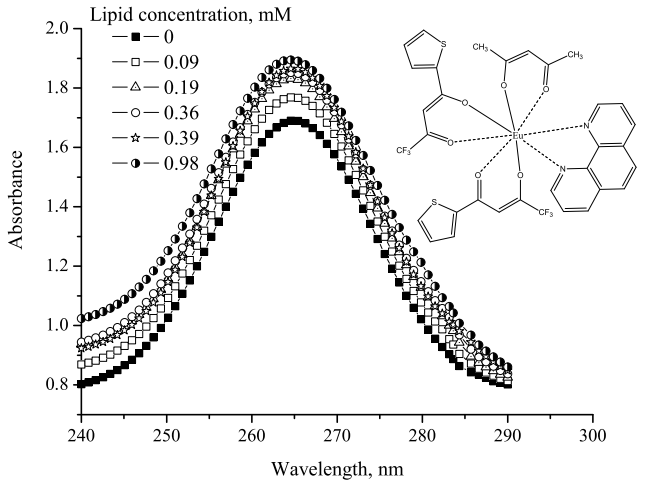

Fig. 1. Absorption spectra of europium complex in suspension of PC liposomes at varying lipid concentration. Absorbance was measured using 0.2-cm-path-length cuvettes and then multiplied by a factor of 5 to obtain the values, corresponding to standard optical length 1 cm. LC concentration was 40 μM. Shown in inset is chemical structure of Eu(III) tris-β-diketonato coordination complex.

3. Results and discussion

As seen in Fig. 1, LC has broad absorption spectrum in the range 240–320 nm with maximum around 266 nm. Titration of LC with PC liposomes was followed by the absorbance increase suggesting that this europium chelate tends to partition into lipid phase. To interpret this effect quantitatively, the observed dependency of absorbance increase at 266 nm on lipid concentration (Fig. 2) was further analyzed in terms of partition coefficient defined as [7]:

$$K_p = \frac{N'_L V_W}{N'_W V_L} \tag{1}$$

where N'_L, N'_W are the moles of LC in lipid and aqueous phases, respectively; V_L, V_W are the volumes of these phases. The volume of lipid phase was calculated as:

$$V_L = N_A C_L v_{PC} \tag{2}$$

here C_L is the molar lipid concentration, v_{PC} is molecular volume of hydrated PC molecule taken as 1.58 nm^3 [8], N_A is Avogadro's number. Under the employed experimental conditions ($C_L \leqslant 1$ mM) the V_L value is much less than total volume of the system ($V_t = 1$ dm^3), so that $V_W \approx V_t$. The relationship between K_p and the absorbance increase ($\Delta A_{266}(C_L)$) can be written as [7]:

$$\Delta A_{266}(C_L) = A_L - A_W = \frac{K_p V_L (A_{max} - A_W)}{1 + K_p V_L} = \frac{K_p N_A C_L v_{PC} (A_{max} - A_W)}{1 + K_p N_A C_L v_{PC}} \tag{3}$$

where A_L is LC absorbance at a certain lipid concentration C_L, A_W is LC absorbance in buffer, A_{max} is a limit absorbance in lipidic environment. Approximation of the experimental curve ($\Delta A_{266}(C_L)$) by Eq. (3) yields partition coefficient $(6.7 \pm 1.4) \times 10^3$, the values corresponding to free energy changes of ca. –5 kcal/mol. Allowing for zwitterionic nature of PC molecule and the fact that lanthanide complex under study is highly hydrophobic compound bearing no charge, it seems reasonable to consider partitioning process as being a consequence of hydrophobic effect which reflects the tendency of nonpolar molecule to avoid the contact with the water by escaping to a less polar phase. Upon binding to the membrane, water molecules are released from the solute hydration shell leading to an entropy-driven association process [9]. However, one should bear in mind that membrane-partitioning is inevitably accompanied by structural reorganization of a lipid bilayer, referred to as 'bilayer effect'. Accordingly, energetics of partitioning may be

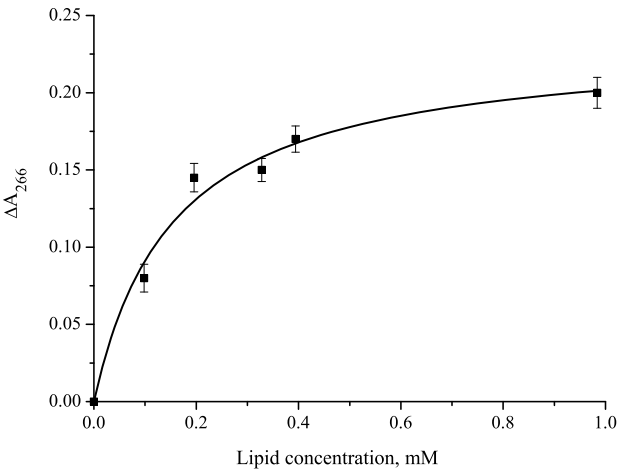

Fig. 2. Increase of LC absorbance at 266 nm as a function of lipid concentration. Solid line represents theoretical curve calculated from Eq. (3) with partition coefficient 6.7×10^3 and $A_{max} - A_W = 0.23$.

essentially controlled by the changes in bilayer free energy related to its perturbations. Specific interactions of lanthanide molecular groups with PC headgroups may disturb lipid packing, increase free volume and facilitate LC partitioning into lipid bilayer. These rationales point to scenario in which LC *per se* perturbs lipid bilayer promoting thereby self-partitioning in membrane interior as it was observed by Custodio et al. for 4-hydroxytamoxifen [10].

In view of the above considerations, at the next step of the study we addressed the question of whether LC is capable of modifying structural state of PC bilayer. To answer this question, fluorescent probe pyrene sensitive to the changes in membrane polarity and dynamic properties [11,12] was employed. Emission spectrum of pyrene monomers is featured by a well-defined vibronic structure with five major vibronic bands between 370 and 400 nm, generally numbered I–V, from the lowest wavelength (Fig. 3). Relative intensities of vibronic transitions exhibit clear dependence on solvent polarity (Ham effect) [13]. Particularly, the intensity ratio of the third to the first vibronic bands, $R_{III} = I_{III}/I_I$, is reported to be about 2 in a saturated perfluorinated solvent, 1.7 in *n*-hexane and 0.50.6 in water [14]. Notably, the R_{III} values recovered here for PC bilayer (*ca.* 0.9–1.2) are in harmony with those reported previously by other authors [15].

As illustrated in Fig. 4A, this parameter exhibits tendency to increase with LC concentration, suggesting that polarity of pyrene microenvironment decreases on lanthanide incorporation into the lipid bilayer. As follows from ^{1}H NMR data, pyrene molecule whose longest dimension is *ca.* 0.9 nm tends to align its principal long axis along the bilayer normal, residing in the region of 413 carbons [16]. The observed rise in R_{III} value implies that LC either brings about bilayer dehydration or causes pyrene to take deeper location within the membrane interior.

Another parameter recovered from pyrene spectra, excimer-to-monomer fluorescence intensity ratio (*E*/*M*), also increased in the presence of europium complex (Fig. 4B), but in this case there was no clear dependency on LC concentration. Formation of pyrene excimers (excited-state dimers) upon interaction between the ground-state and excited-state monomers is displayed in appearance of characteristic peak in emission spectra around 490 nm (Fig. 3). In general, increase in the extent of pyrene excimerization reflects the changes of the three main parameters: (i) pyrene concentration, (ii) pyrene lifetime, and (iii) probe diffusion rate constant. In our case, the first possibility can be ruled out since pyrene concentration was kept constant in all experiments. Rise in the probe lifetime resulted from LC-induced decrease in bilayer

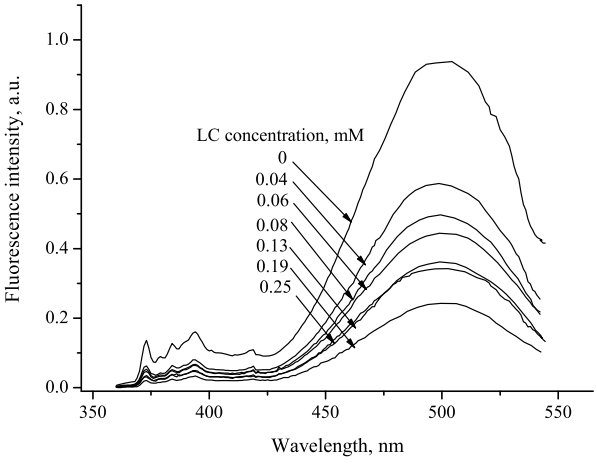

Fig. 3. Pyrene emission spectra in suspension of PC liposomes at varying LC concentration. Pyrene concentration was 20 μM.

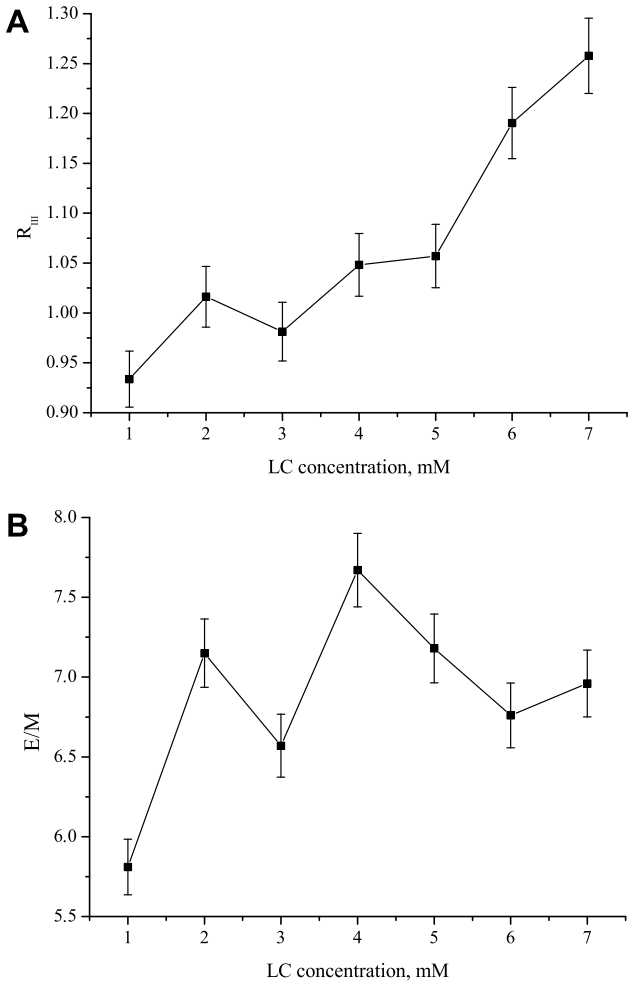

Fig. 4. Intensity ratio of the third to first vibronic peaks (A) and excimer-to-monomer fluorescence intensity ratio (B) plotted vs. LC concentration. Pyrene concentration was 20 μM. Lipid concentration was 0.5 mM.

polarity may, in principle, account for the observed increase in *E*/*M* ratio, however, it is unlikely to significantly contribute E/M value the following reasons. If modification of membrane polarity profile is the only determinant of the changes in the extent of pyrene excimerization, then the magnitudes of *E*/*M* and R_{III} increase are expected to be comparable. However, as seen in Fig. 4, formation of drug–lipid complexes led to increase in *E*/*M* ratio by ~23% while R_{III} increased only by ~9%. Hence, it seems highly probable that the revealed *E*/*M* increase is largely determined by the enhanced probe diffusion, which, in turn, depends on the membrane structural and dynamical properties and is commonly described in terms of bilayer free volume model. In this model, diffusion of membrane constituents or guest molecules is considered as proceeding over three-major steps: (i) formation of dynamic defects (kinks) in the acyl chains followed by opening the cavities in a lipid monolayer; (ii) jump of diffusing molecules into the cavities resulting in generation of a voids; and (iii) sealing the voids by the movement of defects along the adjacent hydrocarbon chains. Appearance of dynamic defects in membrane interior is associated with *trans-gauche* isomerization of hydrocarbon chains initiated by thermal motion and packing constraints. Membrane free volume, produced by lateral displacements of hydrocarbon chains after kink formation, characterizes the difference between effective and van der Waals volumes of lipid molecules. The revealed increase of *E*/*M* ratio points to that LC is capable of increasing

membrane free volume, most likely, via elevating the rate of *trans-gauche* isomerization of hydrocarbon chains. This conclusion is also corroborated by the finding that LC binding to PC bilayer induces the 14%-decrease in fluorescence anisotropy of membrane-incorporated probe 1,6-diphenylhexatriene (data not shown), stemming from the disordering of lipid molecules. Notably, the observed effect proved virtually independent of LC-to-lipid molar ratio, suggesting the existence of interfering bilayer-modifying processes of opposite sign.

Cumulatively, the results presented here strongly suggest that Eu(III) tris-β-diketonato coordination compound can be efficiently entrapped by lipid phase of PC vesicles, with partition coefficient being *ca.* $(6.7 \pm 1.4) \times 10^3$. Analysis of pyrene fluorescence spectra measured at varying LC-to-lipid molar ratios revealed the reduction in polarity of the probe microenvironment coupled with increase in the extent of excimer formation. These findings can be rationalized in terms of perturbing effect of europium complex on membrane structure, presumably through the generation of structural defects, promotion of *trans-gauche* isomerization of acyl chains and altering packing density of PC molecules.

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