Fluorescence study of interactions between a europium coördination complex and model membranes

Andrey Yudintsev,[1,*] Valeriya Trusova,[1] Galyna Gorbenko,[1] Todor Deligeorgiev,[2] Aleksey Vasilev,[2] Stefka Kaloianova[2] and Nedjalko Lesev[2]

[1] *Department of Biological and Medical Physics, V. N. Karazin Kharkiv National University, 4 Svobody Sq., 61077 Kharkiv, Ukraine*
[2] *Department of Applied Organic Chemistry, Faculty of Chemistry, University of Sofia, 1164 Sofia, Bulgaria*

Membrane partitioning and bilayer-modifying properties of a Eu(III) coördination complex (drug) were evaluated using equilibrium dialysis and absorption and fluorescence spectroscopies. To gain insight into the drug's influence on the physical parameters and molecular organization of the lipid bilayer, several fluorescent probes have been employed: pyrene, 1,6-diphenyl-1,3,5-hexatriene (DPH), 4-p-(dimethylaminostyryl)-1-dodecylpyridinium (DSP-12) and 6-propionyl-2-(dimethylamino)naphthalene (Prodan). The high lipophilicity of the Eu(III) coördination complex together with its relatively weak membrane-modifying propensities are prerequisites for the development of liposomal formulations of this compound.

Keywords: bilayer modification, drug-lipid interactions, Eu(III) coördination complex, membrane partitioning

1. INTRODUCTION

Owing to their unique physicochemical and photophysical properties, lanthanide chelates have found application in a variety of areas including laser techniques (as transformers of light energy), immunofluorescence assays and NMR spectroscopy (as shift reagents) [1,2]. The luminescence of lanthanides enhanced by ligands makes it possible to use these compounds as probes in bioanalytical assays [3]. A major rôle of the ligands in this case is the absorption of ultraviolet light and the transfer of excited state energy to the lanthanide (III) ion [4,5], the direct excitation of which is inefficient.

Besides, the use of lanthanides in biomedical practice requires synthesis of chelate systems possessing specific bioactivity (e.g. specific binding to biomolecules). During the last decades considerable research efforts have been focused on the characterization of lanthanide complexes bearing β-diketone ligands [1,5,6]. Particularly, it was found that europium (III) tris-β-diketonates exert cytotoxic effects against different tumour cell lines. The structure of these compounds can be described with the general formula $Eu(L)_3Int$, where L are β-diketones (e.g., acetyl acetone, thenoyltrifluoro-acetone etc.) and Int is 1,10-phenanthroline or 2,2'-bipyridine. It has been demonstrated that the presence of DNA-intercalating motifs (such as 2,2'-bipyridine or 1,10-phenanthroline) in the structure of europium complexes is the key to the augmentation of their cytotoxicity [7].

Given that high cytotoxic activity is often followed by the side effects, developing special delivery systems for such medicinal drugs seems to be very important [8]. An accessible and effective drug carrier system is based on the use of liposomes—spherical self-closed vesicles in which the lipid bilayer encloses an aqueous compartment. Liposomes possess the following advantages: biocompatibility, complete biodegradability, low toxicity and the capability of incorporating both hydrophilic and lipophilic agents. Hence, liposomes represent promising drug carrier systems. The concept of their use for delivery of antineoplastic drugs has gained increasing interest in recent years. Several liposomal formulations for anticancer drugs are currently employed in clinical practice: amphothericin B, benzoporphyrin [9], doxorubicin and daunorubicin [8–10].

This work focuses on the examination of interactions between a Eu(III) coördination complex (a potential anticancer drug) and a lipid bilayer. The membrane-partitioning properties of the investigated compound were evaluated using absorption spectroscopy and equilibrium dialysis. To gain insight into the drug influence on physical parameters and molecular organization of the model membranes several fluorescent probes, have been employed, viz. pyrene, 1,6-diphenyl-1,3,5-hexatriene (DPH), 4-p-(dimethylaminostyryl)-1-dodecylpyridinium (DSP-12) and 6-propionyl-2-(dimethilamino)naphthalene (Prodan).

2. MATERIALS AND METHODS

2.1. Materials

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

* Corresponding author. E-mail: ayudintsev@yahoo.com.

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chloroform: methanol:acetic acid:water, 25:15:4:2 v/v, was found to exceed 90%. The Eu(III) coördination complex (inset to Fig. 1), referred to here as LC, was synthesized as described previously [7]. DSP-12 was obtained from Zonde (Latvia); pyrene, DPH and Prodan were from Sigma (Germany).

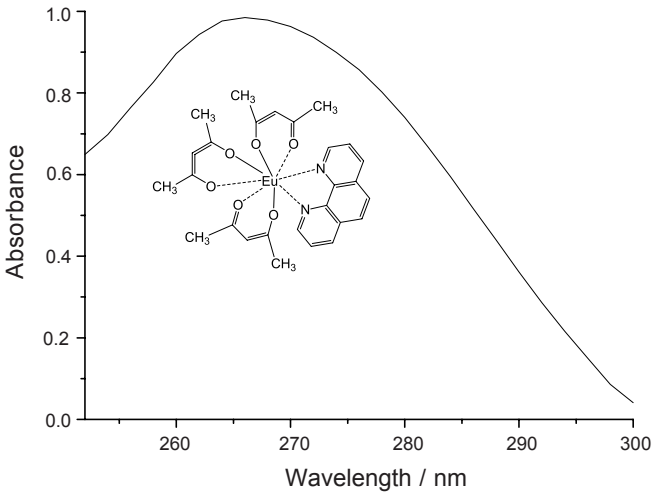

Figure 1. Absorption spectrum of the Eu(III) coördination complex LC. Shown in the inset is its chemical structure.

2.2. Preparation of lipid vesicles

Unilamellar lipid vesicles composed of PC were prepared by the extrusion technique. The thin lipid film was obtained by evaporation of lipid ethanol solutions and then hydrated with 1.2 mL of 5 mM Na-phosphate buffer (pH 7.4) at room temperature. Thereafter, the lipid suspension was extruded through a 100 nm pore polycarbonate filter (Nucleopore, Pleasanton, California).

2.3. Liposome–buffer partitioning

Partitioning of europium complex into a lipid phase was determined using the equilibrium dialysis technique at room temperature. The dialysis cells were divided into two chambers by semipermeable cellulose membranes. The volume of each compartment was 3 mL. The dialysis samples were prepared by loading buffer solution into the first compartment and a drug–lipid mixture into the second compartment. The lipid and drug concentrations were 90 μM and 48 μM, respectively. After the equilibrium had been established, the LC concentration in the solution from the first compartment was determined by absorption spectroscopy, using the extinction coefficient $\varepsilon_{266} = 1.1 \times 10^4\ M^{-1}cm^{-1}$.

2.4. Spectroscopic measurements

Absorption measurements were conducted using a SF-46 spectrophotometer (LOMO, Russia) against solvent blanks. Fluorescence spectra were recorded with a CM 2203 spectrofluorimeter equipped with a magnetically stirred, thermostated cuvette holder (SOLAR, Belarus). Excitation wavelengths were 340 nm for pyrene, 350 nm for DPH and Prodan and 460 nm for DSP-12. Excitation and emission slit widths were set at 2 nm for pyrene, 5 nm for DSP-12 and Prodan, and 10 nm for DPH. The excimer-to-monomer pyrene fluorescence intensity ratio (E/M) was determined by measuring fluorescence intensity at the monomer (392 nm) and excimer (465 nm) peaks.

2.5. Statistical analysis

Statistical data analysis was performed using common methods of variation statistics including calculations of the mean and standard deviation. The significance of differences between the values of observed parameters in control and drug-containing systems was estimated using Student's *t*-test for the lowest probability (p) and critical *t*-value (t_c), equal to 0.05 and 2.18, respectively.

3. RESULTS AND DISCUSSION

3.1. Membrane partitioning of the Eu(III) complex

LC is an asymmetric Eu(III) coördination complex with acetyl acetone ligands and a 1,10 phenanthroline motif. The organic chromophores of this complex are responsible for absorbing the excitation light and transferring the energy to the lanthanide [3]. The optical absorption spectrum of LC features a maximum at 266 nm (Fig. 1). Taking into account the high hydrophobicity of this compound and its relatively small size (approximately 11 Å) in comparison with the lipid bilayer thickness (46 Å) LC is expected to be efficiently incorporated into the lipid phase. To corroborate this idea, we employed the equilibrium dialysis technique. This technique is based on physical separation of free (in solution) molecules and membrane-bound ones [11]. (Equilibrium dialysis is often used as a reference method for the determination of liposome/water partition coefficients [11,12].)

Generally, the drug mole-fraction partition coefficient is defined as [12]:

$$K_p = \frac{C_{bound}/[L]}{C_{free}/[W]}, \qquad (1)$$

where C_{free} and C_{bound} are the equilibrium molar concentrations of the drug free (in solution) and bound to the lipid vesicles. [W] and [L] stand for water and lipid molar concentrations. At room temperature [W] = 55.3 M [12]. Considering that C_{free} and C_{bound} values can be determined spectrophotometrically using the drug extinction coefficient, experimental partition coefficients can be practically calculated from:

$$K_p = \frac{(A^{eq}_{free} - A^{eq}_{bound})[W]}{[L]A^{eq}_{bound}}, \quad (2)$$

where A^{eq}_{free} and A^{eq}_{bound} are the absorbances at 266 nm of our drug free in solution and bound to the lipid vesicles, respectively. The partition coefficient determined in this way was found to be *ca* $5 \times 10^4 \pm 2.5 \times 10^3$. This quantity is related to the Gibbs free energy of drug-lipid binding as:

$$\Delta G = -RT \ln K_p \quad (3)$$

where R and T represent the gas constant and the absolute temperature. The obtained ΔG value (–26.3 ± 1.3 kJ/mol) lies in a range which is characteristic for the transfer of a hydrophobic molecule from an aqueous to a membrane phase [13]. For comparison, the free energies of membrane partitioning of cyclosporin A is –31.4 kJ/mol and of paclitaxel, –33.1 kJ/mol [9]. According to modern theories of membrane electrostatics the partition coefficient mentioned above can be described as a sum of electrostatic and nonelectrostatic terms [13–15]:

$$K_p = \exp(\{\omega_{el} + \omega_{ct} + \omega_h + \omega_n + \omega_d\}/kT) \quad (4)$$

where ω_{el}, ω_{ct}, ω_h, ω_n and ω_d characterize the electrostatic interactions, charge transfer, hydration, neutral and dipole potential contributions, respectively. In the case of hydrophobic drugs the contribution of electrostatic interactions is negligibly small, and partitioning into the lipid phase is determined predominantly by the last three terms.

Taking into consideration the high hydrophobicity of our drug and allowing for the zwitterionic nature of the PC molecule, it seems reasonable to suggest that hydrophobic interactions play an essential rôle in LC association. The high K_p value confirms that our compound can be efficiently incorporated into a PC bilayer. Next, it seemed of importance to evaluate whether drug incorporation into a lipid phase is followed by the modification of the membrane structural state. It is well known that the more lipophilic the drug is, the deeper it will become immersed into the hydrocarbon core of a membrane. Nevertheless, its embedding into the lipid bilayer can result in a change of both acyl chain conformations and, to a certain extent, the polar lipid headgroups. In this connexion it logical to suppose that the zone in which the complex is located will be subjected to the greatest modifications. To check this hypothesis, several environmentally-sensitive membrane fluorescent probes, differing in their bilayer location and responsiveness to a membrane perturbation, have been employed (Fig. 2).

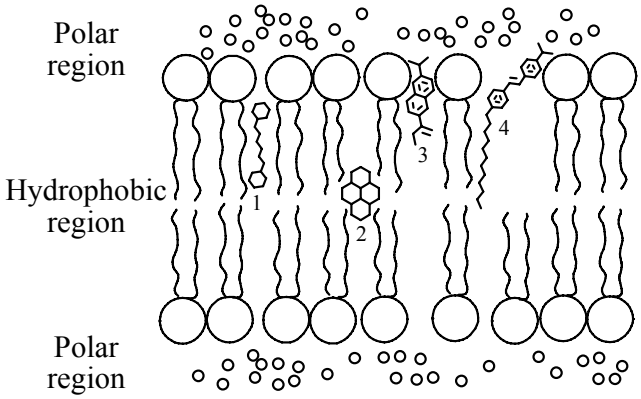

Figure 2. Schematic representation of fluorescent probe locations in a lipid bilayer. 1: DPH; 2: pyrene; 3: Prodan; 4: DSP-12.

3.2. Effect of lanthanide on pyrene spectral characteristics

Pyrene is a well known fluorescent probe used extensively in membrane studies [16–19]. The emission spectrum of pyrene monomers features a vibrational band structure between 370 and 400 nm (Fig. 3), which exhibits strong sensitivity to the changes in polarity of the probe environment. In particular, the intensity ratio of the first to the third vibronic bands, I_I/I_{III}, increases with solvent polarity (e.g., this parameter is equal to 0.6 in n-hexane and to 1.35 in methanol) [17,18]. The formation of pyrene excimers (excited state dimers) upon interaction between ground state and excited state monomers leads to the appearance of a characteristic peak in the emission spectrum at 465 nm (Fig. 3) [16]. The pyrene excimer-to-monomer intensity ratio (E/M) reflects the rate of probe lateral diffusion within the membrane plane and variations in the membrane free volume (the difference between effective and van der Waals volumes of the lipid molecules) [19–22].

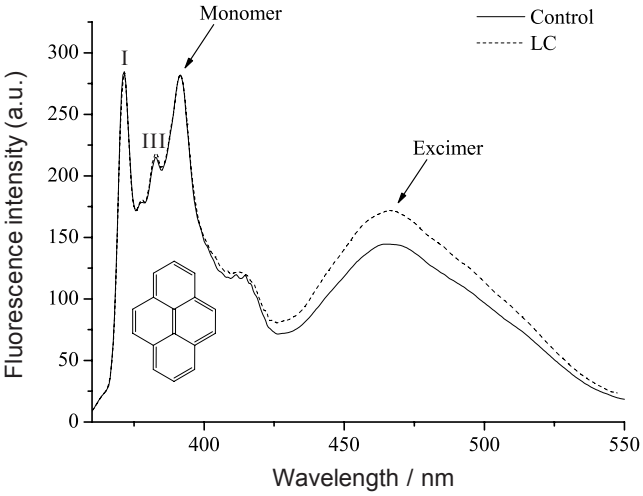

Figure 3. Typical emission spectra of pyrene in PC liposomes with and without LC. Pyrene and lipid concentrations were 0.7 and 3.3 μM, respectively. LC concentration was 1.1 μM.

The analysis of pyrene emission in PC and PC+LC systems showed that I_I/I_{III} remains virtually invariant upon drug association with the lipid bilayer, the observed changes of this parameter did not exceed 5%. This finding suggests that the drug does not affect the distribution of pyrene monomers and, consequently, exerts no influence on liposome polarity. In contrast, the formation of drug–lipid complexes resulted in a statistically significant increase of the pyrene excimer-to-monomer intensity ratio. As illustrated in Fig. 4, the extent of the relative changes of pyrene degree of excimerization $\Delta E/M$ caused by LC:

$$\Delta E/M = \frac{E/M_{+LC} - E/M_{control}}{E/M_{control}} \cdot 100\%, \qquad (5)$$

where $E/M_{control}$ and E/M_{+LC} are pyrene excimer-to-monomer intensity ratios in the control and drug-containing systems, respectively, depends on the lipid-to-bound drug molar ratio (L/D_{bound}). D_{bound} was calculated using the above K_p value as:

$$D_{bound} = \frac{D_T K_p V_L}{1 + K_p V_L}, \qquad (6)$$

where D_T is the total drug concentration, K_p the partition coefficient and V_L the volume of the lipid phase. V_L was determined as:

$$V_L = N_A C_L v_{PC}, \qquad (7)$$

where C_L is the molar lipid concentration, v_{PC} is the PC molecular volume, taken as 1.58 nm^3, and N_A is Avogadro's number.

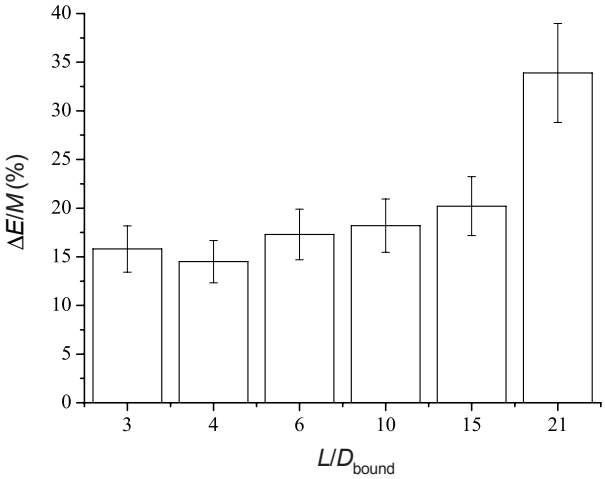

Figure 4. Excimer-to-monomer fluorescence intensity ratio *versus* drug to lipid ratio. Pyrene concentration was 1.8 μM, lipid concentration was 40 μM.

Notably, the $\Delta E/M$ dependence on the L/D_{bound} ratio appeared to display a nonmonotonic behaviour—the addition of a small amount of LC (L/D_{bound} = 21) to the liposomes leads to an appreciable E/M change (increase by 34%), while upon increasing the drug concentration to L/D_{bound} = 15, $\Delta E/M$ decreased by ~ 15%. Further increase of the amount of LC in the membrane region virtually did not affect $\Delta E/M$. The fact that $\Delta E/M$ increases with the rise in L/D_{bound} indicates that the drug hampers the probe lateral diffusion within the membrane plane. LC incorporation into the lipid phase is likely to bring about modification of the bilayer structure, which may involve, particularly, an increase of trans–gauche isomerization of the acyl chains. This is followed by kink formation coupled with the appearance of local free volume (voids) in the lipid bilayer [23]. The pyrene molecule jumps into this cavity, thus forming a void at the previous position [20,21]. However, at a higher LC concentration (L/D_{bound} increased from 15 to 3), the drug can cause tighter lipid packing, which manifests itself in the lower degree of excimerization. The observed E/M pattern provides evidence for LC embedding into the hydrophobic membrane region. To corroborate this idea we have carried out a series of experiments with the classical fluidity probe DPH.

3.3. Effect of lanthanide on DPH anisotropy

The characterization of drug–membrane interactions seems to be incomplete without elucidation of the drug's effect on the mobility of the fatty acyl chains. The importance of drug influence on membrane dynamic properties (fluidity) was emphasized in a number of studies [23,24]. According to Goldstein [23], the term "fluidity" includes flexibility of the acyl chains, lateral diffusion within the membrane plane, transverse diffusion of molecules between the monolayers, phase transitions and phase separations. One of the most widely used ways of obtaining information about a drug's influence on the dynamical membrane properties is based on examining the spectral characteristics of the membrane probe DPH. Due to its low solubility and fluorescence quenching in water, emission from this probe is only observed from molecules bound to a membrane [16]. DPH is preferentially located in the hydrophobic region of a lipid bilayer (Fig. 2) [25]. The probe molecules absorb light polarized along their long axis [16]. Fluorescence polarization (anisotropy) of bilayer-embedded DPH reflects the rotational diffusion rate of this probe, which in turn is related to the extent of lipid ordering; that is, the packing of the fatty acyl chains [23,26,27]. The smaller the DPH anisotropy value, the faster the probe rotates, implying that the lipid environment has become less ordered.

It was found that incorporation of the europium complex into PC liposomes is followed by a small (around 9%) but

statistically significant increase of DPH anisotropy within the L/D_{bound} range from 28 to 58 (Fig. 5). This effect can be interpreted in terms of the drug's ability to modify the molecular organization of a lipid bilayer. Drug insertion into the membrane interior is likely to induce some restrictions on acyl chain motion. It can be assumed that LC can alter trans–gauche isomerization of the lipid acyl chains due to its specific orientation in the hydrophobic membrane region. This may be the reason for the observed increase of DPH fluorescent anisotropy. This process is likely to resemble the condensing effect of chlorpromazine on the hydrophobic membrane region [22]. However, our present results seem to contradict the pyrene excimerization data. The apparent discrepancy may be explained by the differences between pyrene and DPH in their sensitivity to the changes in membrane free volume. Because of the highly anisotropic nature of a lipid bilayer the membrane free volume is determined by the translational, rotational, orientational, and conformational degrees of freedom of the lipid molecules. In contrast to pyrene, whose excimerization originates from alterations in its lateral movements, the DPH anisotropy reflects the changes in its rotational mobility. Lateral displacement of the hydrocarbon chains (caused by LC insertion) generates a local free volume. Evidently, the increase of free volume overall in the bilayer bulk will be accompanied by tighter lipid packing in certain membrane regions, and the observed changes of DPH anisotropy depend on the relative contributions of DPH molecules located in the voids and in the condensed regions to the measured anisotropy.

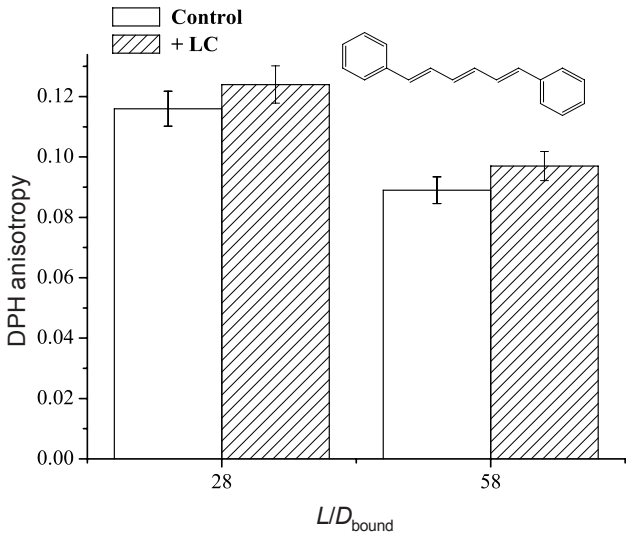

Figure 5. Effect of LC on DPH anisotropy. DPH concentration was 2 μM.

As was discussed above, at low concentrations of membrane-bound LC the appearance of local voids in the membrane interior facilitates pyrene excimerization. Drug molecules probably move the hydrophobic tails of neighbouring lipids apart. This leads to tighter lipid packing on each side of the drug and the appearance of local free volume deep in the membrane, where pyrene excimers are formed. On the other hand, considering that the long DPH molecule is preferentially oriented parallel to the acyl chains (Fig. 2), it is straightforward to infer that any changes of lipid packing should led to the restriction of DPH mobility (due to probe confinement by the acyl tails) even if the overall free volume of the membrane increases. This process is reflected in the observed increase of DPH anisotropy.

3.4. Effect of lanthanide on DSP-12 fluorescence

To evaluate whether LC membrane incorporation is followed by the changes in bilayer polarity, we employed two polarity-sensitive probes—DSP-12 and Prodan. The fluorescent probe DSP-12 possesses a charged hydrophilic fluorophore moiety and a long hydrophobic tail. The alkyl tail of DSP-12 tends to reside in the hydrophobic membrane region, whereas the aromatic group is located at the lipid–water interface. This probe has poor water solubility, which can engender the formation of probe aggregates (micelles) [28].

Deconvolution of the fluorescence spectra of DSP-12 in PC liposomes (Fig. 6) revealed two Gaussian components, corresponding to emission from the two probe populations that differ in their transverse membrane location [29]:

$$I(\lambda) = \sum_{i=1}^{2} a_i \frac{1}{w\sqrt{\pi/2}} \exp\left[\frac{-2(\lambda - \lambda^i_{max})}{w^2}\right], \qquad (8)$$

where a_i, w_i and λ^i_{max} stand for area, width and maximum position of the ith component, respectively. The correlation coefficient (R^2) for the spectral fitting exceeded 0.99. The relative contributions of the short- and long-wavelength components (f_1 and f_2) in the overall fluorescence spectrum were determined as

$$f_{1,2} = \frac{a_{1,2}}{a_1 + a_2}. \qquad (9)$$

Analysis of the data presented in Table 1 shows that the differences between the spectral parameters are statistically insignificant for the control and drug-containing systems. This finding indicates that LC does not influence the membrane interfacial region, probably because of the deeper localization of the drug molecule compared to the probe location.

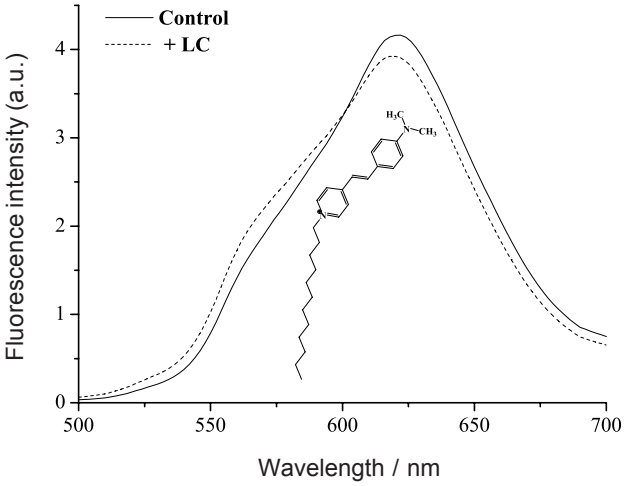

Figure 6. Emission spectra of DSP-12 in the control and drug-containing systems. DSP-12 concentration was 1.9 μM; lipid and LC concentrations were 71 μM and 5 μM, respectively.

Table 1. Spectroscopic parameters obtained from the deconvolution of DSP-12 fluorescence spectra in phospholipid vesicles.

Parameter	Control	+ LC
λ_1 / nm	568.8 ± 0.45	567.3 ± 0.39
a_1	32 ± 1.65	46 ± 1.9
w_1	29.4 ± 0.88	32.8 ± 0.7
λ_2 / nm	620.4 ± 0.23	618.8 ± 0.27
a_2	258 ± 1.83	251 ± 2.03
w_2	57.1 ± 0.41	58.2 ± 0.43
f_1	0.11 ± 0.05	0.15 ± 0.05
f_2	0.89 ± 0.05	0.85 ± 0.05

3.5. Effect of lanthanide on Prodan spectral characteristics

The fluorescent probe Prodan is localized in the phospholipid headgroup region (Fig. 2). The emission spectrum of Prodan is sensitive to the polarity of the probe environment, due to dipolar relaxation processes. Prodan has a large dipole moment in the excited state because of intramolecular charge transfer. After the excitation, the solvent molecules undergo a reorientation around the excited state dipole of the probe. This process is followed by the energy consumption that leads to a red shift of emission maximum [17,30]. A large shift of the Prodan emission maximum is observed upon changes in the environmental polarity of this probe [31,32]. For example, the fluorescence emission maximum of Prodan in water is 520 nm, while in benzene it is 428 nm [31,33]. The lower the dielectric constant of the solvent, the shorter the wavelength of the emission maximum [34].

To analyse the differences in the Prodan emission spectra and to explore the drug effect on the lipid bilayer polarity we evaluated the generalized polarization (*GP*). This parameter can be calculated from the Prodan emission spectra according to [31]:

$$GP = \frac{I_{1M} - I_{2M}}{I_{1M} + I_{2M}}, \qquad (10)$$

where I_{1M} and I_{2M} are the Prodan fluorescence intensities at 420 nm and 480 nm in PC liposomes (Fig. 7), obtained after subtraction of Prodan emission in buffer. *GP* reflects Prodan's microenvironmental polarity: the more negative the value of *GP*, the higher the polarity. *GP* was found to be *ca* –0.23 ± 0.01 and –0.22 ± 0.01 for the control (i.e., without drug addition) and the drug-containing systems, respectively. The slight (statistically insignificant) difference between the *GP* values points to the absence of any appreciable change of Prodan's microenvironmental polarity in the presence of LC. This result is in good agreement with the behaviour of the pyrene spectral parameters discussed above.

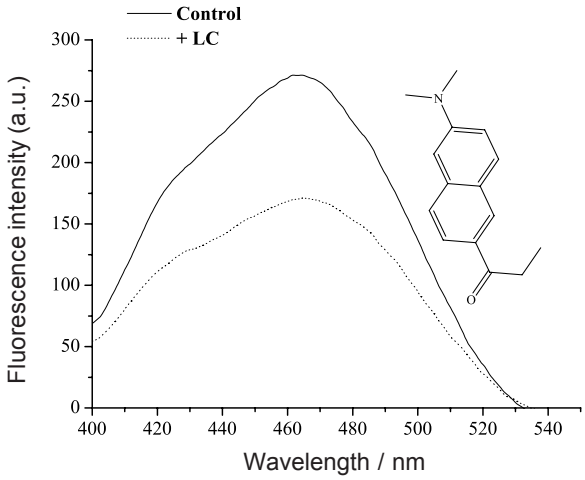

Figure 7. Emission spectra of Prodan. LC concentration was 8 μM; Prodan concentration was 0.35 μM, lipid concentration was 40 μM.

4. CONCLUSIONS

Our results demonstrate the principal possibility of incorporating europium coördination complexes into the lipid phase of model membranes. Analysis of pyrene, DPH, DSP-12 and Prodan spectral parameters (Table 2) has revealed that LC does not exert any influence on bilayer polarity. In the meantime, LC incorporation into the membrane interior is followed by modification of the lipid bilayer structural state, presumably involving alterations in trans–gauche isomerization of the lipid acyl chains. These findings suggest that the investigated compound is located in the hydrophobic region of lipid bilayers. The fact that the observed bilayer-modifying effects are relatively small in their magnitude creates a background for the development of liposome-based formulations of the Eu(III) coördination complex, as a potential anticancer drug.

Table 2. Summary of the results obtained.

Probe	Parameter	Control	+ LC	*t*-value[a]	LC effect
Influence on the polar membrane region					
Pyrene	I_I/I_{III}	1.08 ± 0.05	1.05 ± 0.05	1.45	insignificant
DSP-12	f_1	0.11 ± 0.05	0.15 ± 0.05	1.79	insignificant
	f_2	0.89 ± 0.05	0.85 ± 0.05	1.79	insignificant
Prodan	*GP*	–0.23 ± 0.01	–0.22 ± 0.01	1.79	insignificant
Influence on the hydrophobic membrane region					
Pyrene	*E/M*	5.71 ± 0.29	7.65 ± 0.38	12.86	increased about 34%
DPH	anisotropy	0.089 ± 0.004	0.097 ± 0.005	3.58	increased about 9%

[a] The larger the *t*-value compared to t_c= 2.18, the more significant the effect of LC on the corresponding parameter.

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