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LIPOSOMAL FORMULATIONS OF ANTITUMOR DRUGS. II. EFFECT OF LIPID COMPOSITIONS ON MEMBRANE INTERACTIONS OF EUROPIUM COORDINATION COMPLEXES

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Currently there is a growing interest in screening of new drugs, capable of destroying cancer cells effectively, without damaging health tissues. In this context the potential of liposomes as a drug carrier system is extensively investigated [1-3]. Liposomes are nanosize particles in which lipid bilayer encloses an aqueous internal compartment. Size, charge and surface properties of liposomes can be easily changed simply by adding new ingredients to the lipid mixture before liposome preparation or by variation of preparation techniques. Another important feature is that lipid vesicles can entrap both hydrophilic and hydrophobic pharmaceutical agents. Liposome delivery systems can enhance drug solubility, reduce toxicity associated with free anticancer drugs and improve stability of the drug by protecting the compound from chemical degradation or transformation. However, the therapeutic and toxic effects of drug are strongly determined by the degree or efficiency of its loading into the liposomes. For this reason, while using liposomes as delivery systems for hydrophobic drugs, it is necessary to know the character of a drug effect on the structure and physicochemical properties of a lipid bilayer. The aim of this work was to investigate the effect of lipid composition on membrane interactions of europium coordination complexes, V3 and V4, the potential antineoplastic drugs. Liposomes were formed by egg yolk phosphatidylcholine (PC) and its mixture with cardiolipin (CL) and cetyltrimethylammonium bromide (CTAB). The membrane-partitioning properties of the investigated drugs were evaluated using the equilibrium dialysis technique in combination with absorption spectroscopy. To gain insight into the drug influence on physical parameters and molecular organization of lipid bilayer, two fluorescent probes have been employed, viz. pyrene and 1,6-diphenyl-1,3,5-hexatriene (DPH). It was found that inclusion of anionic lipid cardiolipin and cationic detergent CTAB into PC bilayer gives rise to decrease of the drugs partition coefficients. The drug incorporation into liposomal membrane is accompanied by the alterations of pyrene spectral parameters and DPH anisotropy. The observed effects suggest that the influence of europium compounds on bilayer structural state can be modulated by CL and CTAB.

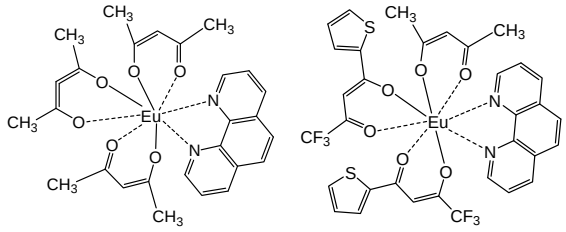
KEY WORDS: europium complexes, liposome, cardiolipin, CTAB.

Over the past decades, the concept of liposome use for the delivery of antineoplastic drugs has gained increasing interest. Liposomes are spherical self-closed particles, consisting of a bilayer enclosing an aqueous compartment. For drug payload purposes liposomes are commonly prepared from phospholipids, cholesterol, cholesterol esters and triglycerides. Owing to biodegradable and biocompatible nature of liposomal constituents such lipid nanocarriers offer a number of advantages making it an ideal drug delivery vehicle. The amphiphilic character of lipid vesicles enables encapsulation of both hydrophobic and hydrophilic compounds. However, similar to other carrier systems, the use of liposomes in drug delivery also has disadvantages and difficulties. One of them concerns their chemical and biological stability. The stability of liposomal formulations is largely determined by the drugs membrane-partitioning properties as well as by physicochemical characteristics of the lipid vesicles [1-3]. The present study was undertaken to evaluate the effect of lipid composition on membrane interactions of the new potential antineoplastic drugs europium coordination complexes (EC), referred here to as V3 and V4.

MATERIALS AND METHODS

Egg yolk phosphatidylcholine and beef heart cardiolipin were purchased from Biolek (Kharkov, Ukraine). DSP-12 was obtained from Zonde (Latvia), pyrene, DPH and CTAB were from Sigma (Germany). Eu(III) coordination complexes (Fig. 1) were synthesized as described previously [4]. Lipid vesicles composed of PC and its mixtures with CL and CTAB were prepared using the extrusion technique [5]. The thin lipid film was 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. Phospholipid concentration was determined according to the procedure of Bartlett [6]. Absorption measurements were conducted using SF-46 spectrophotometer. Fluorescence measurements were performed with CM 2203 spectrofluorimeter equipped with magnetically stirred, thermostated cuvette holder (SOLAR, Belarus). Pyrene fluorescence measuring was recorded under the following conditions: pyrene concentration – 22 μM, V3 and V4 concentrations – 0.49 and 0.36 μM in PC:CL liposomes, 2.8 and 0.35 μM in PC:CTAB, respectively. Lipid concentration was 0.16 mM. While measuring DPH anisotropy, the concentrations employed were: DPH – 4μM, lipid – 0.15 mM, V3 and V4 – 7.1 and 5.1 μM in PC:CL liposomes, 2.8 and 0.35 μM in PC:CTAB, respectively.

RESULTS AND DISCUSSION



V3 V4

Fig. 1. Structure of europium coordination complexes.

Efficient and stable encapsulation of drugs is one of the most difficult tasks in the development of liposomal drug formulations. At the first step of the study we focused on investigation of EC partitioning into the lipid bilayer. To evaluate the lipid association ability of the drugs in questions the equilibrium dialysis technique was used. This method is based on the diffusion of small molecules through a semi-permeable membrane. The dialysis cells are composed of two chambers separated by cellulose membrane. One half-cell is filled with the buffer and the other half-cell with the drug solution or drug-liposome mixture. In our experiments the volume of liquid in each compartment was 3 ml. The loading of dialysis cells was made as follows: 3 mL of the buffer solution were placed on one side of the membrane (compartment 1) and 3 mL of drug-lipid mixture, containing 2.7 ml of liposomes (lipid concentration – 0.9 mM) and 0.3 ml of the investigated drugs (V3 and V4 concentration were 4.8×10^{-5}M, 3.5×10^{-5}M in PC and PC:CL liposomes, respectively, V3 concentration in PC:CTAB – 6.4×10^{-5}M), on the other side (compartment 2). After the equilibrium was established, the V3 and V4 concentrations in the solution from the first compartment were determined by absorption spectroscopy, using extinction coefficients $\varepsilon_{266} = 1.1\times10^4 M^{-1}cm^{-1}$ for V3 and $\varepsilon_{266} = 2.8\times10^4 M^{-1}cm^{-1}$ for V4.

According to the methodology described previously [8, 11] the experimental values of mole fraction membrane-water partition coefficients (K_p) can be determined as [7, 8]:

$$K_p = \frac{(A_0^c - A_{out}^{eq})[water]}{[lipid]A_{out}^{eq}} \quad (1)$$

where A_0^c and A_{out}^{eq} are the drug optical densities at 266 nm in liposome-free and liposome-containing systems, respectively, [*water*] and [*lipid*] represent water and lipid molar concentrations, respectively.

Table 1. Partition coefficients of europium coordination complexes in different lipid systems

	PC	PC:CTAB(5%)	PC:CL(20%)
V3	$5\times10^4 \pm 1.5\times10^4$	$1.3\times10^4 \pm 3.9\times10^3$	$1.9\times10^4 \pm 5.7\times10^3$
V4	$3.2\times10^4 \pm 9.6\times10^3$	$3.2\times10^3 \pm 9.6\times10^2$	not determined

As seen from the Table 1, relatively high K_p values were obtained for all systems under study, suggesting the efficient drug encapsulation into the liposomal membrane. These data are in good accordance with our previous results obtained for PC and PC-cholesterol liposomes. It seemed also of interest to compare the K_p values recovered for neat PC, PC:CTAB and PC:CL model membranes. The inclusion of anionic lipid cardiolipin and cationic detergent CTAB into PC bilayer gives rise to decrease of EC partition coefficients. This finding suggests that CL and CTAB molecules can limit the drug partitioning into the lipid phase. One of the possible reasons for such limitation may include modification of lipid packing by CL and CTAB molecules. [9]

At the next step of this study the bilayer-modifying properties of V3 and V4 were evaluated using fluorescent probes pyrene and DPH. Pyrene is one of the most commonly used membrane probes. It allows investigation of the membrane dynamic properties and its polarity. The fluorescence spectra of this probe possess a well defined vibrational band structure between 370 and 400 nm (monomer emission), and a characteristic peak around 465 nm (excimer emission). The relative intensities of the first and third vibronic peaks (I_I/I_{III}, where I_I and I_{III} are fluorescent intensities at 374 and 384 nm, respectively) undergo significant changes upon going from nonpolar to polar solutions being a polarity-sensitive parameter. In particular, I_I/I_{III} increases from 0.64 to 1.45 on going from cyclohexane to ethanol; in water, $I_I/I_{III} = 1.95$ [10].

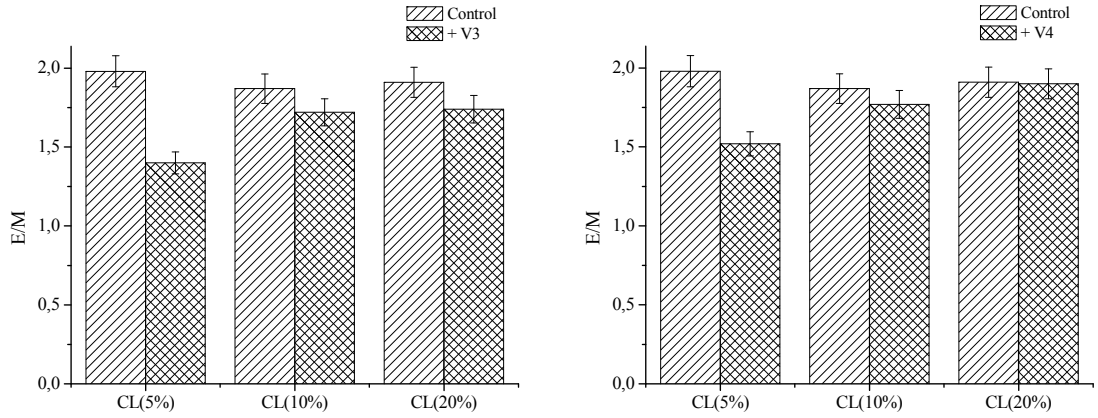
As was found in our previous study the intensity ratio of the first to the third pyrene vibronic bands [10], remains virtually unchanged upon V3 and V4 association with PC, and PC:Chol vesicles. similar behavior of I_I/I_{III} ratio was observed in PC:Cl and PC:CTAB systems (Table 2). In this context it is interesting to draw attention to the fact that I_I/I_{III} values observed in our experiments correspond to the intensity ratio of pyrene vibronic bands in a hydrophobic environment. These results corroborate our assumption that EC do not affect the distribution of pyrene monomers and exert no influence on polarity of lipid bilayers.

Table 2. Effect of europium coordination complexes on pyrene spectral parameters in different lipid-drug systems.

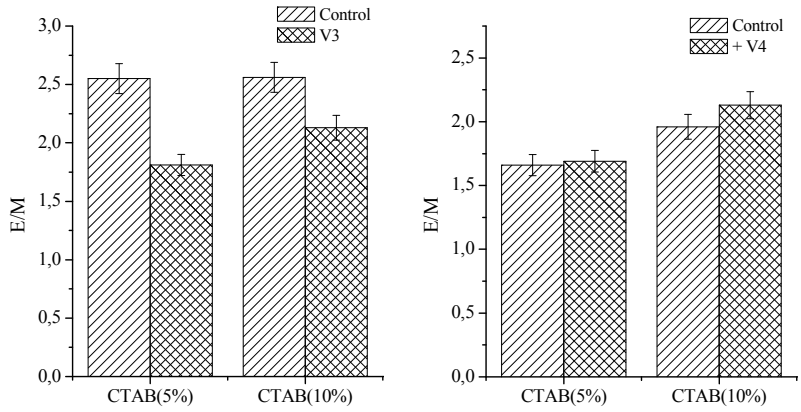
Liposome composition	**Without V3**	**+V3**	**Without V4**	**+V4**
	I_I / I_{III}	I_I / I_{III}	I_I / I_{III}	I_I / I_{III}
PC	1.04	1.02	1.04	1.04
PC:CL(5%)	1.02	1.01	1.02	1.01
PC:CL(20%)	1.03	0.98	1.03	0.65
PC	0.97	0.91	0.87	0.9
PC:CTAB(5%)	0.92	0.94	0.87	0.86
PC:CTAB(10%)	0.94	0.91	0.91	0.87

On the contrary, EC incorporation into the lipid phase of model membranes brings about the changes of another parameter under examination – pyrene excimer-to-monomer intensity ratio (E/M) which reflects the rate of probe lateral diffusion within the membrane plane (Fig 2). It was found that both V3 and V4 reduce E/M in PC:CL liposomes (Fig. 1, A). The same

effect was observed for V3 in PC:CTAB liposomes (Fig. 1, B), while in V4+PC:CTAB system no significant E/M changes were detected. These results indicate that EC incorporation into liposomal membrane is accompanied by the restriction of pyrene lateral diffusion, which in turn, may reflect the reduction of lipid bilayer free volume.



A)



B)

Fig. 2. Effect of europium coordination complexes on pyrene spectral parameters: A) PC:CL, and B) PC:CTAB lipid vesicles.

These data seem to be contradict the results of DPH anisotropy measurements. Polarization and anisotropy of this probe are defined by the extent to which its fluorophore rotates during the excited-state lifetime. In other words, anisotropy value is inversely proportional to the probe rotation rate: the smaller is anisotropy value, the faster probe rotates.

Table 3. The change of DPH anisotropy induced by europium coordination complexes, %.

Investigated systems	CL(5%)	CL(10%)	CL(20%)	CTAB(5%)	CTAB(10%)
V3	-13.8%	-13.5	-8.5	-10.6	-22
V4	+36.4	+45	+51.8	-9.7	-31.9

As shown in the Table 3 EC inclusion into PC:CTAB liposomes were followed by the decrease of DPH anisotropy. In keeping with our previous results obtained for PC:Chol liposomes the observed discrepancy in pyrene and DPH data may be explained by the difference in the mechanisms by which these probes respond to the alterations in their

microenvironment. While the changes in pyrene excimerization arise from the modifications in probe lateral movements, variations in DPH anisotropy mirrors the changes in probe rotational mobility. However it should be noted that the opposite changes of DPH anisotropy were observed – decrease for V3 to and increase for V4. Opposite behaviour of V3 and V4 in CL liposomes can be interpreted in terms of both distinctions in EC structure and different pattern of their interaction with the given type of lipid bilayer.

CONCLUSIONS

The results of the present study confirm the possibility of incorporation of europium coordination complexes into the lipid vesicles. The obtained information about cardiolipin and cetyltrimethylammonium bromide effect on membrane interactions of the drugs in question can be used in the designing of their effective liposomal formulations.

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