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LIPOSOMAL FORMS OF NEW ANTITUMOR DRUGS BASED ON EUROPIUM CHELATES EXAMINED BY *p*-TERPHENYL FLUORESCENCE QUENCHING

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Europium chelates have been previously shown to possess pronounced cytotoxic activity. These compounds are of great interest for biomedical investigations and diagnostics, because their spectral characteristics are optimal for visualization of the occurred processes. Application of these pharmaceutical compounds in the free form is limited by their high toxicity and metabolic instability. In view of this, the development of the delivery systems for europium chelates becomes actual. Liposomes represent one of the most promising delivery systems, which allows to increase the efficiency of pharmacological agents. The use of liposomal formulations of antitumor drugs is currently in a focus of biomedical and biophysical research due to the following advantages: complete biocompatibility, ability to carry both lipophilic and hydrophilic compounds, protecting them from chemical degradation and transformation, decreased toxicity and increased therapeutic index of drug, *etc*. In the present work we explore the interaction between europium chelates (here referred to as V6 and V8) and model lipid membranes. Fluorescence intensity of membrane-incorporated probe *p*-terphenyl was found to decrease with enhancement of drugs concentration. The obtained results indicate that *p*-terphenyl fluorescence is quenched upon europium chelate incorporation into phosphatidylcholine liposomes. Quantitative characteristics of *p*-terphenyl fluorescence quenching by the drugs under consideration have been determined.
KEY WORDS: europium chelate, liposomes, fluorescence quenching, partition coefficient.

ИССЛЕДОВАНИЕ ЛИПОСОМАЛЬНЫХ ФОРМ НОВЫХ ПРОТИВООПУХОЛЕВЫХ ПРЕПАРАТОВ НА ОСНОВЕ ХЕЛАТОВ ЕВРОПИЯ МЕТОДОМ ТУШЕНИЯ ФЛУОРЕСЦЕНЦИИ *п*-ТЕРФЕНИЛА

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Ранее в ряде работ было показано, что хелаты европия обладают ярко выраженными цитотоксическими свойствами. Данные соединения представляют особый интерес для биомедицинских исследований и диагностики, поскольку их спектральные характеристики являются оптимальными для визуализации протекаемых процессов. Применение подобных лекарственных препаратов в свободной форме ограничено их высокой токсичностью и метаболической нестабильностью. В связи с этим, актуальной становится разработка новых систем доставки таких препаратов. Одним из многочисленных видов систем, которые позволяют повысить эффективность противоопухолевых препаратов, являются липосомы. Применение липосомальных нанопереносчиков фармакологических препаратов в настоящее время является предметом многих биомедицинских и биофизических исследований благодаря их следующим преимуществам: полной биосовместимости, способности переносить как липофильные, так и гидрофильные соединения, защищая их от химической деградации и трансформации, уменьшению токсичности и увеличению терапевтического индекса препаратов и т. д. В данной работе исследовалось взаимодействие хелатов европия (в работе они обозначены как V6 и V8) с липидным бислоем модельных мембран. Было обнаружено, что интенсивность флуоресценции локализованного в мембране зонда *п*-терфенила уменьшается при увеличении концентрации препаратов. Анализ полученных результатов показал, что флуоресценция *п*-терфенила в фосфатидилхолиновых липосомах тушится хелатами европия. Были определены количественные характеристики тушения флуоресценции *п*-терфенила исследуемыми препаратами.
КЛЮЧЕВЫЕ СЛОВА: хелат европия, липосомы, тушение флуоресценции, коэффициент распределения.

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ДОСЛІДЖЕННЯ ЛІПОСОМАЛЬНИХ ФОРМ НОВИХ ПРОТИПУХЛИННИХ ПРЕПАРАТІВ НА ОСНОВІ ХЕЛАТІВ ЄВРОПІЮ МЕТОДОМ ГАСІННЯ ФЛУОРЕСЦЕНЦІЇ *n*-ТЕРФЕНІЛА

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Раніше у ряді робіт було показано, що хелати європію мають виражені цитотоксичні властивості. Дані сполуки становлять особливий інтерес для біомедичних досліджень та діагностики, оскільки їхні спектральні характеристики є оптимальними для візуалізації досліджуваних процесів. Застосування подібних лікарських препаратів у вільній формі обмежено їхньою високою токсичністю та метаболічною нестабільністю. У зв'язку з цим, актуальною стає розробка нових систем доставки таких препаратів. Одним із численних видів систем, які дозволяють підвищити ефективність протипухлинних препаратів, є ліпосоми. Застосування ліпосомальних нанопереносчиків фармакологічних препаратів на сьогодні є предметом багатьох біомедичних і біофізичних досліджень завдяки наступним їхнім перевагам: повній біосумісності, здатності переносити як ліпофільні, так і гідрофільні сполуки, захищаючи їх від хімічної деградації та трансформації, зменшенню токсичності та збільшенню терапевтичного індексу препаратів, тощо. У даній роботі вивчалася взаємодія хелатів європію (у роботі вони позначені як V6 і V8) з ліпідним бішаром модельних мембран. Було виявлено, що інтенсивність флуоресценції локалізованого у мембрані зонда *n*-терфеніла зменшується при зростанні концентрації препаратів. Аналіз отриманих результатів показав, що флуоресценція *n*-терфеніла у фосфатидилхолінових ліпосомах гаситься хелатами європію. Були визначені кількісні характеристики гасіння флуоресценції *n*-терфеніла досліджуваними препаратами.

КЛЮЧОВІ СЛОВА: хелат європію, ліпосоми, гасіння флуоресценції, коефіцієнт розподілу.

INTRODUCTION

The application of the antitumor drugs in the free form is often limited by undesirable side effects, such as high cardiotoxicity, negative exposure on the healthy tissues, accumulation in the organs of reticuloendothelial system, *etc*. Thus, development of drug delivery systems represents the hot topic of modern biochemical and biophysical studies. Existing drug nanocarriers involve formulations based on polymer materials (polymer conjugates, microspheres and wafers) [1], osmotic pumps, microchips and different lipid-based carrier systems (lipid microbubbles, microspheres and microtubules, solid lipid nanoparticles, oily suspensions, lipid implants, submicron lipid emulsions, liposomes) [2]. Among them, liposomes, spherical, closed structures composed of lipid bilayers enclosing an aqueous compartment, represent the most promising low toxic, biodegradable drug nanovehicles. Highly adaptable liposome-based nanosystems currently attract increasing attention, because of their indisputable advantages, *viz.* complete biodegradability, ability to carry both hydrophilic and lipophilic payloads and protect them from chemical degradation and transformation, increased therapeutic index of drug, flexibility in coupling with targeting and imaging ligands, improved pharmacodynamic profiles compared to free drugs, *etc*. Liposome-based formulations of several antineoplastic drugs such as daunorubicin, amphotericin-B, doxorubicin, lurtotecan, nystatin, annamycin *etc.* [3] are currently available on the market. Recently, a novel class of antitumor drugs has been discovered. This class is represented by europium coordination complexes (EC). EC belong to a wide group of lanthanide complexes, which are of particular interest for biomedical investigations and diagnostics. The antineoplastic activity of EC was shown to be determined by their unique structural properties – they contain 1,10-phenantroline or 2,2'-bipyridine, the DNA-intercalating motifs, responsible for EC antineoplastic activity. We have shown previously, that these drugs do not affect significantly lipid bilayer properties [4] accentuating the possibility of the successful design of EC liposomal formulations. Another prerequisite is strong lipid-associating ability of the drug which would ensure the high stability of the complex during the transportation to the target tissue. The present work has been undertaken

to assess the affinity of EC for the model lipid membrane composed of phosphatidylcholine (PC). Fluorescence quenching of *p*-terphenyl has been utilized as an analytical tool.

MATERIALS AND METHODS

Egg yolk phosphatidylcholine was purchased from Biolek (Kharkov, Ukraine). Fluorescent probe *p*-terphenyl was from Signe (Latvia). Eu (III) coordination complexes, V6 and V8 (Fig. 1), were synthesized as described previously [5]. Lipid vesicles composed of PC were prepared using the extrusion technique [6]. Appropriate amounts of lipid stock solution in ethanol:methanol mixture was evaporated to dryness under vacuum for 1.5 h to remove any residual solvent. The obtained lipid films were subsequently 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 (Nucleopore, Pleasanton, CA). Fluorescence measurements were performed with LS-55 spectrofluorimeter (Perkin Elmer, Great Britain) equipped with magnetically stirred, thermostated cuvette holder. *p*-terphenyl excitation and emission wavelengths were 280 and 358 nm, respectively.

RESULTS AND DISCUSSION

The chemical structure of analyzed europium chelates is presented in Fig. 1.

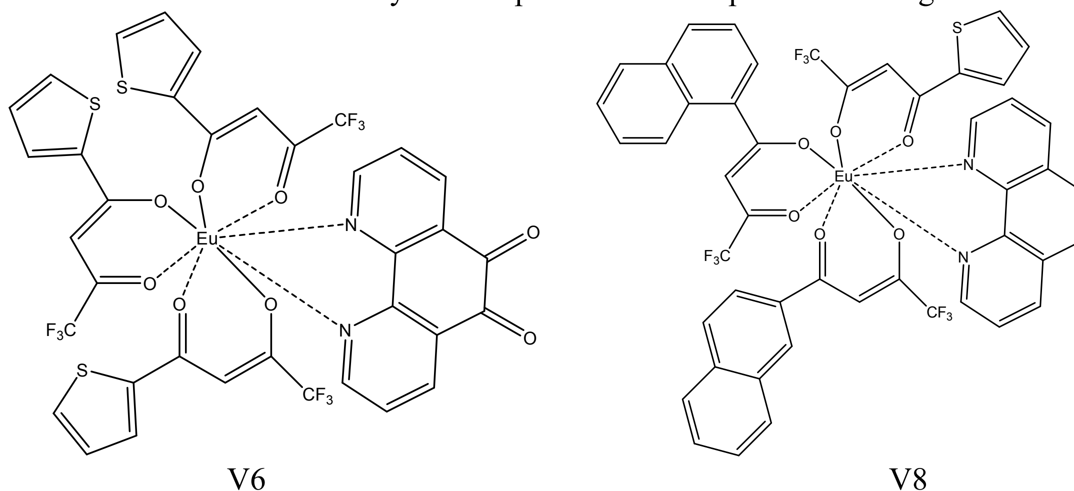

Fig. 1. Chemical structures of europium chelates.

To explore the interactions between the drugs under study and PC lipid bilayer we used the fluorescence quenching of membrane probe *p*-terphenyl (*p*-TF) (Fig. 2).

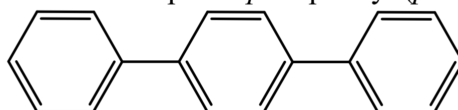
Fig. 2. Chemical structure of fluorescent probe *p*-terphenyl.

p-TF is a highly lipophilic compound, residing in the hydrophobic region of the membrane. At the first stage of our study the efficiency of *p*-terphenyl incorporation into PC bilayer was examined. Partition coefficient was obtained from the probe fluorimetric titration with liposomes and was found to be *ca.* $(1.5\pm0.4)\times10^4$. Such value of probe partition coefficient suggests high *p*-terphenyl affinity for the lipid phase.

As seen from Fig. 3, increasing concentration of EC resulted in the decrease of fluorescence of membrane-incorporated *p*-TF, with the magnitude of this effect being dependent on lipid concentration. These data indicate that europium chelates V6 and V8 represent the effective quenchers of *p*-terphenyl fluorescence. The mechanism of the revealed

quenching is, most probably, spin-orbit coupling and intersystem crossing to the triplet state [7].

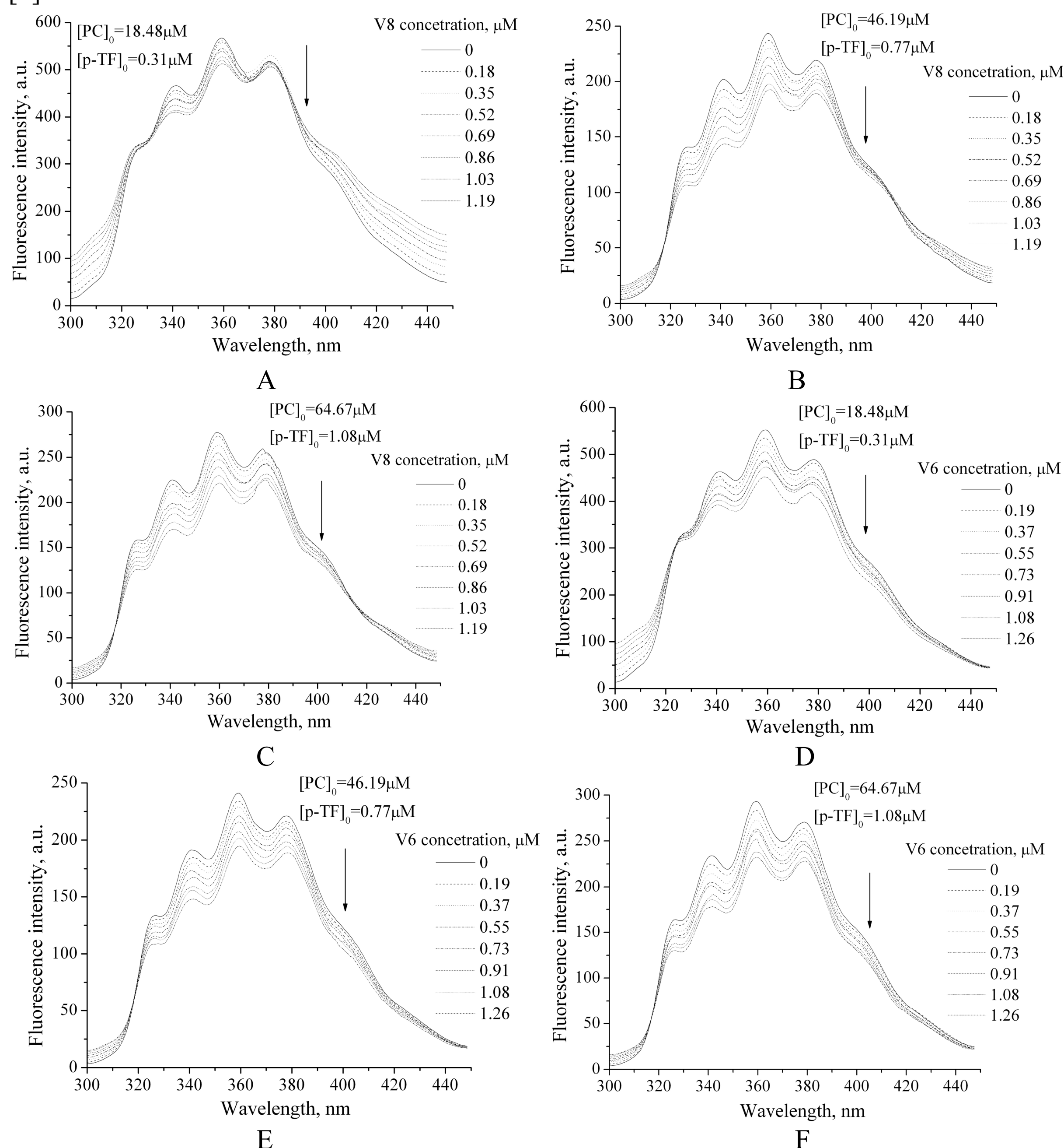

Fig. 3. Fluorescence spectra of *p*-terphenyl in suspension of PC liposomes at varying V8 (A, B, C) and V6 (D, E, F) concentrations.

Fluorescence quenching provides a valuable source of information on the different properties of biosystems. Quenching studies can be used to reveal the localization of fluorophores in proteins and membranes, and their permeabilities for quenchers. Both static and dynamic quenching requires the molecular contact between the fluorophore and quencher. In the case of collisional quenching, the quencher must diffuse to the fluorophore during the lifetime of the excited state. Upon the interactions between the probe and the quencher, the fluorophore returns to the ground state, without the emission of a photon. In static quenching a complex is formed between the fluorophore and the quencher, and this complex is

nonfluorescent. Collisional quenching of fluorescence is described by the Stern-Volmer equation:

$$\frac{F_0}{F} = 1 + k_Q \tau_0 [Q], \tag{1}$$

where F_0 and F are the fluorescence intensities in the absence and presence of a quencher, respectively; k_Q is the bimolecular quenching constant; τ_0 is the lifetime of the fluorophore in the absence of a quencher, and $[Q]$ is the concentration of a quencher.

To clarify the observed quenching of membrane-bound probe, it is necessary to evaluate the lipid-water partition coefficient of EC. V6 and V8 are highly hydrophobic complexes, tending to locate in the nonpolar membrane region. This process results in the increase in frequency of collisions between the drug and probe molecules. EC molecules tend to distribute between water and lipid phase. According to a commonly used methodology [6, 7], partition coefficient is determined as a ratio between quencher concentrations in lipid ($[Q_L]$) and water ($[Q_W]$) phases:

$$P = [Q_L]/[Q_W]. \tag{2}$$

The concentration of quencher in lipid phase can be determined according to the following equation:

$$[Q_L] = \frac{P[Q]}{P\alpha_m + 1 - \alpha_m}, \tag{3}$$

where $\alpha_m = V_m / V_T$ is the volume fraction of membrane phase, V_T is the total volume of the system. Substitution of this expression into the Stern-Volmer equation (eq. (1)) gives the expression:

$$\frac{F_0}{F} = \frac{\tau_0}{\tau} = 1 + \frac{\tau_0 k_m P[Q]}{P\alpha_m + 1 - \alpha_m} = 1 + \tau_0 k_{app}[Q], \tag{4}$$

where k_m is the bimolecular quenching constant for the membrane-bound fluorophore, which reflects the efficiency of the quenching or the accessibility of the fluorophore to the quencher; $k_{app} = k_m P / (P\alpha_m + 1 - \alpha_m)$ is the apparent quenching constant. It should be mentioned, that this parameter depends on the volume fraction of membrane phase α_m (and lipid concentration):

$$\frac{1}{k_{app}} = \alpha_m \left(\frac{1}{k_m} - \frac{1}{k_m P} \right) + \frac{1}{k_m P}. \tag{5}$$

Taking into account the last expression k_m and P can be obtained from the dependence of k_{app}^{-1} on α_m.

The obtained Stern-Volmer plots for V6 and V8 are shown in Fig. 4. The linearity of these plots suggests that *p*-terphenyl fluorescence quenching results from collisions between probe and quencher molecules (dynamic quenching) [7].

Approximation of the experimental results by eq. (4) allowed us to determine the apparent Stern-Volmer constants ($k_{SV}^{app} = \tau_0 k_{app}$) as a slope of linear curve. The values of k_{SV}^{app} were divided on the *p*-terphenyl fluorescence lifetime (1 ns, [7]) to calculate k_{app}. This parameter exhibits nearly linear dependence on lipid concentration. The results of $k_{app}(\alpha_m)$ approximation by eq. (5) are presented in the Table 1.

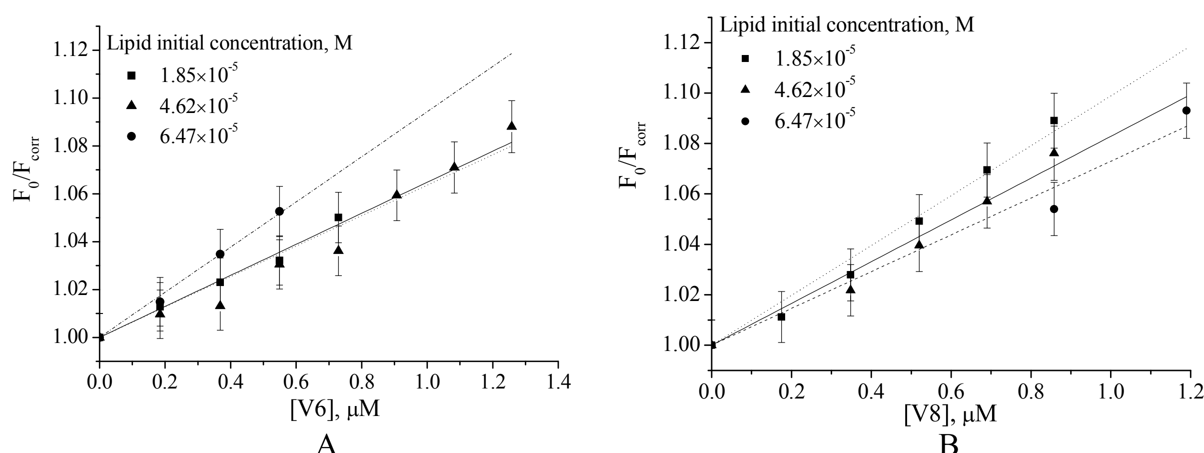

Fig. 4. Stern-Volmer plots of *p*-terphenyl quenching by V6 (A) and V8 (B) in PC liposomes.

Table 1. Parameters of *p*-terphenyl quenching by europium chelates

Europium chelate	Bimolecular quenching constant, $M^{-1}s^{-1}$	Partition coefficient
V6	$(9.99 \pm 2.49)\times10^{9}$	$(5.55 \pm 1.39)\times10^{3}$
V8	$(2.44 \pm 0.61)\times10^{10}$	$(2.13 \pm 0.53)\times10^{4}$

As judged from Table 1, partition coefficients of europium chelates into PC bilayer appear to be rather high. The values of bimolecular quenching constants and partition coefficients for V6 and V8 differ because of distinctions in their chemical structures. Since EC are nonpolar compounds, their embedment into the hydrophobic bilayer region seems to be the most probable mode of drug-membrane interactions. EC can be efficiently incorporated into PC liposomes, increasing thereby drug efficiency and stability.

CONCLUSIONS

Fluorescence quenching technique has been used to analyze the interaction between europium chelates – highly promising potential antineoplastic drugs – and model lipid membranes. Quantitative characteristics of drugs partitioning into membrane were determined. It was demonstrated that quenching of *p*-terphenyl fluorescence by europium coordination complexes occured according to dynamic mechanism. The obtained results suggest a possibility of designing liposome-based EC nanocarriers, thereby opening new perspectives for effective therapy of oncological diseases. To summarize, our results provide direct evidence for incorporation of europium chelates into nonpolar region of PC model membranes. The recovered ability of EC to quench *p*-terphenyl fluorescence creates a basis for development of the novel drug screening assays.

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