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ORIGINAL ARTICLE

Liposomal Co-Encapsulation of Two Novel Europium Complexes and Doxorubicin: Fluorescence Study

Valeriya M. Trusova[1,2] · **Todor Deligeorgiev**[3] · **Galyna Gorbenko**[1]

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Abstract The present study was undertaken to design the novel liposomal drug formulation containing doxorubicin and europium coordination complexes. It was shown that co-encapsulation of the drugs facilitates the partitioning and permeation of lanthanides into the lipid bilayer. The obtained results suggest that new drug platform may have potential application in the design of novel antitumor agents.

Keywords Liposomal drug formulation · Drug combination · Doxorubicin · Lanthanides

Introduction

During the last decades biomedical research has been revolutionized by the development of novel nanostructured molecular systems for targeted delivery of various drugs [1, 2]. Of these, liposomes represent the most promising drug carrier owing to their biocompatibility, biodegradability and the lack of immunogenicity [3, 4]. Encapsulation of drugs into the lipid vesicles reduces the distribution volume, prolongs circulation lifetime of the drugs, decreases toxic side effects in healthy tissues, ensures targeted delivery of the pharmaceuticals, etc. [5]. Recently, the accent in the field of drug delivery has shifted from single-drug systems to the development of formulations combining several active agents. This strategy is widely used in anticancer therapy and is based on the design of liposomal nanovehicles containing two or more co-encapsulated drugs with different mechanisms of action. It was demonstrated that liposomal co-encapsulation of the drugs results in several beneficial consequences: i) synchronized drug distribution; ii) delivery of drug combinations at a specific ratio; iii) supra-additive anticancer activity due to therapeutic synergism of the loaded agents; iv) targeting of multiple oncogenic routes [6–8]. Promising clinical potential has been reported for a variety of drug combinations, among which are alendronate/doxorubicin [9], cisplatin/daunorubicin [10], irinotecan/floxuridine [11], doxorubicin/C_{60} fullerene [12], just to name a few. In the present study we concentrated our efforts on evaluating the possibility of liposomal co-encapsulation of classical anticancer drug doxorubicin and novel antineoplastic agents, the representatives of europium chelates, referred to here as V6 and V9 (Fig. 1). Liposomes were composed of zwitterionic lipid phosphatidylcholine (PC) and its mixture with anionic lipid cardiolipin (CL) in a molar ratio 9:1.

Materials and Methods

Chemicals

1-palmitoyl −2-oleoyl-*sn*-glycero-3-phosphocholine (PC) and beef heart cardiolipin (CL) were from Avanti Polar Lipids (Alabaster, AL). Europium complexes V6 and V9, and squaraine dye SQ-1 were synthesized at the Faculty of Chemistry, University of Sofia. Laurdan (6-Lauroyl- 2-dimethylaminonaphthalene) was from Invitrogen Molecular Probes (Eugene, OR, USA).

✉ Valeriya M. Trusova
valeriya.m.trusova@gmail.com

[1] Department of Nuclear and Medical Physics, V.N. Karazin Kharkov National University, 4 Svobody Sq, Kharkov 61072, Ukraine

[2] 19-32 Geroyev Truda Str, Kharkov 61072, Ukraine

[3] Department of Applied Organic Chemistry, Faculty of Chemistry, University of Sofia, 1164 Sofia, Bulgaria

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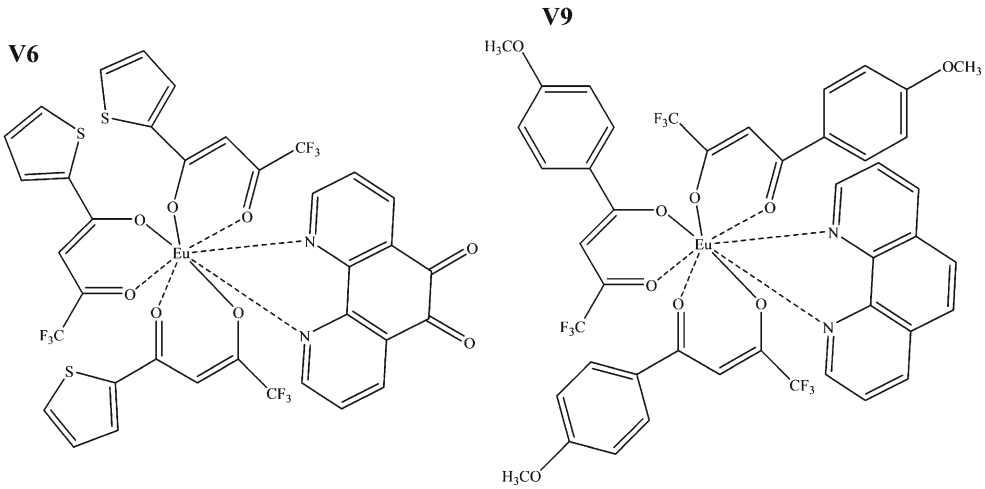

Fig. 1 Chemical structures of V6 and V9

Preparation of Lipid Vesicles

Large unilamellar vesicles were prepared by extrusion from PC mixtures with CL (10 mol%). A thin lipid film was 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 5 mM sodium phosphate buffer, pH 7.4 at room temperature to yield lipid concentration of 1 mM. Thereafter, the sample was subjected to 15 passes through a 100 nm pore size polycarbonate filter (Millipore, Bedford, USA), yielding liposomes of desired composition.

Fluorescence Measurements

Steady-state fluorescence spectra were recorded with LS-55 spectrofluorometer equipped with a magnetically stirred, thermostated cuvette holder (Perkin-Elmer Ltd., Beaconsfield, UK). Fluorescence measurements were performed at 20 °C using 10 mm path-length quartz cuvettes. Emission spectra were recorded with excitation wavelengths of 364 nm (Laurdan), 640 nm (SQ-1) and 450 nm (Dox). Excitation and emission band passes were set at 10 nm.

Results and Discussion

While doxorubicin (Dox) is a mainstay in cancer therapy, europium (Eu) tris-β-diketonates belong to the novel class of antitumor drugs. Pioneering studies of Momekov et al. showed that the major determinant of cytotoxic activity of europium chelates is the abundance of DNA-intercalating motifs [13]. The first step of our study was directed towards the analysis of lanthanide partitioning into the neat and Dox-loaded liposomes. To this end, fluorescence quenching method was utilized. As illustrated in Fig. 2a, fluorescence intensity of the membrane probe Laurdan decreased upon addition of lanthanides, suggesting that these compounds are capable of partitioning into lipid phase where they serve as the quenchers for membrane-embedded Laurdan. The representative Stern-Volmer plots are given in Fig. 2b. Quantitative

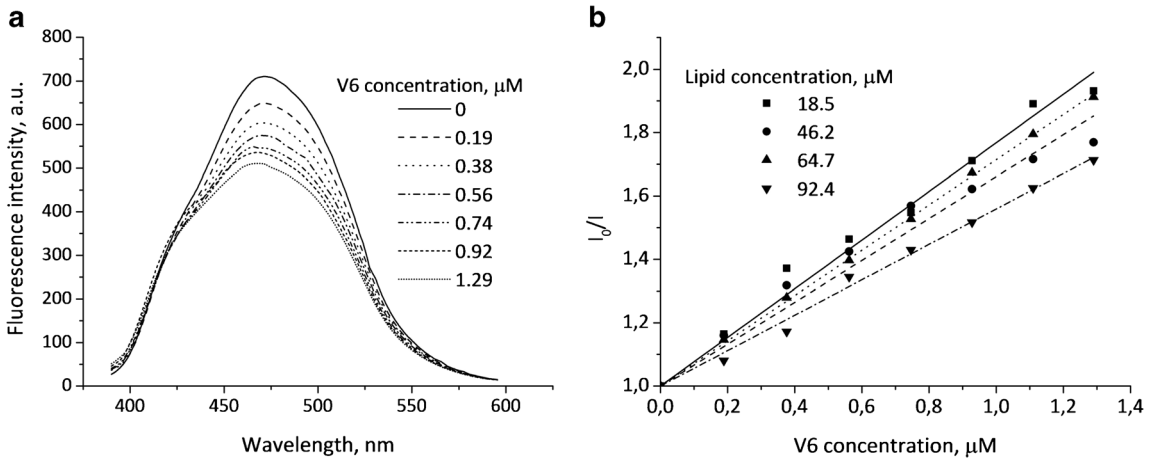

Fig. 2 Fluorescence spectra of Laurdan in PC lipid vesicles (**a**). Lipid concentration was 92.4 μM. Stern-Volmer plots for Laurdan quenching by V6 (**b**). Laurdan concentration 0.01 μM

Table 1 Parameters of lanthanide-lipid complexation. The subscript $_{Dox}$ denotes lipid vesicles doped with doxorubicin. CL10 corresponds to liposomes containing 10 mol% CL

Drug	Liposomes	K_p	k_m, M^{-1} s^{-1}	P_e, cm s^{-1}
V6	PC	$(9.13 \pm 0.91) \times 10^3$	$(3.71 \pm 0.62) \times 10^9$	$(7.2 \pm 0.66) \times 10^{-5}$
	CL10	$(2.48 \pm 0.28) \times 10^4$	$(9.12 \pm 0.45) \times 10^8$	$(1.8 \pm 0.11) \times 10^{-4}$
	PC_{Dox}	$(2.28 \pm 0.52) \times 10^4$	$(8.28 \pm 0.93) \times 10^8$	$(2.3 \pm 0.15) \times 10^{-4}$
	$CL10_{Dox}$	$(9.62 \pm 0.73) \times 10^4$	$(7.11 \pm 1.01) \times 10^8$	$(8.1 \pm 0.74) \times 10^{-4}$
V9	PC	$(1.13 \pm 0.17) \times 10^4$	$(2.15 \pm 0.27) \times 10^9$	$(9.7 \pm 0.89) \times 10^{-5}$
	CL10	$(3.44 \pm 0.96) \times 10^4$	$(7.43 \pm 0.85) \times 10^8$	$(2.1 \pm 0.17) \times 10^{-4}$
	PC_{Dox}	$(4.72 \pm 0.97) \times 10^4$	$(5.11 \pm 0.74) \times 10^8$	$(3.6 \pm 0.42) \times 10^{-4}$
	$CL10_{Dox}$	$(9.81 \pm 0.84) \times 10^4$	$(4.13 \pm 0.37) \times 10^8$	$(9.4 \pm 0.93) \times 10^{-4}$

analysis of the obtained results in terms of the combined quenching/partitioning model [14], described in details in [15], allowed extracting the coefficient of lanthanide partitioning into the lipid phase (K_P) and bimolecular rate constant (k_m) for Laurdan fluorescence quenching.

Next, the obtained k_m values were used for evaluating the permeability coefficients (P_e) of the examined Eu chelates based on Smoluchowski equation and solubility-diffusion model [16]. The recovered values of K_P, $k_m$and P_e are summarized in Table 1. As can be seen, partition coefficients of V6 and V9 have a magnitude about 10^3–10^4 indicating that the examined Eu chelates can be efficiently entrapped by the lipid phase. In addition, the values of permeability coefficients (10^{-5}–10^{-4} cm s^{-1}) suggest that liposomal membranes are highly permeable for lanthanides. Pre-incubation of lipid vesicles with Dox resulted in the rise in $K_P$and P_e of V6 and V9 (Table 1) with the magnitude of this effect being more significant in CL-containing membranes.

In the present context it should be noted that the observed impact of Dox on the membrane affinity of V6 and V9 may be induced either by direct interactions between Dox and Eu chelates or via influence of Dox on the lipid bilayer structure. To differentiate between these two possibilities, in a separate set of experiments we tried to characterize the relative location of the examined drugs within the lipid vesicles. To this end, liposomal formulations of Dox were titrated with V6. It appeared that fluorescence intensity of Dox undergoes very slight changes upon adding the Eu chelate (Fig. 3).

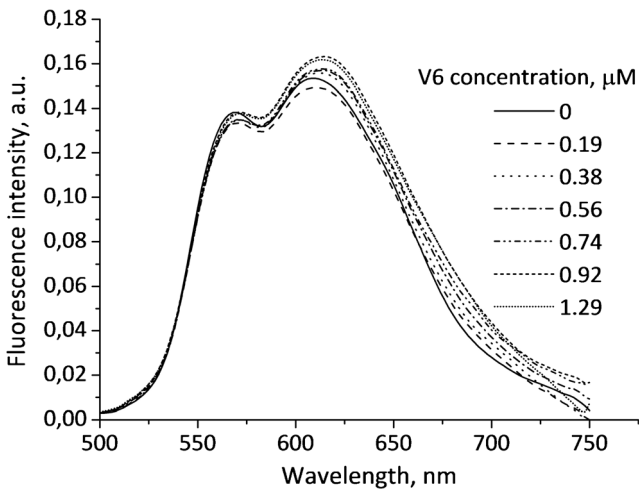

Fig. 3 Doxorubicin fluorescence spectra in PC membranes at varying concentration of V6. Lipid and doxorubicin concentrations were 64.7 and 2.3 μM, respectively

The absence of Dox fluorescence quenching by V6 may indicate that one of these agents resides in the interior water phase of the liposomes rather than within the lipidic one. According to our previous studies [15], due to their hydrophobic nature the lanthanides tend to distribute in the nonpolar region of lipid bilayer. Therefore, we assume that Dox accumulates in the aqueous phase of the lipid vesicles, presumably at the lipid/water interface. To gain additional argument in favor of this idea, emission spectra of Dox-doped lipid membranes were recorded at varying concentrations of squaraine fluorescent probe SQ-1. Since the absorption spectrum of SQ-1 overlaps with the fluorescence spectrum of Dox, one might expect the occurence of the Förster resonance energy transfer (FRET) between these compounds. The addition of SQ-1 to the Dox + liposome system was accompanied by the appearance of emission peak at 680 nm without the changes in the Dox fluorescence intensity (Fig. 4a). Analogous effect was observed upon the titration of the neat lipid vesicles with SQ-1 (Fig. 4b). Thus, it may be concluded that the observed spectral changes reflect direct excitation of SQ-1, rather than FRET.

The absence of energy transfer between Dox and SQ-1 implies that these compounds are located at a distance exceeding the characteristic distance for FRET (~10 nm). This scenario can be realized when either donor or acceptor resides preferentially in the aqueous phase of the lipid vesicles. Taken together with the above quenching data, the results obtained for Dox-SQ-1 systems suggest that Dox and lanthanide molecules are far enough from each other for direct interactions between them to occur, so Dox-induced facilitation of the membrane partitioning of Eu complexes most probably arises from the Dox effect on lipid bilayer structure. de Wolf et al. reported that Dox membrane binding is followed by the decrease in lipid order parameter, concomitant with the increase in the membrane free volume [17]. The authors supposed that the mechanism underlying this effect involves specific interactions between Dox and lipid headgroups. An analogous

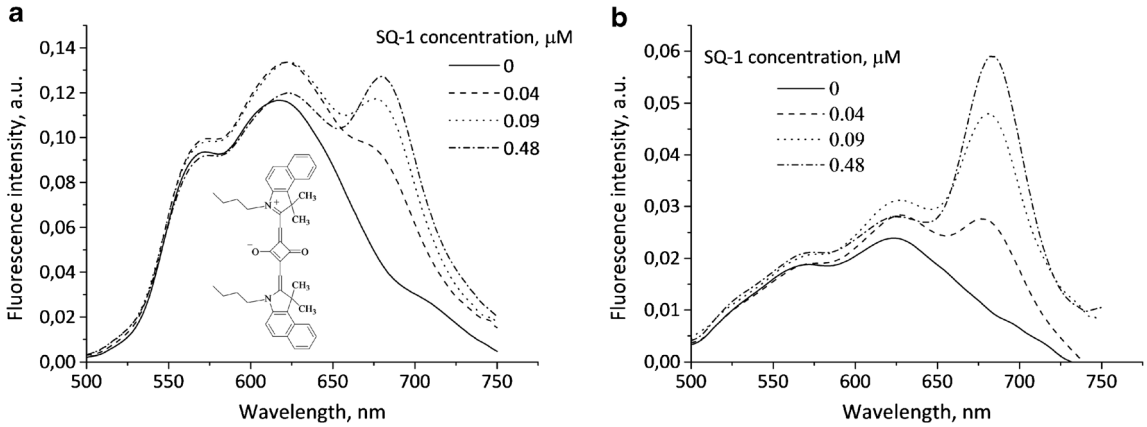

Fig. 4 Fluorescence spectra of SQ-1 bound to PC/CL bilayers in the presence (**a**) and absence (**b**) of doxorubicin. Given in the inset is the chemical structure of SQ-1. Lipid and doxorubicin concentrations were 64.7 and 2.33 μM, respectively

conclusion has been drawn by Yakoub and co-workers [18]. Specifically, based on the results of molecular dynamics simulation, it was shown that Dox specific complexation with lipid headgroups significantly perturbs the lipid bilayer structure resulting in creation of local defects, membrane curving and disordering, and enhancement of water permeation. It can be supposed that the same mechanism lies behind Dox effect on lanthanide-membrane binding observed in our systems. Apparently, accumulation of Dox in the liposome interior at the lipid/water interface is followed by the membrane destabilization allowing more extensive partitioning and permeation of Eu-chelates.

Another interesting observation is that the magnitude of Dox impact on $K_P$and P_e is more pronounced in CL-containing vesicles. Dox is known to form complexes with a variety of lipids, but the affinity of this drug for zwitterionic lipids is much lower than that for anionic lipids [17, 19, 20]. Evidently, incorporation of CL into PC bilayer increases the extent of Dox binding to liposomes, that, in turn, destabilizes the membrane structure to a greater extent, and allows more lanthanide molecules to penetrate into the lipid bilayer.

In conclusion, the present study points to the possibility of the development of novel formulation of anticancer drugs based on liposomal co-encapsulation of doxorubicin and lanthanides. Our pre-clinical data demonstrate that the presence of Dox facilitates the partitioning and permeation of Eu chelates V6 and V9 into the lipid nanocarriers. The proposed drug platform may be of particular interest in cancer therapy due to potential synergistic antitumor effect of the examined antineoplastic agents.

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