БІОФІЗИКА КЛІТИНИ

LANTHANIDE EFFECT ON PHYSICOCHEMICAL PROPERTIES OF LIPID BILAYER AS REVEALED BY INDICATOR DYE BROMOTHYMOL BLUE

A.V.Yudintsev[1], V.M.Trusova[1], G.P.Gorbenko[1], T.Deligeorgiev[2], A.Vasilev[2], N.Gadjev[2]

[1]*V.N. Karazin Kharkov National University, 4 Svobody Sq., Kharkov, 61077*
[2]*Department of Applied Organic Chemistry, Faculty of Chemistry, University of Sofia, Bulgaria*
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Development of new formulations of antineoplastic drugs currently represents extensively growing research area. Efficiency of existing anti-tumor drugs is frequently limited by their high general toxicity, metabolic instability in an organism and bad penetration into a cancer cell. Besides, insignificant direct influence on tumoral growth also limits the application of antineoplastic drugs in a free form. One efficient way of drug delivery is based on the use of lipid vesicles (liposomes). Liposomes are spherical, self-closed structures formed by one or several concentric lipid bilayers with an aqueous phase inside and between the lipid bilayers. The lipid bilayer favors solubilization of hydrophobic compounds, whereas internal aqueous phase of lipid vesicles is suitable for encapsulation of hydrophilic drugs. Design of liposomal carriers is heavily based on the evaluation of bilayer-modifying properties of the drug. This is important not only for achieving maximum payload without compromising liposome stability, but also for 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. In the present work the effect of the two potential antineoplastic drugs – europium coordination complexes (LC) – on the physicochemical properties of phosphatidylcholine (PC) model membranes has been investigated using the environmentally-sensitive pH indicator dye bromothymol blue (BTB). This dye responds to the changes in environmental conditions by the shifts of its protolytic and partition equlibria. Incorporation of LC into the lipid vesicles was found to exert no influence on the effective electrostatic potential of model membranes, i.e. the mean potential at location of the dye prototropic moiety in the interfacial region. In contrast, BTB membrane partitioning markedly enhanced in the presence of drugs, indicating that europium coordination complexes can affect molecular organization of a lipid bilayer, presumably through generation of structural defects and altering the conformation of PC headgroups. High lipophilicity of Eu(III) coordination complexes together with their relatively weak membrane-modifying propensities create prerequisites for the development of liposomal formulations of these compounds.

KEY WORDS: lanthanide complexes, liposomes, bromothymol blue

Among a wide variety of drug nanocarriers developed to date, 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, improved pharmacokinetic and pharmacodynamic profiles compared to free drugs, reduced side effects, etc [1]. The physicochemical characteristics of liposomes (size, lamellarity, surface charge, pH- and thermosensitivity, bilayer dynamic properties) can be readily manipulated. Furthermore, the possibility of bringing together therapeutic, targeting and imaging compounds makes liposomes an ideal candidate for producing nanocarriers with a number of coordinated specialized functions, optimum pharmacokinetic parameters and controlled drug release in the pathological zone in response to pH, temperature, photo or other stimuli [2]. Development of liposomal carriers is heavily based on the evaluation of bilayer-modifying properties of the drug. This is important not only for achieving maximum payload without compromising liposome stability, but also for 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 [3,4].

Considerable efforts are currently focused on liposome loading with effective antitumor agents. Of particular significance in this regard is the development of liposomal formulations of new classes of antineoplastic drugs with alternative mode of cytotoxic action and nonoverlapping mechanisms of drug resistance. One of such classes is represented by lanthanide coordination complexes whose high cytotoxic potential has been demonstrated very recently [5]. In the present work we concentrated our efforts on clarifying the effects of two Eu(III) coordination complexes, referred to here as LC1 and LC2, on physicochemical properties of the model lipid membranes. In approaching this goal pK_a-probe bromothymol blue has been employed. Phosphatidylcholine, zwitterionic lipid, abundant in most cellular membranes was used to prepare monolamellar lipid vesicles.

MATERIALS AND METHODS

Egg yolk phosphatidylcholine was purchased from Biolek (Kharkov, Ukraine). Lipid vesicles composed of PC were formed using extrusion technique [6]. 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.
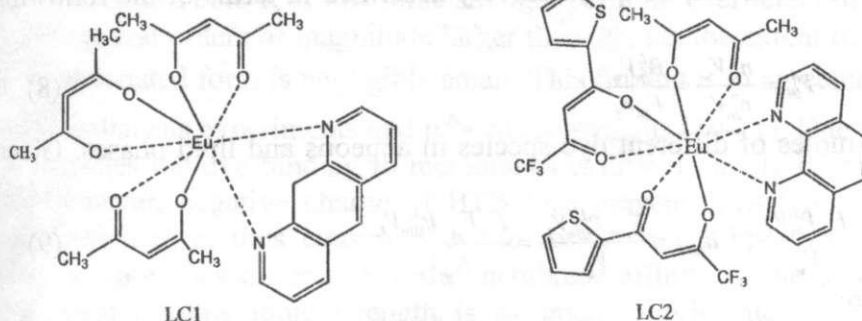

Fig. 1. Structure of lanthanide complexes.

Phospholipid concentration was determined according to the procedure of Bartlett [7]. Absorption measurements were conducted using SF-46 spectrophotometer against solvent blanks. LC1 and LC2 (Fig. 1) were synthesized as described previously [5].
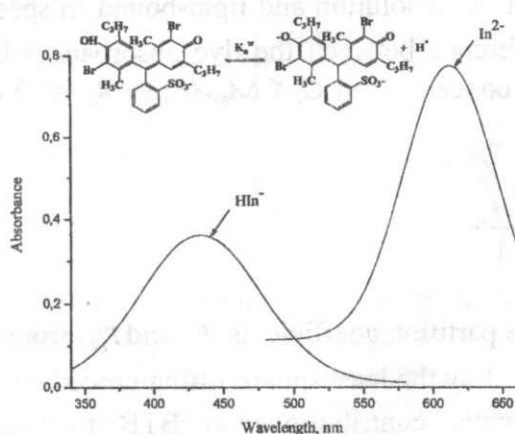
RESULTS AND DISCUSSION

Fig. 2. Absorption spectra of deprotonated and protonated forms of bromothymol blue

Acid-base indicator bromothymol blue has long been employed to probe the alterations in the structure and physicochemical properties of the proteins [8], phospholipid vesicles [9, 10-11] and biomembranes [12]. This dye responds to the changes in environmental conditions by the shifts of its protolytic and partition equilibria.

The acid-base equilibrium of bromothymol blue $HIn^- \Leftrightarrow In^{2-} + H^+$ free in solution is described by the following thermodynamic constant [10-11]:

$$K_a^w = \frac{a_{H^+} a_{In}}{a_{HIn}} \cong \frac{F_{H^+} F_{In}^0}{F_{HIn}^0} \tag{5}$$

where a_{H^+}, a_{In}, a_{HIn} are the activities of the protons, deprotonated (In) and protonated (HIn) dye forms, respectively; $F_{H^+}, F_{In}^o, F_{HIn}^o$ are the concentrations (mol dm^{-3}) of the corresponding dye species. In a buffer solution the following relationships hold:

$$D_0 = F_{In}^o + F_{HIn}^o; \qquad F_{In}^o = \frac{D_o}{1 + \frac{F_{H^+}}{K_a^w}} \tag{6}$$

where D_o is the total dye concentration. Since pK_a^w is ca. 7 [10-11], at neutral pH BTB solution represents a mixture of protonated (HIn$^-$) and deprotonated (In^{2-}) forms having absorption maxima at 420 and 617 nm, respectively (Fig. 2). In a suspension of lipid vesicles the above protolytic equilibrium is shifted due to the dye redistribution between aqueous (w) and lipid (L) phases. Accordingly, D_o can be written as a sum of concentrations of free in solution (F) and lipid-bound (B) dye species:

$$D_0 = F_{In} + F_{HIn} + B_{In}^L + B_{HIn}^L \tag{7}$$

Membrane association of BTB can be quantitatively described in terms of the following partition coefficients:

$$P_{HIn}^L = \frac{n_{HIn}^L V_w}{n_{HIn}^w V_L} = \frac{B_{HIn}^L V_w}{F_{HIn} V_L}; \qquad P_{In}^L = \frac{n_{In}^L V_w}{n_{In}^w V_L} = \frac{B_{In}^L V_w}{F_{In} V_L} \tag{8}$$

where n is the number of moles of different dye species in aqueous and lipid phases. Given that

$$F_{HIn}^w = \frac{F_{In} F_{H^+}}{K_a^w}; \qquad B_{In}^L = \frac{F_{In} P_{In}^L V_L}{V_w}; \qquad B_{HIn}^L = \frac{F_{HIn} P_{HIn}^L V_L}{V_w} = \frac{F_{In} F_{H^+} P_{HIn}^L V_L}{K_a^w V_w} \tag{9}$$

Eq. 7 can be transformed to:

$$D_0 = F_{In} + \frac{F_{In} F_{H^+}}{K_a^w} + \frac{F_{In} P_{In}^L V_L}{V_w} + \frac{F_{In} F_{H^+} P_{HIn}^L V_L}{K_a^w V_w} = F_{In}\left(1 + \frac{F_{H^+}}{K_a^w} + \frac{P_{In}^L V_L}{V_w} + \frac{P_{HIn}^L F_{H^+} V_L}{K_a^w V_w}\right) \tag{10}$$

The process of BTB partitioning into lipid phase can be examined through monitoring the absorbance changes of the In or HIn species. The results presented here have been obtained by performing BTB spectrophotometric measurements at the wavelength 617 nm, where the absorbances measured in buffer solution (A_o) or liposomal suspension (A_L) are determined exclusively by the deprotonated dye form:

$$A_o = \varepsilon_f F_{In}^o; \qquad A_L = \varepsilon_f F_{In} + \varepsilon_b^L B_{In}^L \tag{11}$$

where $\varepsilon_f, \varepsilon_b^L$ are the extinction coefficients of the free in solution and lipid-bound In species at 617 nm. By combining the Eqs. 6–11 the difference between the dye absorbances in a buffer and liposomal suspension at a certain lipid concentration C_L ($\Delta A_{617}(C_L) = A_o - A_L$) can be written as:

$$\Delta A_{617}(C_L) = \frac{\left(\varepsilon_f - \varepsilon_b^L\right) D_o P_{In}^L \dfrac{V_L}{V_w} + \left(\varepsilon_f P_{HIn}^L - \varepsilon_b^L P_{In}^L\right) D_o \dfrac{F_{H^+}}{K_a^w} \dfrac{V_L}{V_w}}{\left(1 + \dfrac{F_{H^+}}{K_a^w}\right)\left(1 + \dfrac{F_{H^+}}{K_a^w} + P_{In}^L \dfrac{V_L}{V_w} + P_{HIn}^L \dfrac{F_{H^+}}{K_a^w} \dfrac{V_L}{V_w}\right)} \tag{12}$$

here $F_{H^+} = 10^{-pH}$. Eq. 12 was employed to derive the partition coefficients P_{In}^L and P_{HIn}^L from the experimental dependencies $\Delta A_{617}(C_L)$ shown in Fig. 3 by the least square fitting procedure. To separate Coulomb electrostatic and nonelectrostatic contributions to BTB membrane partitioning, spectrophotometric titrations of BTB with liposomes have been conducted under the conditions of low (5mM) and high (2M) ionic strength.
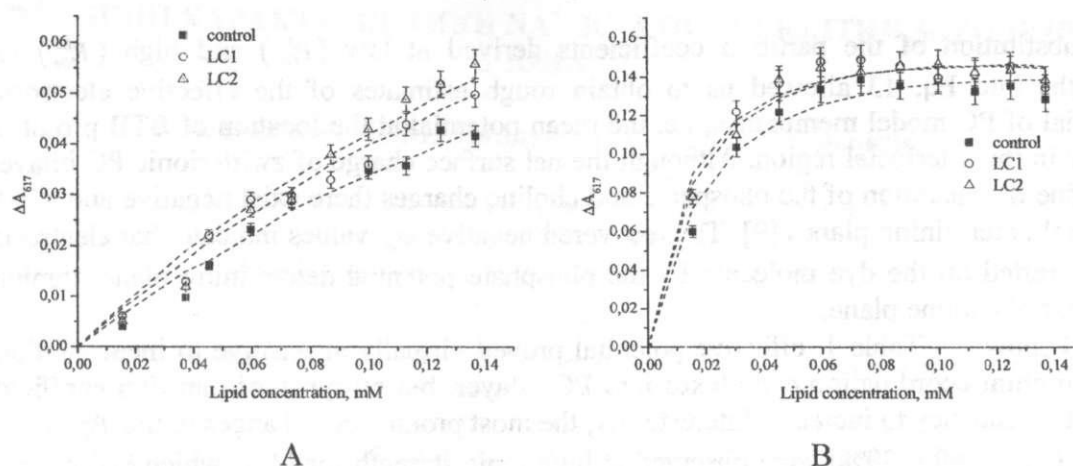
Fig. 3. Absorbance changes of the protonated BTB form as a function of lipid concentration at ionic strengths 5 mM (A) and 2 M (B). Drug concentration was 8 μM (LC1) or 6 μM (LC2). Dash lines represent theoretical curves calculated from Eq. 12

As seen in Table 1, at low ionic strength partition coefficient of the protonated dye form, P_{HIn}^{L}, is several orders of magnitude larger than P_{In}^{L}, i.e. the extent of membrane association of the deprotonated form is negligibly small. This finding is in agreement with the results of our previous dialysis experiments and pK_a measurements [10-11]. Due to high hydrophobicity of BTB species the dye binding to membranes is driven mainly by non-ionic interactions [10-12]. However, negative charge of BTB ions prevents penetration of the dye species into hydrophobic core, thus causing them to reside in the interfacial bilayer region [9]. The observed enormous difference in the membrane affinity of the deprotonated and protonated BTB forms at low ionic strength is assumed to originate from the nearly even charge distribution over the In^{2-} ions, which renders this dye form incapable of forming hydrophobic contacts [12].

Table 1

Effect of europium coordination complexes on partition coefficients of the protonated and deprotonated forms of bromothymol blue The accuracy of the partition coefficients falls in the range 12-15%.

	P_{In}^{L}	P_{HIn}^{L}	P_{In}^{L}	P_{HIn}^{L}	Ψ, V
	Ionic strength 5 mM		Ionic strength 2 M		
PC liposomes	4.3	5.6×10^3	2.1×10^4	1.2×10^5	-0.079
PC + LC1	0.02	6.7×10^3	3.2×10^4	1.7×10^5	-0.085
PC + LC2	1.02	7.3×10^3	2.7×10^4	1.5×10^5	-0.079

Nevertheless, membrane affinity of In species drastically enhances upon Debye-Huckel screening of the interacting charges by elevating ionic strength. Likewise, partition coefficient of HIn form increases by more than order of magnitude upon changing monovalent salt concentration from 5mM to 2M. The partition coefficient of this form can be represented as a product of non-electrostatic (P_{HIn}^{Lo}) and electrostatic terms:

$$P_{HIn}^{L} = P_{HIn}^{Lo} \exp\left(-z_{HIn}e\psi_{el} / kT\right) \tag{13}$$

Substitution of the partition coefficients derived at low (P_{HIn}^{L}) and high (P_{HIn}^{Lo}) ionic strengths into Eq. 13 allowed us to obtain rough estimates of the effective electrostatic potential of PC model membranes, i.e. the mean potential at the location of BTB prototropic moiety in the interfacial region. Although the net surface charge of zwitterionic PC bilayer is zero, due to separation of the phosphate and choline charges there exist negative and positive potential-determining planes [9]. The recovered negative ψ_{el} values indicate that electrostatic force exerted on the dye molecule by the phosphate potential-determining plane dominates over that of choline plane.

As shown in Table 1, effective potential proved virtually insensitive to incorporation of the europium coordination complexes into PC bilayer, but all types of partition coefficients exhibited tendency to increase. Interestingly, the most pronounced changes in the P_{HIn}^{L} and P_{HIn}^{Lo} values (by ca. 40 – 50%) were observed at high ionic strength for LC1, which is featured by much higher membrane partitioning efficiency. The modifying effect of LC on BTB-lipid interactions can be attributed to the drug-induced bilayer disordering coupled with increased accessibility of hydrophobic membrane region to the dye. On the other hand, allowing for possible contributions to energetics of membrane partitioning (Eq. 4), it cannot be ruled out that enhanced association of HIn^{-} and In^{2-} ions with PC bilayer arises from the drug effect on the membrane dipole potential. Incorporation of LC into the lipid bilayer could alter the orientation of ester carbonyl and phosphocholine dipoles and exert influence on the interfacial hydration through the formation of structural defects, thereby increasing positive value of the dipole potential.

CONCLUSIONS

Examining the lanthanide effect on the physicochemical properties of PC bilayer with pH indicator bromothymol blue revealed that the agents under investigation do not exert influence on the mean potential at location of the dye prototropic moiety in the interfacial region. Yet, BTB membrane partitioning markedly enhanced in the presence of drugs, indicating that europium coordination complexes can affect molecular organization of a lipid bilayer, presumably through generation of structural defects and altering the conformation of PC headgroups.

High lipophilicity of Eu(III) coordination complexes together with their relatively weak membrane-modifying propensities create prerequisites for the development of liposomal formulations of these compounds.

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