



## Chlorpromazine-membrane interactions as revealed by $pK_a$ and fluorescent probes

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The effect of the cationic drug chlorpromazine (CPZ) on the structural state and physico-chemical properties of model lipid membranes composed of the zwitterionic phospholipid phosphatidylcholine (PC) and the anionic phospholipid cardiolipin (CL) at molar ratios 19:1, 9:1 and 4:1 has been investigated using the  $pK_a$  probe Neutral Red (NR) and the fluorescent membrane probes pyrene and 1,6-diphenyl-1,3,5-hexatriene (DPH). CPZ incorporation into the PC:CL lipid bilayers was followed by an increase of the NR partition coefficients. This effect was interpreted in terms of drug-induced enhancement of membrane hydration and alterations in headgroup molecular packing. Analysis of pyrene excimerization and DPH anisotropy data provided further evidence for the ability of CPZ to promote bilayer condensation.

**Keywords:** chlorpromazine, DPH, lipid bilayer,  $pK_a$  probes, pyrene

### 1. INTRODUCTION

Despite extensive research efforts, the exact molecular mechanisms of anaesthesia are far from being fully understood. A wide variety of existing theories diverge along two apparently incompatible lines: one, predicted by the Meyer-Overton rule, assumes that membrane lipids are the target for anaesthetic compounds and that drug potency strongly correlates with their solubility in the lipid environment, while the other suggests that the drugs bind directly to the proteins whose altered conformation subsequently determines the anaesthetic action [1, 2]. However, the above hypotheses could not provide a satisfactory explanation for the exact mechanism of anaesthesia. During the last decades the idea implying the indirect action of drugs on the proteins via perturbations of the lipid bilayer including phase separation, change in order parameter, curvature, lateral pressure, etc. is becoming generally recognized [3].

To develop a unique conception of anaesthesia further investigations of drug-membrane interactions are required. In clarifying the molecular mechanisms of anaesthesia, model lipid and protein-lipid membrane systems appear to be particularly suitable. The present study was undertaken to explore the effect of the amphipathic phenothiazine derivative chlorpromazine (CPZ) on the structural state of model lipid membranes composed of the zwitterionic phospholipid phosphatidylcholine (PC) and the anionic phospholipid cardiolipin (CL). To evaluate the drug effect on the polar region of lipid bilayers, the  $pK_a$  probe Neutral Red (NR), whose acid-base behaviour is environmentally sensitive, has been employed, while the CPZ influence on the bilayer's hydrophobic part has

been explored using the fluorescent membrane probes pyrene and 1,6-diphenyl-1,3,5-hexatriene (DPH).

CPZ is an antipsychotic, antagonistic drug, which apart from its traditional medical usage has also been employed as an anticancer agent [4]. Accumulating evidence indicates that chlorpromazine association with membranes is a versatile process. Specifically, anaesthetic action on microsomal cells was reported to involve membrane protection against loss of fluidity, while in erythrocytes CPZ causes concave membrane bending with formation of stomatocytes [5]. It is assumed that this drug exerts its influence via the perturbations of bilayer integrity and modulation of membrane physico-chemical characteristics. The vast majority of studies suggests that CPZ-induced modification of membrane properties may include changes in the conformation of lipid acyl chains (*trans-gauche* isomerization), membrane curvature, microheterogeneity (phase separation, domain formation) and membrane thickness, just to name a few. The magnitude and sign of all these effects varies with lipid bilayer composition [1]. Deeper understanding of CPZ-lipid interactions is also crucial for the design of liposome-based drug carriers with reduced undesirable side effects, because CPZ lipophilicity favours its nonspecific association with biological membranes.

### 2. MATERIALS AND METHODS

#### 2.1. Materials

Egg yolk phosphatidylcholine and beef heart cardiolipin were purchased from Bielek (Kharkov, Ukraine). Both phospholipids gave single spots by thin layer chromatography in the solvent system chloroform:methanol:acetic acid:water, 25:15:4:2 v/v. Chlorpromazine was from Sigma (St. Louis, USA). NR was from Zonde (Latvia). All other chemicals were of analytical grade.

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## 2.2. Preparation of lipid vesicles

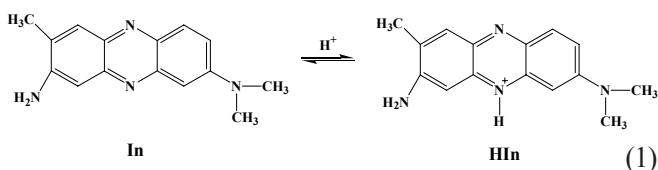
Unilamellar lipid vesicles composed of PC mixtures with 5, 10 and 20 mol % CL were prepared by the extrusion method [6]. Appropriate amounts of lipid stock solutions were mixed in ethanol, evaporated to dryness under a gentle nitrogen stream, and then left under reduced pressure for 1.5 h to remove any residual solvent. The obtained thin lipid films were hydrated with 1.2 mL of 5 mM Na-phosphate buffer (pH 7.4) at room temperature to yield a final lipid concentration of 10 mM. Thereafter lipid suspensions were extruded through a 100 nm pore size polycarbonate filter (Nucleopore, Pleasanton, California). The phospholipid concentration was determined according to the procedure of Bartlett [7]. To incorporate CPZ into the lipid bilayers, liposomal suspensions were incubated with the anaesthetic for 30 min at room temperature to yield a final drug:lipid molar ratio of 0.1.

## 2.3. Spectroscopic measurements

The spectrophotometric and fluorescence measurements were performed in 5 mM sodium phosphate buffer, pH 7.4, at room temperature. Absorption measurements with NR were conducted using an SF-46 spectrophotometer (Kharkov, Ukraine) against solvent blanks. NR concentration was determined spectrophotometrically using the extinction coefficients of the dye in protonated (HIn) and deprotonated (In) forms at 525 nm  $\epsilon_{HIn} = 2.6 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$  and  $\epsilon_{In} = 2.4 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ . Fluorescence measurements were performed with a CM 2203 spectrofluorimeter (Solar, Belarus) equipped with a magnetically stirred, thermostated cuvette holder. Excitation wavelengths were 340 nm and 350 nm for pyrene and 1,6-diphenyl-1,3,5-hexatriene (DPH), respectively. Excitation and emission slit widths were set at 2 nm for pyrene and 10 nm for DPH anisotropy measurements. The excimer-to-monomer pyrene fluorescence intensity ratio (E/M) was determined by measuring the fluorescence intensities at the monomer (394 nm) and excimer (480 nm) peaks.

## 3. THEORETICAL BACKGROUND

At physiological pH there exists an equilibrium between the protonated (HIn) and deprotonated (In) NR forms:



The thermodynamic dissociation constant for NR in buffer solution can be written as:

$$K_d = \frac{F_{H^+} F_{In}^0}{F_{HIn}^0} \quad (2)$$

where  $F_{H^+}$ ,  $F_{In}^0$ ,  $F_{HIn}^0$  are the concentrations of the protons and the deprotonated and protonated NR forms ( $\text{mol dm}^{-3}$ ), respectively. Denoting the total dye concentration by  $D_0$  ( $4 \times 10^{-5} \text{ M}$  in our experiments) one obtains:

$$D_0 = F_{In}^0 + F_{HIn}^0, \quad (3a)$$

$$F_{In}^0 = \frac{D_0}{1 + \frac{F_{H^+}}{K_d}} \quad (3b)$$

and

$$F_{HIn}^0 = \frac{D_0}{1 + \frac{K_d}{F_{H^+}}} \quad (3c)$$

In a liposome suspension the above protolytic equilibrium is shifted due to the NR distribution between the aqueous ( $w$ ) and lipid ( $L$ ) phases. In this case  $D_0$  is given by:

$$D_0 = F_{In} + F_{HIn} + B_{In}^L + B_{HIn}^L \quad (4)$$

where  $F_{In}$  and  $F_{HIn}$  are the concentrations of deprotonated and protonated dye forms free in solution, and  $B_{In}^L$  and  $B_{HIn}^L$  are the concentrations of the deprotonated and protonated dye species, respectively, bound to the lipid vesicles. The dye partition coefficients are defined as [8]:

$$P_{HIn}^L = \frac{B_{HIn}^L v_w}{F_{HIn}^L v_L} \quad (5a)$$

and

$$P_{In}^L = \frac{B_{In}^L v_w}{F_{In}^L v_L} \quad (5b)$$

where  $v_w$  and  $v_L$  are the volumes of the aqueous and lipid phases. The volume of the lipid phase was calculated as:

$$v_L = N_A C_L \sum V_i f_i; \quad (6)$$

here  $C_L$  is the molar lipid concentration,  $f_i$  is the mole fraction of the  $i$ th bilayer constituent, and  $V_i$  is its molecular volume (taken as  $1.58 \text{ nm}^3$  and  $3 \text{ nm}^3$  for PC and CL, respectively [9]). Under the experimental conditions employed ( $C_L \leq 1 \text{ mM}$ )  $v_L$  is much less than the total volume of the system ( $v_t = 1 \text{ dm}^3$ ), so that  $v_w \approx v_t$ .

Taking into account that:

$$F_{HIn} = \frac{F_{In} F_{H^+}}{K_d}, \quad (7a)$$

$$B_{In}^L = \frac{F_{In} P_{In}^L v_L}{v_w} \quad (7b)$$

and

$$B_{HIn}^L = \frac{F_{HIn} P_{HIn}^L v_L}{v_w} = \frac{F_{In} F_{H^+} P_{HIn}^L v_L}{K_d v_w} \quad (7c)$$

eqn (4) may be rewritten as:

$$D_0 = F_{In} \left( 1 + \frac{F_{H^+}}{K_d} + P_{In}^L v_L + \frac{F_{H^+} P_{HIn}^L v_L}{K_d} \right). \quad (8)$$

NR absorbance in the buffer ( $A_0$ ) or lipid phase ( $A_L$ ) is given by:

$$A_0 = D_0 G \quad (9a)$$

and

$$A_L = (F_{In} + F_{HIn})G + \varepsilon_{HIn}^b B_{HIn}^L + \varepsilon_{In}^b B_{In}^L \quad (9b)$$

where  $G = \frac{\varepsilon_{HIn}}{1 + (K_d / F_{H^+})} + \frac{\varepsilon_{In}}{1 + (F_{H^+} / K_d)}$ , and  $\varepsilon_{HIn}^b$  and  $\varepsilon_{In}^b$

are the extinction coefficients of lipid-bound protonated and deprotonated NR species. Assuming that  $\varepsilon_{HIn}^b \cong \varepsilon_{HIn} = 2.64 \times 10^4$ ,  $\varepsilon_{In}^b \cong \varepsilon_{In} = 2.4 \times 10^3$ , and  $K_d = 2.5 \times 10^{-7} \text{M}^{-1}$ , the dye partitioning of dye into the lipid phase from the buffer solution can be detected by monitoring the change in NR absorbance ( $\Delta A$ ). Combining eqns (3–9) one obtains:

$$\Delta A = A_L - A_0 = \frac{D_0 v_L \left[ \frac{P_{In}^L (G - \varepsilon_{In})}{1 + \frac{F_{H^+}}{K_d} + P_{In}^L v_L} - \frac{P_{HIn}^L F_{H^+} (G - \varepsilon_{HIn})}{K_d} \right]}{1 + \frac{F_{H^+}}{K_d} + P_{In}^L v_L + \frac{F_{H^+} P_{In}^L v_L}{K_d}} \quad (10)$$

#### 4. RESULTS AND DISCUSSION

At the first step of the study we examined the CPZ effect on the polar region of lipid bilayers using the indicator dye NR. The binding of NR to lipid vesicles is followed by an increase of the dye absorbance, the magnitude of this effect being dependant on membrane surface potential. Approximation of the experimental  $\Delta A(C_L)$  dependences (Fig. 1) by eqn (10) allowed us to determine the dye-membrane partition coefficients for the protonated ( $P_{HIn}^L$ ) and deprotonated ( $P_{In}^L$ ) NR species in the absence and presence of anaesthetic. The estimates obtained are summarized in Table 1. The observed higher magnitudes of  $P_{HIn}^L$  relative to  $P_{In}^L$  can be explained by the fact that electrostatic interactions between the positively charged protonated NR form and anionic phospholipids favour dye partitioning into the lipid phase. For energetic reasons positive HIn species would, apparently, reside near the vesicle surface while neutral In species seem to prefer the polar/apolar interface. Interestingly, an increase of membrane surface potential also led to an enhancement of bilayer partitioning of the NR deprotonated form, indicating that the changes in surface charge are coupled with perturbation of the hydrophobic bilayer region.

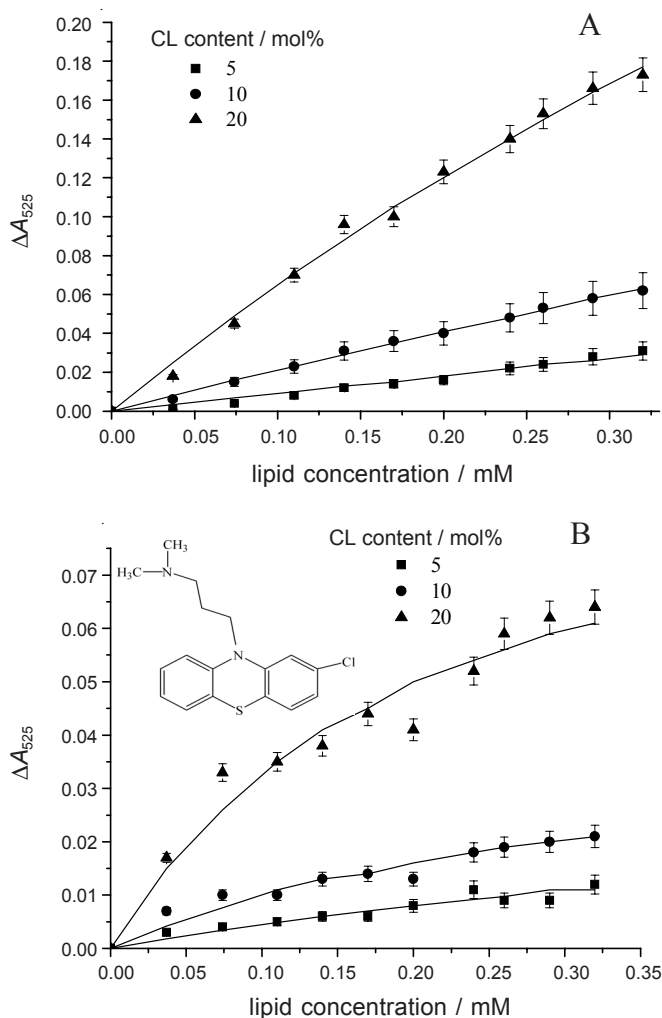


Figure 1. Changes in NR absorbance upon the dye association with PC/CL lipid vesicles in the absence (A) and presence (B) of CPZ. Shown in the inset is CPZ structure. Dye concentration was  $39 \mu\text{M}$ . CPZ concentration was  $32 \mu\text{M}$ .

As seen in Table 1, drug incorporation into the model membranes gives rise to a considerable increase both in  $P_{HIn}^L$  and  $P_{In}^L$ : upon formation of drug-lipid complexes the change in partition coefficients of the deprotonated NR form (and hence the CPZ effect on the bilayer hydrophobic core) attenuates with an increase of membrane surface potential. The most probable explanation for this observation lies in different drug locations in lipid vesicles differing in CL content. Since chlorpromazine has a  $pK$  of about 9.3 [10], under the experimental conditions employed (pH 7.4), it should exist in the protonated, positively charged form. Therefore, electrostatic CPZ-CL interactions are expected to significantly contribute to anaesthetic complexation with the liposomes. According to the model of CPZ location in the lipid bilayer proposed by Nerdal et al. [11], the tricyclic ring system of the drug penetrates the membrane hydrophobic core, adopting an orientation parallel to the acyl chains, while the positive

side group of CPZ resides in proximity to the anionic lipid headgroups. Electrostatic interaction anchors the drug molecule close to the membrane surface, preventing deep intercalation of the ring system into the hydrophobic interior of the bilayer. It may be expected that this model is adequate for the PC/CL membranes under study. Obviously, with increasing CL content the number of binding sites for the drug on the bilayer surface significantly increases, the CPZ position becomes closer to the lipid headgroups, and anaesthetic influence on the membrane nonpolar region is suppressed.

Table 1. Chlorpromazine effect on NR bilayer partitioning.

Lipid system	without CPZ		in the presence of CPZ	
	$P_{In}^L$	$P_{HIn}^L$	$P_{In}^L$	$P_{HIn}^L$
PC/CL (5 mol%)	0.31 ± 0.02	2118 ± 350	2.507 ± 0.87	3062 ± 250
PC/CL (10 mol%)	380.2 ± 23	4634 ± 520	2773 ± 420	5636 ± 845
PC/CL (20 mol%)	600 ± 100	6200 ± 800	3600 ± 940	17000 ± 650

The increase of NR partition coefficients observed upon CPZ inclusion in PC/CL vesicles could result from the increased hydration of the bilayer polar region, which favours membrane partitioning of the water-soluble dye species. It may be supposed that CPZ incorporation into the lipid bilayer changes the packing density of lipid headgroups, allowing a greater number of water molecules to penetrate the membrane polar region. Possibly, the positive side group of CPZ, sticking out into the head-group region of the bilayer, moves the neighbouring lipid molecules apart, thereby increasing membrane hydration.

Evidence for CPZ embedment into the nonpolar bilayer region comes from the pyrene fluorescence and DPH anisotropy studies. Analysis of pyrene excimer formation is an approach that has been successfully employed for examining lateral diffusion in membranes [12, 13]. Pyrene is a fluorescent probe whose excited species can interact with non-excited ones, forming excited-state dimers—excimers (this process is frequently referred to as self-quenching of pyrene fluorescence) [14–16]. Fig. 2 represents typical pyrene fluorescence spectra in the suspensions of PC/CL liposomes in the absence and presence of anaesthetic. These spectra are featured by well-defined bands of monomer and excimer emission. Relative intensities of vibronic transitions exhibit clear dependence on solvent polarity (the so-called “Ham effect”) [17]. As illustrated in Fig. 3A, the intensity ratio of monomer peaks at 374 and 384 nm ( $I_{374}/I_{384}$ ) remains virtually unchanged upon CPZ association with the lipid vesicles. This means that the drug does not affect the distribution of pyrene monomers across the membrane. In contrast, formation of CPZ-lipid complexes brings about a decrease of the excimer-to-monomer intensity

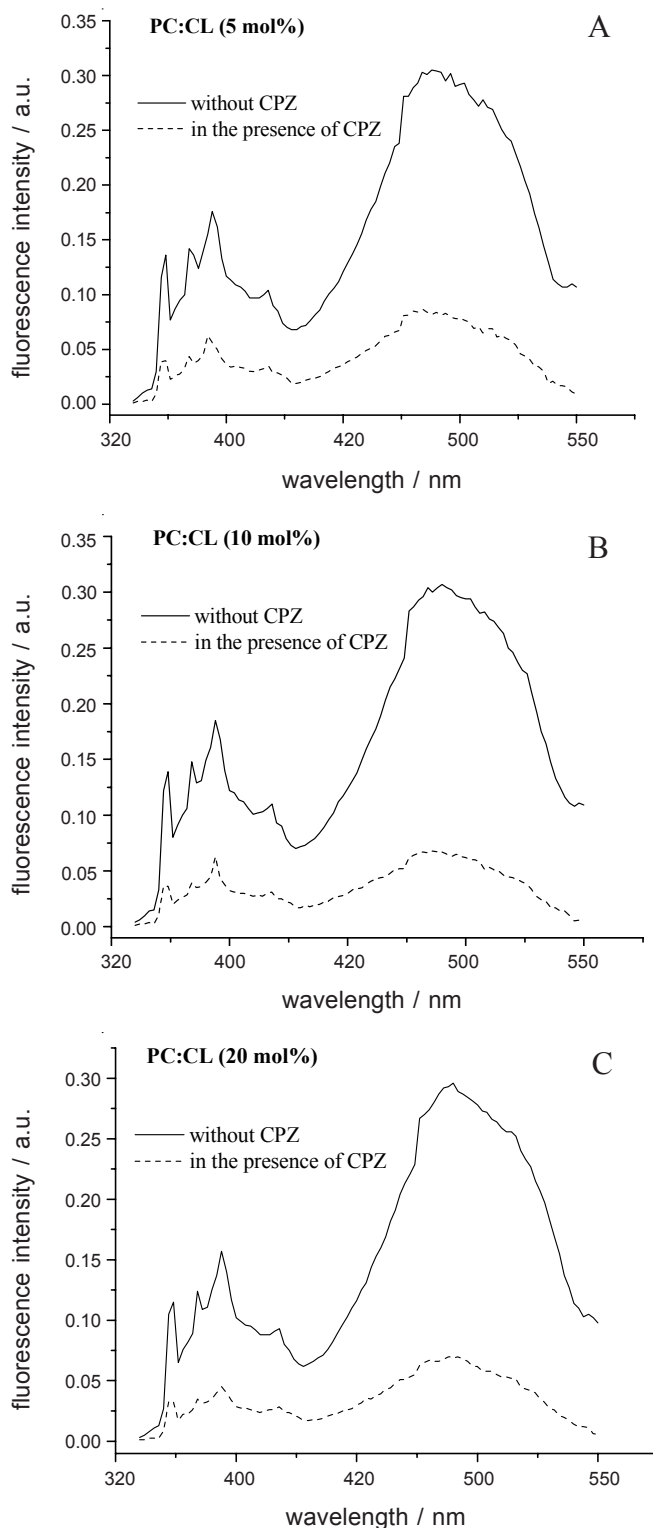


Figure 2. Emission spectra of pyrene incorporated in lipid and CPZ-lipid systems: PC/CL = 19:1 (A); PC/CL = 9:1 (B); and PC/CL = 4:1 (C). Lipid concentration was 0.16 mM. Pyrene concentration was 22  $\mu$ M.

ratio (E/M), reflecting the extent of pyrene excimerization which, in turn, largely depends on the monomer lateral distribution controlled by the membrane structural and dynamical properties (Fig. 3B). The observed effect



was interpreted within the framework of the concept of bilayer free volume. Membrane free volume characterizes the difference between the effective and van der Waals volumes of lipid molecules. Packing constraints and thermal motion give rise to *trans-gauche* isomerization of hydrocarbon chains and appearance of dynamic defects in the membrane interior. A local free volume arises from the lateral displacement of the hydrocarbon chain following kink formation. The free volume of a lipid bilayer depends on its composition, lipid lateral distribution, degree of acyl chain saturation, extent of hydration, temperature, etc. [18]. The free volume model considers diffusion of membrane constituents or guest molecules as a three-step process: (i) opening a cavity in the lipid monolayer due to the formation of kinks in the hydrocarbon chains; (ii) a jump of the diffusing molecule into a cavity leading to the appearance of a void; and (iii) closing the void by movement of a defect in an adjacent chain. The CPZ-induced decrease of E/M ratio reflects a reduction of membrane free volume, which most likely originates from an anaesthetic-promoted increase of bilayer structural order. These findings are in good harmony with the DPH anisotropy data. As seen in Fig. 4, CPZ addition to the liposome suspension resulted in a rise of DPH anisotropy, suggestive of the drug's condensing effect on the hydrophobic part of the lipid bilayer. Such ordering of the lipid bilayer (or reduction of the membrane free volume) upon CPZ incorporation may imply augmented thickness of the membrane hydrocarbon region and an increased number of lipid molecules per unit area. Alterations in molecular volume occupied by lipids are associated with the ordering and diminished rate of *trans-gauche* isomerization of the lipid acyl chains, due to specific orientation of the drug tricyclic moiety in the region of the lipid acyl chains. It may be supposed that CPZ rings, localized in the membrane hydrophobic interior, rigidify the tails of the lipid molecules, reducing their mobility and increasing the lipid packing density. Such modifications in bilayer properties impose restrictions on DPH rotation and decrease the frequency of pyrene monomer collisions.

In this context it is tempting to discuss the possible CL effects on the molecular organization of PC bilayers. As seen in Fig. 3B, the E/M dependence on CL content exhibits a dip (both in the absence and presence of CPZ) at 10 mol% CL. The same effect was observed in our earlier study [19]. The decrease of the extent of excimerization recovered at CL contents varying from 0 to 10 mol% suggests that CL brings about the reduction of lipid bilayer free volume. This effect may be explained by CL's ability to modify the structure of the PC bilayer via a decrease of its water permeability, enhancement of hydration of the ester C=O groups and initiation of cooperative

conformational changes in the PC head groups, rearrangement of the water bridges at the bilayer surface and stabilization of the intermolecular hydrogen-bonded network [20]. All the above phenomena may account for the reduction of PC/CL bilayer free volume deduced here from the decreased extent of pyrene excimerization observed upon increasing the CL content from 0 to 10 mol%. A further increase of the E/M value at 20 mol% CL can be attributed to the free volume increase resulting from repulsion of the negatively charged CL headgroups. Interestingly, DPH anisotropy exhibited an unambiguous dependence on CL content (Fig. 4), suggesting dissimilar sensitivities of pyrene and DPH to the changes in membrane dynamical properties. This may be a consequence of different photophysical mechanisms (bimolecular reaction of excimer formation in the case of pyrene, and probe rotation in the anisotropic medium for DPH) underlying the spectral responses of the employed probes to binding to the neat lipid and CPZ-lipid bilayers.

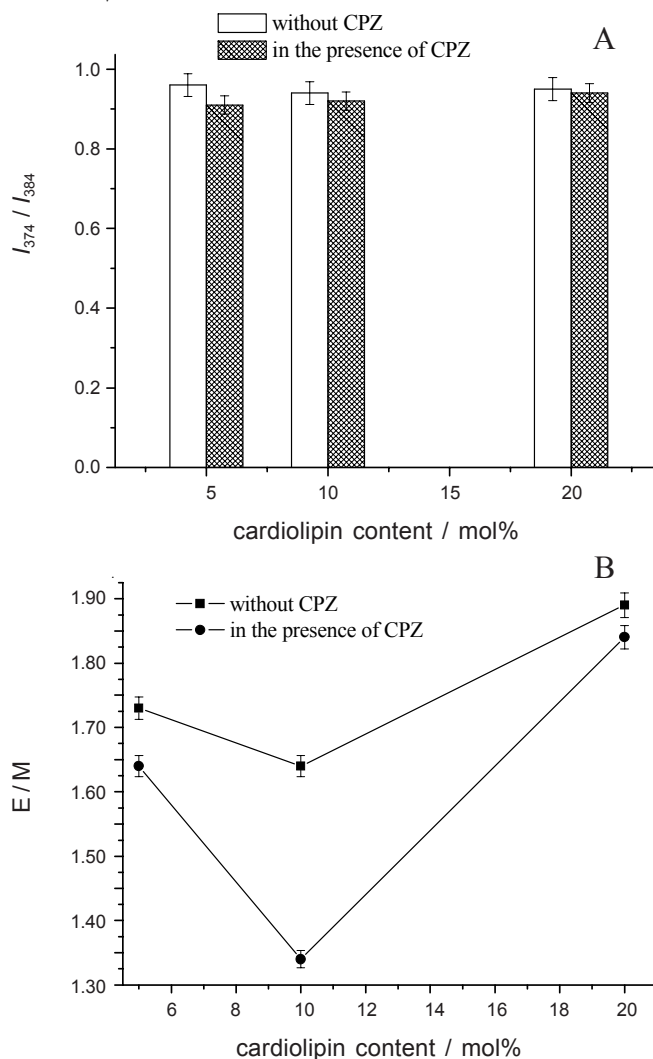


Figure 3. CPZ effect on pyrene vibronic structure (A), and excimer-to-monomer intensity ratio (B).

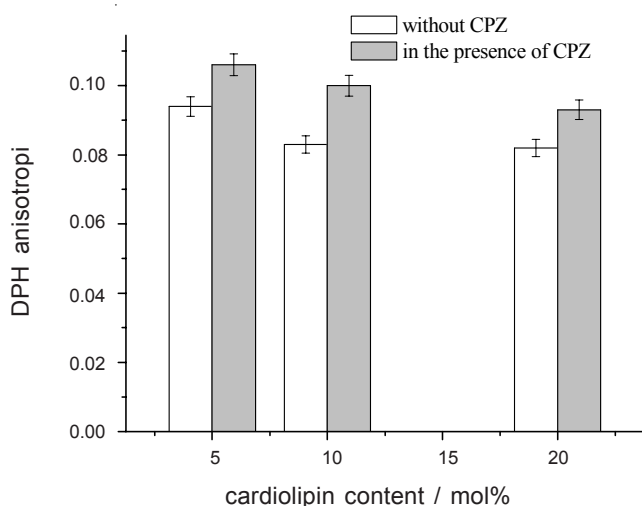


Figure 4. Changes in DPH fluorescence anisotropy upon formation of CPZ-lipid complexes. Lipid and DPH concentrations were 0.16 mM and 3.8  $\mu$ M, respectively.

An alternative explanation for free volume variations in PC/CL bilayers is provided by the lattice model of lipid lateral distribution [21, 22]. In the case of two-component bilayers there exist three major types of lateral distribution of the membrane constituents: domain segregated, random and regular. The contribution from one or another type of distribution depends on lipid geometry and intermolecular interactions. In the case of a lipid-segregated distribution the minor component tends to form clusters when attraction between similar components is much stronger than between different species. Lipid separation is driven by the membrane elastic deformation stemming from the difference in the cross-sectional areas between the guest and matrix lipids. A random distribution is inherent to ideal binary mixtures. It occurs when the interaction energy between the similar components is equal to that between the different lipids, and when the geometrical dimensions of guest and matrix lipid molecules are identical. A regular distribution suggests that the minor components are maximally separated because they attract each other more strongly than the major ones. The extent of regular distribution varies periodically with mole fraction of the guest lipid, thereby giving rise to corresponding variations in the membrane free volume, which is less in regular regions than in irregular ones. This model proved successful in explaining the origin of dips and kinks in the E/M dependence on mole fraction of pyrene-labelled lipid in different binary mixtures [23]. It may be assumed that CL molecules tend to distribute regularly in the PC matrix. In this case membrane free volume variations accompanied by E/M dips and kinks might be expected to occur with varying CL content.

It is noteworthy that CPZ addition to the lipid membranes led to an increase in the observed pyrene dip by a factor of *ca* 3.5. This finding suggests that CPZ may affect the lipid regular distribution in PC/CL bilayers by perturbations of the membrane free volume. However, it is important to note that possible CL effects on lipid organization cannot be fully explained in terms of the lattice model of lipid lateral distribution. Unlike other naturally occurring glycerophospholipids, CL is a quadruple-chained amphiphile composed of two phosphatidyl moieties esterified to the 1- and 3-hydroxyl groups of a single glycerol molecule [24]. Consequently, the CL polar headgroup is a relatively rigid, mobility-restricted entity, the cross-sectional area of which is smaller than the combined cross-sectional areas of the four hydrocarbon chains. Such a geometry of the CL molecule makes this lipid capable of forming an inverted nonlamellar phase.

The transition from the lamellar ( $L_{\alpha}$ ) to the hexagonal ( $H_{II}$ ) phase occurs when the negatively charged phosphate groups at the surface of CL-containing bilayers are protonated at low pH or screened by dispersal in solutions of high ionic strength, or if divalent metal cations such as  $Ca^{2+}$  or  $Mg^{2+}$  are added [25]. This transition has often been rationalized by invoking electrostatic effects, particularly the neutralization or shielding of the repulsive negative charges of the anionic phospholipid headgroups at neutral pH, in the absence of cations or at high salt concentrations respectively, which would favour the formation of the  $H_{II}$  over the  $L_{\alpha}$  phase, because of the greater electrostatic repulsion in the more tightly packed headgroups in the inverted phase [26]. It has been suggested that these properties of CL may determine the capacity of certain microorganisms to regulate the lamellar/nonlamellar phase propensity of their membrane lipids [27, 28]. However, the experimental conditions employed in this study do not favour  $H_{II}$  formation, therefore we concluded that the lamellar-hexagonal phase transition cannot account for the observed dip in the E/M pyrene intensity ratio.

To summarize, the results of NR binding, pyrene excimerization and DPH anisotropy studies revealed that CPZ exerts influence on the structural and dynamical properties and the molecular organization of the model lipid membranes, with the nature of these effects being different for polar and nonpolar bilayer regions. More specifically, it was shown that the formation of CPZ-lipid complexes gives rise to an increase of NR partition coefficients, presumably originating from the increased hydration of the bilayer headgroup region. The magnitude of these changes was found to depend on CL content. The most probable explanation for this finding involves the different depths of CPZ intercalation into the lipid

matrix of the lipid bilayers differing in their proportion of CL. Analysis of pyrene fluorescence spectra showed that CPZ does not exert an influence on the monomer distribution in the bilayer plane. However, the rate of pyrene lateral diffusion tends to decrease upon drug binding to the liposomes, suggesting that CPZ causes the bilayer condensation. This conclusion is strongly corroborated by the rise of DPH fluorescence anisotropy reflecting the ordering of lipid tails, a reduction of their mobility, and an increase in the molecular packing density. Better understanding of CPZ-lipid interactions is crucial for the design of liposome-based drug carriers since CPZ lipophilicity makes biological membranes sensitive target sites for drug pharmacological action.

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