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A New Fluorescent Squaraine Probe for the Measurement of Membrane Polarity

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The present study was undertaken to evaluate the sensitivity of newly synthesized squaraine dye **1** to the changes in lipid bilayer physical properties and compared it with the well-known dye **2**. Partitioning of the dye **1** into lipid bilayer was found to be followed by significant increase of its fluorescence intensity and red-shift of emission maximum, while intensity of the dye **2** fluorescence increased only slightly on going from aqueous to lipidic environment. This suggests that dye **1** is more sensitive to the changes in membrane properties as compared to dye **2.** Partition coefficients of the dye **1** have been determined for the model membranes composed of zwitterionic phospholipid phosphatidylcholine (PC) and its mixtures with positively charged detergent cetyltrimethylammonium bromide (CTAB), anionic phospholipid cardiolipin (CL), and sterol (Chol). The spectral responses of the dye **1** in different liposome media proved to correlate with the increase of bilayer polarity induced by Chol and CL or its decrease caused by CTAB. It was concluded that dye **1** can be used as fluorescent probe for examining membrane-related processes.

KEY WORDS: Squaraines; liposomes; degree of lipid bilayer hydration.

INTRODUCTION

Membrane physical properties are known to control a variety of biological processes such as partitioning of proteins and peptides into lipid bilayer, membrane fusion, modulating the enzyme activity, just to name a few [1]. One of the most important membrane characteristics is its polarity (or hydrophobicity). It is well recognized that the degree of bilayer hydration strongly affects structural, dynamical, and mechanical properties of lipid membranes. Moreover, membrane polarity is involved in lipid-ion association, ion transport and binding and has an influence on chemical reactivity and electrical conductivity of membrane surface [2]. One powerful physical technique for detecting the alterations in hydrophobicity is based on the use of bilayer-embedded extrinsic fluorophores [3,4]. To date, a wide variety of fluorescent probes have been synthesized, differing in their structure, spectral properties, and sensitivity to various membrane processes. However, some classes of compounds so far remain poorly examined from the viewpoint of their applicability to membrane studies. One of such classes is represented by squaraine dyes, a subclass of cyanines. The present study was undertaken to fill this gap. A new squaraine dye **1** has been synthesized and tested for its ability to respond to the changes in membrane properties and its response was compared to the well-known dye **2**. The structures of these compounds are given in Fig. 1. The spectral characteristics of the dye **1** have been examined using model membranes composed of egg yolk phosphatidylcholine (PC) and its mixtures with cholesterol (Chol), anionic phospholipid cardiolipin (CL), and cationic detergent cetyltrimethylammonium bromide (CTAB).

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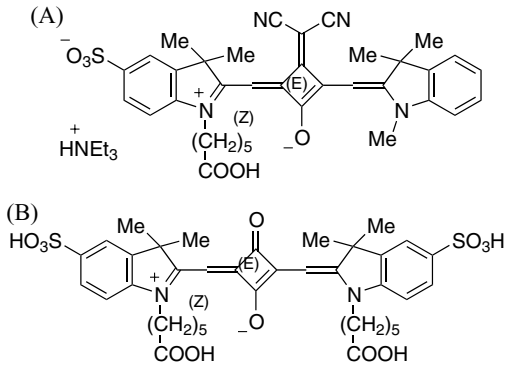

Fig. 1 Structures of the probes **1** (A) and **2** (B).

MATERIALS AND METHODS

Materials

Egg yolk phosphatidylcholine and beef heart cardiolipin were purchased from Biolek (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. CTAB and Chol were from Sigma. Dyes **1** and **2** were synthesized as described previously [5,6].

Preparation of Lipid Vesicles

Unilamellar lipid vesicles composed of pure PC and PC mixtures with (a) 10, 20 or 40 mol% of CL; (b) 5 or 10 mol% CTAB; (c) 10, 30 or 60 mol% of Chol; (d) 10 mol% of CL and 10, 30 or 60 mol% of Chol were prepared by the extrusion method [7]. The thin lipid films were 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. The phospholipid concentration was determined according to the procedure of Bartlett [8]. The dye-liposome mixtures were prepared by adding proper amounts of the probe stock solutions in buffer to liposome suspension. The probe concentration was determined spectrophotometrically, using extinction coefficients $\varepsilon^1_{662} = 2.07 \times 10^5$ M^{-1} cm^{-1} for the dye **1** and $\varepsilon^2_{633} = 2.65 \times 10^5$ M^{-1} cm^{-1} for the dye **2** [6].

Fluorescence Measurements

Fluorescence measurements were performed at 20°C with CM 2203 spectrofluorimeter (SOLAR, Belarus). Excitation wavelengths were 630 nm for dye **1** or 600 nm for dye **2**. Excitation and emission slit widths were set at 5 nm.

RESULTS AND DISCUSSION

At the first step of the study, we compared the lipophilic properties of the two squaraine dyes and their sensitivity to membrane environment. The fluorescence and absorption spectra of these dyes were recorded in buffer solution (5 mM Na-phosphate, pH 7.4) and liposomal suspensions. As seen in Fig. 2, spectral response of dye **1** to membrane association appeared to be much more pronounced than that of dye **2**. The intensity of **1** fluorescence exhibited more than 10-folds increase, while emission maximum (λ_{max}) was shifted from 674 nm in buffer to about 710 nm in the liposome medium with the spectral band halfwidth remaining virtually unchanged (Fig. 2A). On the contrary, binding of the dye **2** to model membranes was followed by insignificant increase of fluorescence intensity without any shift of the emission maximum (Fig. 2B). Therefore, in the further experiments conducted with different kinds of liposomes, we found it reasonable to restrict ourselves to examining spectral behavior of the dye **1**. Interestingly, not only fluorescence, but also absorption spectra of the dye **1** shifted towards longer wavelengths on going from the aqueous solution to less polar membrane environment (Fig. 2C). Since the ground state dipole moment of the dye **1** is rather high (*ca.* 6.5 D), it seems energetically unfavorable for the probe to penetrate into hydrophobic bilayer region. This dye is most probably located at the lipid–water interface being oriented parallel to the membrane surface.

In the next step of the study, we addressed the question of how the dye lipid-associating ability depends on the membrane physical properties being varied by introducing cationic detergent (CTAB), anionic phospholipid (CL) or sterol (Chol) into PC bilayer. The fluorescence spectroscopy methodology was employed to quantify the dye partitioning into a lipid phase. Based on the fluorescence intensity measurements in different lipid systems, we determined the dye partition coefficient defined as [9]

$$K_p = \frac{N'_L V_W}{N'_W V_L} \tag{1}$$

where N'_L,N'_W are the moles of the dye in the lipid and aqueous phases, respectively; V_L, V_W are the volumes of these phases. The volume of lipid phase was calculated as

$$V_L = N_A C_L \sum v_i f_i \tag{2}$$

here C_L is the molar lipid concentration, f_i is mole fraction of the i-th bilayer constituent, v_i is its molecular volume taken as 1.58 nm^3, 3 nm^3, 0.74 nm^3, and 1.1 nm^3 for PC, CL, Chol, and CTAB, respectively [10]. For cholesterol-containing systems condensing effect of this lipid was

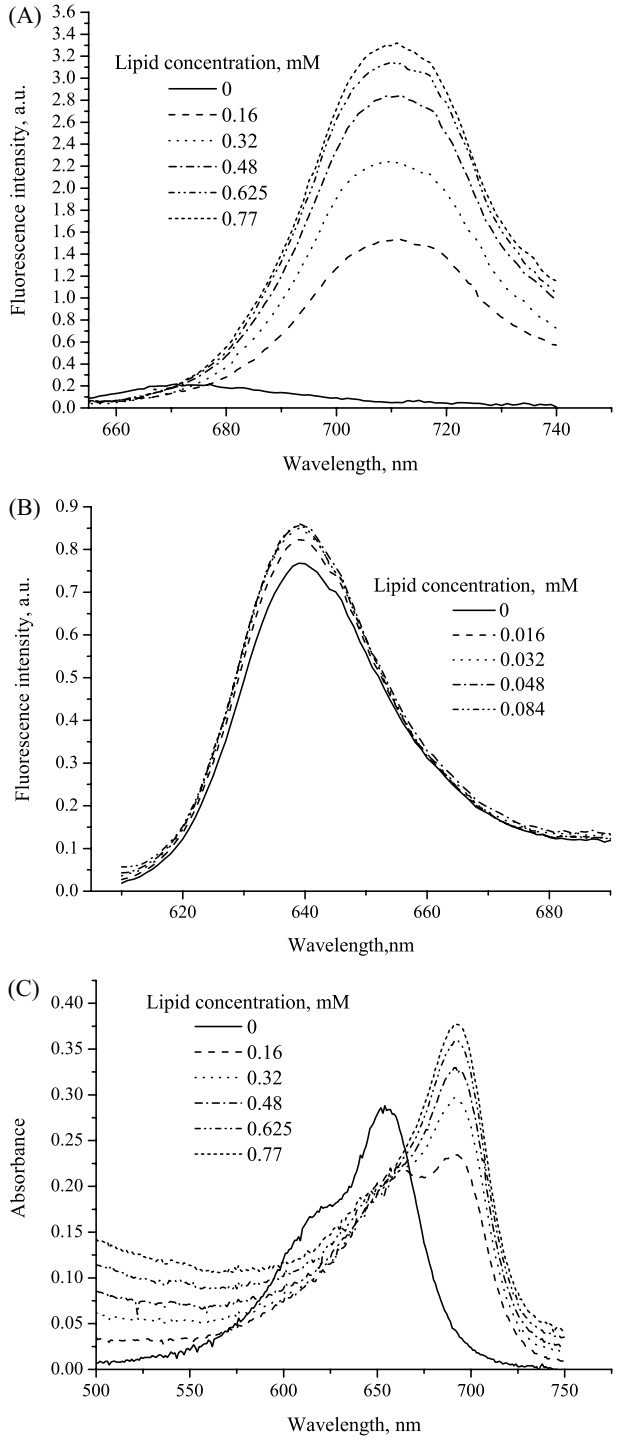

Fig. 2. Emission spectra of the dye **1** in PC liposomes (A) and the dye **2** in PC/CL (8:2) liposomes (B), and absorption spectra of the dye **1** in PC liposomes (C). The probe concentrations were 0.08 μM (**1**) and 0.15 μM (**2**).

taken into account, so that the above v values were reduced by the factors 1.3 and 1.2 for PC and CL, respectively [10]. Under the experimental conditions employed ($C_L \leq 1$ mM), the V_L value is much less than total volume of the system (V_t=1 dm^3), so that $V_W \approx V_t$. The relationship between K_p and fluorescence intensity increase (ΔI) can be written as [9]:

$$\Delta I = I_L - I_W = \frac{K_p V_L (I_{max} - I_W)}{1 + K_p V_L} \quad (3)$$

where I_L is the fluorescence intensity observed in the liposome suspension at a certain lipid concentration C_L, I_W is the probe fluorescence intensity in buffer, I_{max} is the limit fluorescence in the lipidic environment. To derive the dye partition coefficients for different lipid systems the experimental dependencies $\Delta I(C_L)$ presented in Fig. 3 were approximated by Eq. (3). The results obtained (Table I) suggest that partitioning of the examined squaraine into a lipid phase is strongly influenced by the employed modulators of the bilayer physical state. In terms of the modern theories of membrane electrostatics, partition coefficient can be represented as consisting of electrostatic and non-electrostatic terms [2,11]:

$$K_p = \exp(\{ze\psi_{el} + w_{Born} + w_h + w_n + w_d\}/kT) \quad (4)$$

where ze is the dye charge; ψ_{el} is the membrane Coulomb potential; w_{Born} is the free energy of charge transfer between the media having different dielectric constants; w_n is the neutral energy term determined by hydrophobic, van der Waals, and steric factors; w_h is the contribution controlled by the degree of membrane hydration; w_d is the term dependent on the membrane dipole potential (ψ_D), k is Boltzmann constant, and T is the absolute temperature. The dipole potential, arising from the phosphocholine and ester carbonyl dipoles of phospholipids and molecular dipoles of interfacial water, has a magnitude of several hundreds millivolts, being positive inside a bilayer [2,12]. This membrane property is thought to be responsible for the more pronounced ability of hydrophobic anions to associate with lipids and translocate across a bilayer, compared to structurally similar cations [12].

Analysis of the partition coefficients recovered here shows that inclusion of cationic detergent CTAB into PC bilayer gives rise to increase of K_p and limit fluorescence change ($\Delta I_{max} = I_{max} - I_W$) relative to the neat PC membrane. If association of the negatively charged dye **1** into PC/CTAB bilayer was governed exclusively by electrostatic interactions, one might expect an increase in the K_p value with CTAB content. However, partition coefficient remains virtually unchanged on increasing CTAB concentration from 5 to 10 mol%. This finding suggests that the dye partitioning into PC/CTAB bilayer is influenced

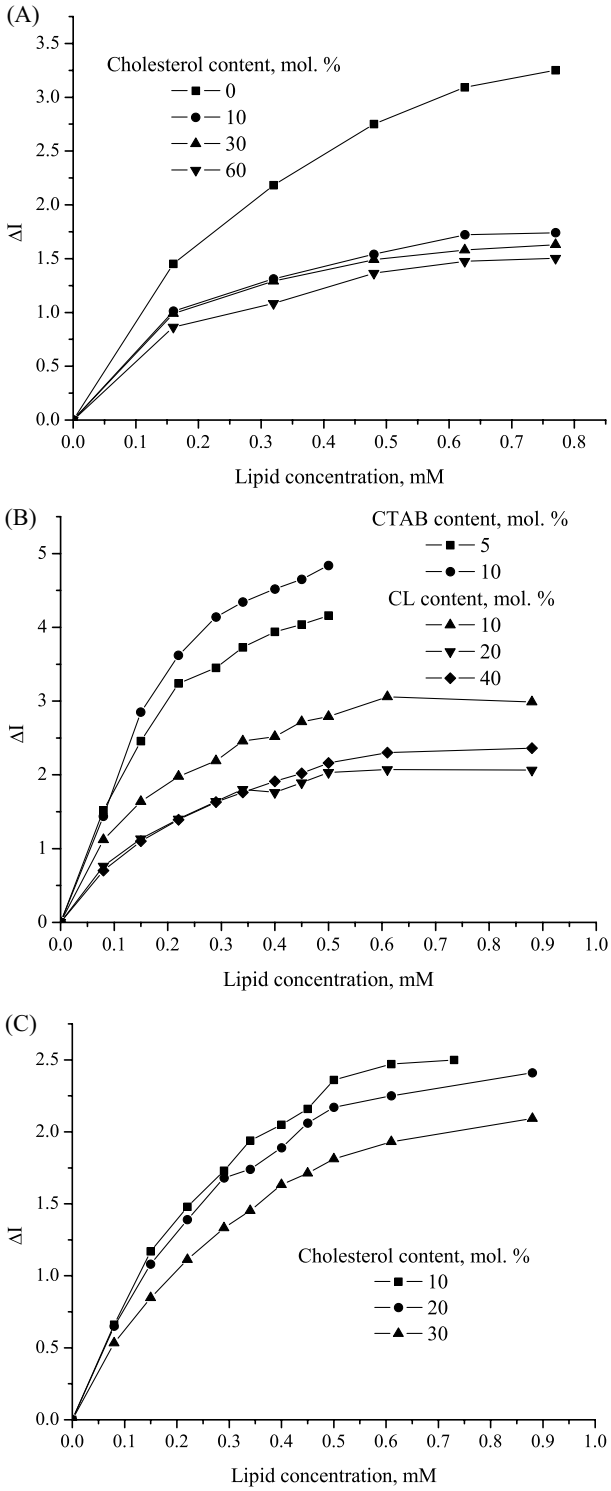

Fig. 3. Binding isotherms of the dye **1** to PC/Chol (A), PC/CTAB and PC/CL (B), and PC/CL/Chol (C) model membranes. Probe concentration was 0.08 μM.

also by nonelectrostatic factors. One of these can be rationalized in terms of lipid geometry. The PC molecule is commonly considered as having a cylindrical shape, while the CTAB molecule can be represented as an inverted cone. Such inconsistency brings about packing effects, schematically illustrated in Fig. 4. To fit the mismatch and curvature strain, the asymmetry of CTAB molecule must be compensated by a lateral headgroup compression. This leads to dehydration of the polar membrane region [13]. Thus, it may be assumed that due to strong electrostatic interactions between oppositely charged CTAB and dye molecules, **1** would tend to reside in the vicinity of the detergent, i.e. in the region with a decreased degree of hydration. This may account for the higher ΔI_{max} values in PC/CTAB systems compared to that in PC. The observed increase in fluorescence intensity (or quantum yield) of the dye **1** in PC/CTAB membranes can be attributed to the decreased rate of nonradiative relaxation processes involving excited state dissipation via vibrations, hydrogen bonding to the solvent cage, and the probe rotation [14, 15]. The above assumption on the CTAB-induced reduction of membrane hydration is in a good agreement with the observed long-wavelength shifts of the emission maximum of dye **1** in PC/CTAB liposomes compared to PC model membranes (Table I). This assumption is further supported by the observation that this shift becomes more pronounced as detergent concentration increases from 5 to 10 mol%.

Contrary to CTAB, anionic phospholipid CL is likely to induce increase in the degree of bilayer hydration, as judged from the shift of the dye **1** emission maximum to shorter wavelengths (Table I). The conical shape of CL molecule induces a negative curvature strain, so that bilayer polar region becomes more accessible to water (Fig. 5) [16]. In the case of CL-containing membranes, electrostatic interactions seem not to be a key factor in the dye partitioning into lipid phase because for liposomes containing 10 or 20 mol% CL, K_p is higher than that for PC liposomes, and only increase of CL content to 40 mol%

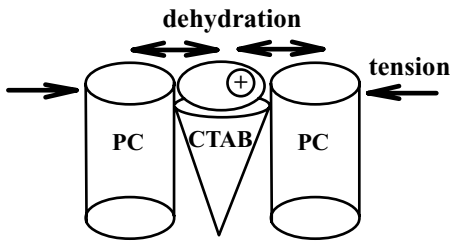

Fig. 4. Schematic illustration of CTAB packing mismatch in PC liposomes.

Table I Partition Coefficients and Emission Maxima of the Dye **1** in Different Lipid Systems

System	Partition coefficient	ΔI_{max}	λ_{max} (nm)
PC	2670 ± 175	4.97 ± 0.144	710
PC:Chol (9:1)	7111 ± 819	2.212 ± 0.079	710
PC:Chol (7:3)	9561 ± 442	1.98 ± 0.026	710
PC:Chol (2:3)	8231±1342	1.95 ± 0.1	710
PC:CTAB (19:1)	5042 ± 473	5.99 ± 0.23	712
PC:CTAB (9:1)	4323 ± 694	7.41 ± 0.52	714
PC:CL:Chol (8:1:1)	3766 ± 311	3.81 ± 0.14	710
PC:CL:Chol (6:1:3)	4570 ± 359	3.352 ± 0.11	708
PC:CL:Chol (3:1:6)	4214 ± 289	3.076 ± 0.097	708
PC:CL (9:1)	4822 ± 476	3.85 ± 0.13	710
PC:CL (4:1)	4778 ± 547.9	2.654 ± 0.1	709
PC:CL (3:2)	2701 ± 222	3.27 ± 0.11	702

leads to lowering the K_p value, probably because of repulsion between negatively charged dye and this anionic lipid. The increase of partition coefficient observed on CL inclusion in PC bilayer can result from the increased bilayer hydration which favors partitioning of the charged probe molecule into membrane.

In cholesterol-containing systems, partition coefficient was found to exhibit less unambiguous behavior. As seen in Table I, Chol addition to PC model membranes resulted in the ΔI_{max} decrease coupled with the increase of K_p values, the effect being most pronounced (3.6-folds K_p increase) at Chol content of 30 mol%. Despite considerable research efforts, Chol distribution in a lipid bilayer is still a matter of controversy. The early investigations suggest that Chol is fully embedded between the acyl chains of amphiphilic lipids [17]. Recent studies [18] support the assumption that it is more energetically favorable for Chol to have only its hydrophobic moiety buried into the nonpolar membrane core (Fig. 6). In such state, Chol stretches over one membrane leaflet with its OH-group sticking out into the polar headgroup region of the lipid bilayer. To date, the consequences of cholesterol inclusion in phospholipid bilayer are rather well characterized. For the liquid–crystalline lipid phase these consequences

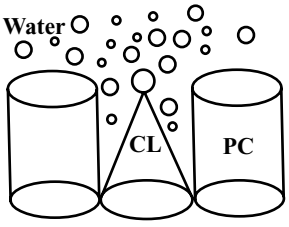

Fig. 5. Negative CL-induced curvature strain.

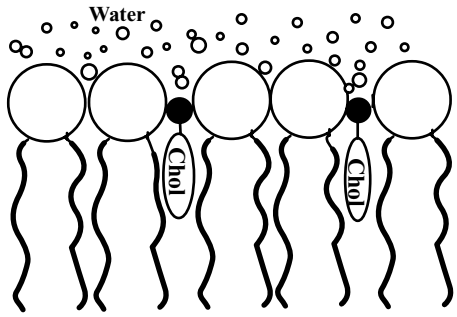

Fig. 6. Cholesterol distribution in lipid bilayer. *Small black circles* represent sterol OH-groups.

include: (i) increase of phospholipid headgroup separation [19, 20]; (ii) increased freedom of motion of phosphorylcholine moiety [20]; (iii) altered headgroup hydration [20–25]; (iv) reduced content of the acyl chain gauche conformations [26, 27]; and (v) tighter lateral packing of hydrocarbon chains (condensing effect) [28]. Depending on the proportion of cholesterol and chemical nature of membrane constituents, a plenty of widely varying effects have been reported [20, 21,29–34,36]. In view of this, in analyzing the results presented here it seemed reasonable to give preference to the studies performed with the lipid systems similar to ours, particularly those including egg PC. Of interest in this context is the recent low frequency impedance study into egg PC–Chol system providing evidence for increase of the hydration of interfacial bilayer region on cholesterol addition [35]. It is assumed that change in the lipid packing density on Chol inclusion allow a greater number of water molecules to penetrate in the headgroup bilayer region. Probably Chol OH-group, which protrudes into carbonyl region of the bilayer, moves the neighbouring lipid molecules apart, thereby increasing membrane hydration. Since, the dye **1** is supposed to be located at the lipid–water interface, it is tempting to interpret the observed enhancement of its partitioning into PC-Chol bilayers in terms of the increased membrane polarity. However, allowing for cholesterol ability to increase the dipole potential of egg PC membrane [37], one cannot rule out the possibility that dye **1** is sensitive to the change of bilayer dipole potential.

It seemed also of interest to compare the K_p values recovered for model membranes containing 10 mol% Chol (liquid-disordered phase) and 60 mol% Chol (liquid-ordered phase). As seen in Table I, no statistically significant difference between these parameters was observed, suggesting that the dye **1** is insensitive to Chol condensing effect, i.e. increase of the packing density of phospholipid acyl chains.

To summarize, the lipophilicity of squaraine dye **1** is much more pronounced than that of the dye **2**. Partitioning of the dye **1** into a lipid bilayer is followed by a significant increase of its fluorescence intensity and a long-wavelength shift of emission maximum, while fluorescence intensity of the dye **2** slightly increases on going from aqueous to liposome environment. The spectral responses of the dye **1** in different liposome media prove to correlate with the increase of bilayer polarity induced by Chol and CL and its decrease brought about by CTAB. These findings allowed us to conclude that squaraine dye **1** may be an effective red emitting fluorescent probe for examining membrane-related processes, especially those coupled with the change in the degree of bilayer hydration.

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