

Lysozyme effect on structural state of model membranes as revealed by pyrene excimerization studies

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Received 28 July 2004; received in revised form 5 November 2004; accepted 16 November 2004

Available online 22 December 2004

Abstract

Steady-state measurements of pyrene fluorescence in the model bilayer membranes composed of phosphatidylcholine (PC) and its mixtures with cardiolipin (CL) have been performed to gain insight into the effect of lysozyme on molecular organization of lipid bilayer. Analysis of vibronic structure of the probe emission spectra revealed no changes in transverse distribution of pyrene monomers on varying CL contents or increasing the extent of lysozyme binding to liposomes. Excimer-to-monomer fluorescence intensity ratio has been found to reduce on lysozyme association with lipids. The magnitude of this effect increased with increasing CL content from 0 to 40 mol%. These results have been interpreted as indicating decrease in the membrane free volume on formation of both electrostatic and hydrophobic protein–lipid contacts.

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Keywords: Lysozyme–lipid interactions; Pyrene excimer formation; Bilayer free volume

1. Introduction

Lipid–protein interactions are currently regarded as an essential factor determining the structural and functional features of biological membranes [1,2]. The problem of lipid–protein interactions has a number of facets. One of them relates to the effect of proteins on molecular organization and physical properties of a lipid bilayer, particularly, on lipid lateral mobility. Lateral motion of lipids, triggered by the fast migration of defects along the acyl chains, creates the prerequisites necessary for lateral diffusion of membrane constituents and membrane-bound compounds in a lipid bilayer. One approach to examining the mechanisms of lateral diffusion in membranes is based on kinetic analysis of the reactions proceeding in lipid phase. Of these, one of the most extensively studied is the bimolecular reaction of pyrene excimer formation. Pyrene is

a fluorescent probe whose excited species can interact with non-excited ones thus forming excited-state dimers—excimers (this process is frequently referred to as self-quenching of pyrene fluorescence) [3–5]. The early studies of Birks et al. revealed that in organic solvents this reaction is diffusion-controlled [6,7]. Based on the steady-state fluorescence measurements, Galla et al. concluded that the same is true for fluid phospholipid membranes [8,9]. However, further kinetic analyses of pyrene fluorescence in a variety of model membranes provided evidence for static rather than collisional mechanism of excimer formation [4]. A compromise model suggests the existence of pyrene monomer subpopulation that does not form excimers [3]. The above ambiguity in the data interpretation can be overcome in part by considering lipid bilayer as a system of reduced dimensionality where excimer formation must be described in terms of two-dimensional (2D) formalism instead of commonly employed 3D Birks's formalism [3,5]. Recently Martins and Melo demonstrated that parameters derived from kinetic analysis of pyrene fluorescence decays in DMPC fluid bilayer have physical meaning only in the case of data treatment in terms of 2D

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model of diffusion-controlled reactions [5]. The lack of consistency between the results obtained so far does not imply pyrene inapplicability to membrane studies. Clearly, the steady-state and time-resolved experimental data cannot be quantitatively interpreted in terms of parameters valid for homogeneous 3D distribution of pyrene molecules, like bilayer viscosity [3,5]. However, pyrene fluorescence studies may prove informative while detecting the changes in bilayer free volume accompanied by membrane redistribution of pyrene species and shift of the monomer–excimer equilibrium.

In the present work pyrene was employed to gain insight into the effect of lysozyme on the structural state of model lipid membranes composed of phosphatidylcholine and its mixtures with cardiolipin. This problem seems to be of interest in two aspects. Firstly, examining the lysozyme–lipid interactions may prove of importance for deeper understanding of the mechanism underlying its action. Lysozyme catalyses hydrolysis of the peptidoglycan layer of bacterial cells, thereby promoting cell aggregation and loss of their viability. It is assumed that lysozyme functioning is controlled by its association with a lipid bilayer. Secondly, due to pronounced lipid-associating ability and well-characterized structure lysozyme can be used in elucidating general principles of protein–lipid interactions. Isoelectric point of lysozyme is ca. 10.7, so that it bears positive charge under pH 10. A number of studies indicate that lysozyme is capable of forming both electrostatic and hydrophobic contacts with lipids [10–14]. Relative contributions of electrostatic and hydrophobic binding components vary depending on chemical nature of lipid molecules and experimental conditions. In the present study electrostatic component of lysozyme–lipid binding was enhanced by increasing the content of anionic phospholipid CL in the model membranes from 0 to 40 mol%. This allowed us to address the question of how lysozyme effect on the lipid bilayer structure is modulated by electrostatic protein–lipid interactions.

2. Materials and methods

2.1. Materials

Egg yolk phosphatidylcholine (PC) and beef heart cardiolipin (CL) were purchased from Bolek (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). Egg white lysozyme and pyrene were from Sigma (Germany).

2.2. Preparation of lipid vesicles

Unilamellar lipid vesicles composed of PC and 5, 10, 20 or 40 mol% of CL were prepared by the ethanol injection method, developed by Batzri and Korn [15]. 1 ml of ethanol

lipid solution containing appropriate amounts of PC and CL was injected into 13 ml of 5 mM phosphate buffer (pH 7.4) under continuous stirring. Ethanol was then removed by dialysis. Phospholipid concentration was determined according to the procedure of Bartlett [16]. Small aliquots (5 μ l) of a concentrated pyrene solution in ethanol were added to the aqueous liposome suspension. To ensure pyrene incorporation into membranes the sample were incubated for 30 min at room temperature. The probe concentration was the same for all experiments (13 μ M). Lysozyme concentration was found spectrophotometrically, using extinction coefficient $\epsilon_{280}=3.3 \cdot 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

2.3. Fluorescence measurements

Fluorescence measurements were performed at 20 °C with CM 2203 spectrometer (SOLAR, Belarus). Pyrene emission spectra were excited at 337 nm. Excitation and emission slit widths were set at 2 nm. The excimer-to-monomer fluorescence intensity ratio (I_E/I_M) was determined by measuring fluorescence intensity at the monomer (389 nm) and excimer (480 nm) peaks.

3. Results and discussion

Fig. 1 shows typical pyrene fluorescence spectra in the suspensions of PC/CL liposomes at varying lysozyme concentrations. These spectra are featured by a well-defined vibronic structure characteristic of pyrene monomer emission (vibronic bands numbered I–V from the lowest wavelength). Relative intensities of vibronic transitions exhibit clear dependence on solvent polarity (“Ham effect”) [17]. For instance, intensity ratio $R_{III}=I_V/I_{III}$ in water is reported to be 1.96, while in the solvents of lower polarity this ratio tends to decrease, reaching the value 0.6 in *n*-

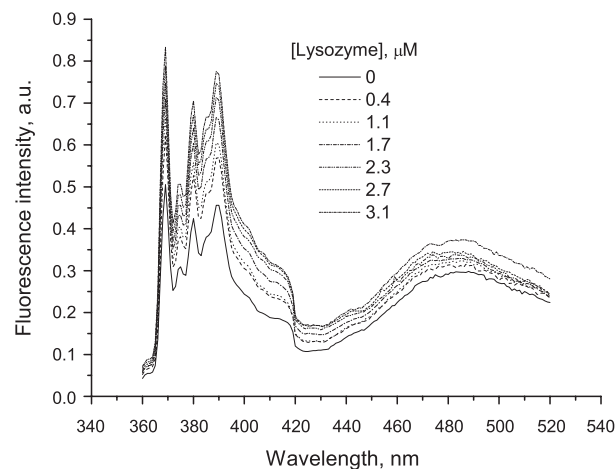


Fig. 1. Uncorrected emission spectra of pyrene incorporated in PC/CL liposomes (40 mol% CL) at varying lysozyme concentrations. Lipid (double acyl chain) concentration was 1.6 mM. Pyrene concentration was 13 μ M. Excitation wavelength was 337 nm.

hexane [18,19]. Vibronic structure of pyrene spectra in liposome medium depends on the probe concentration: parameter R_{III} (whose values in lipid phase are intermediate between *n*-hexane and methanol) increases with pyrene-to-lipid molar ratio, pointing to increased fraction of pyrene monomers located in more polar bilayer regions. As illustrated in Fig. 2A, relative intensity of vibronic band III does not undergo any significant changes with increasing PC/CL molar ratio. Note that R_{III} values recovered here (ca. 1.2) are in good agreement with those observed elsewhere for similar systems (0.9–1.2 (DPPC) [8,20,21], 1.09 (egg PC) [22,23], 1.1 (soya bean PC) [4]). According to ^1H NMR [24] and molecular dynamics data [25], pyrene molecule whose longest dimension is ca. 0.9 nm is likely to align its principal long axis along bilayer normal, residing in the region of 4–13 carbons. As judged from the vibronic structure of emission spectra, transversal distribution of pyrene monomers is similar for the model membranes under study. Furthermore, the data presented in Fig. 3 show that R_{III} value remains virtually unchanged upon lysozyme binding to liposomes, i.e. formation of protein–lipid

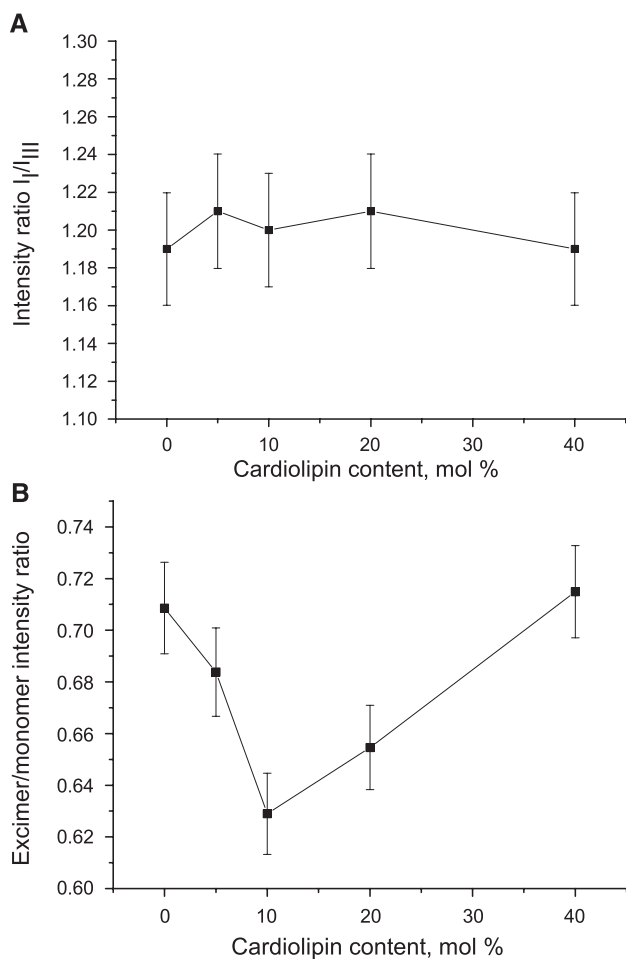


Fig. 2. Relative intensity of vibronic band III (A) and excimer-to-monomer ratio (B) in PC/CL liposomes. Lipid (double acyl chain) concentration was 1.6 mM, pyrene concentration was 13 μM for all types of liposomes.

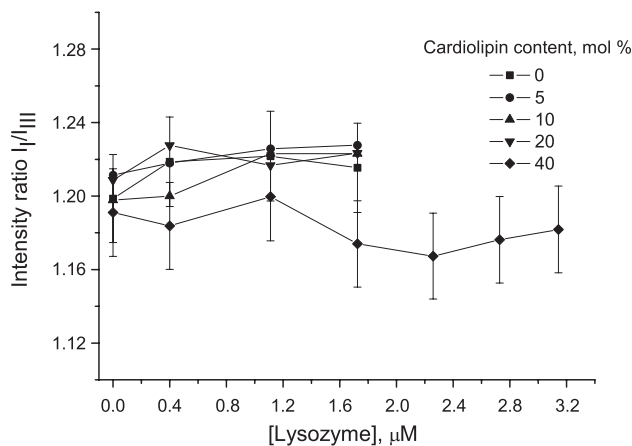


Fig. 3. Lysozyme effect on vibronic structure of pyrene fluorescence spectra in PC and PC/CL liposomes with CL content 5, 10, 20 and 40 mol%. Lipid (double acyl chain) concentration was 1.6 mM, pyrene concentration was 13 μM for all types of liposomes.

contacts do not affect the distribution of pyrene monomers across a lipid bilayer.

Another parameter measured here to characterize lysozyme-induced modification of the model membrane structure was excimer-to-monomer fluorescence intensity ratio (I_E/I_M). This parameter reflects the extent of pyrene excimerization, which, in turn, depends mainly on the monomer lateral distribution in the lipid bilayer. Although pyrene excimerization in membranes has long been extensively studied [3–5,8,9,20–23], the nature of this phenomenon is still a matter of controversy. Existing models consider several possibilities: (i) diffusion-controlled excimer formation resulting from a dynamic collision between a ground-state (M) and excited (M^*) monomers [3,6–9]; (ii) pyrene aggregation in a lipid bilayer ensuring static interaction between M and M^* in close proximity and formation of “static excimers” [3,4,26]; (iii) excimer formation by quantum mechanical coupling of pyrene wave functions at a distance [27]. A number of models considering diffusion in two dimensions have been proposed and employed in pyrene excimerization studies [5–9,20]. For instance, Montroll random-walk model [28] was adapted to excimer formation problem by Galla et al. [9]. More accurate approach to analyzing the kinetics of 2D diffusion-controlled reactions was suggested by Razi Naqvi [29]. Importantly, this approach takes into account time dependence of bimolecular rate constant. The validity of 2D diffusion-controlled kinetic formalism has been proved for $\text{py}_{10}\text{-PC/POPC}$, $\text{py}_{10}\text{-PC/DMPC}$ and free pyrene/DMPC systems [5]. In any case, pyrene excimer formation is a result of interaction between ground-state and excited monomers. The problem is what exactly mode of interaction takes place. If M and M^* are at rather long distance from each other and the lifetime of excited monomers is long enough, M and M^* may diffuse and form excimers due to their collision. By considering pyrene excimerization as 3D reaction, a conclusion that excimer formation is a diffusion-

controlled process can be drawn if the following main criteria are satisfied: (i) the ratio I_E/I_M linearly depends on pyrene concentration; (ii) fluorescence decays of monomer and excimers are characterized by the same lifetimes. By applying these criteria to the data obtained with thylakoid and soya bean PC membranes, Blackwell et al. [4] concluded that excimer formation is rather static than a collisional process. On the contrary, the arguments in favor of collisional mechanisms of pyrene excimerization in egg PC membranes were obtained by Barenholz et al. [3]. Using *N,N*-diethylaniline, a selective quencher of pyrene monomer fluorescence, it was shown that Stern–Volmer constants (K_{SV}) are identical for monomers and excimers ($K_{SV}^M = K_{SV}^E$). Since no actual quenching of excimer fluorescence occurs, the measured K_{SV}^E value reflects dissociation of excimers to monomers in accordance with mass law. This observation suggests that the major part of monomer molecules are in equilibrium with excimers, i.e. contribution of the aggregated pyrene, if any, to monomer fluorescence is negligible. Moreover, increase of pyrene-to-lipid molar ratio was followed by the reduction of monomer fluorescence lifetime, in accordance with collisional mechanism of excimerization.

The results of the steady-state pyrene fluorescence measurements presented here do not allow us to draw any conclusion on the mechanism of pyrene excimerization. Therefore, in interpreting the observed effects we rest on the findings reported elsewhere for similar systems. Evidence for dynamic nature of excimer formation in egg PC membranes comes, particularly, from the studies of Vanderkooi and Callis [20], Binder and Dittes [21], Barenholz et al. [3], to name only a few. As indicated above, static excimers originate from pyrene dimers (or other aggregates) formed prior to excitation. In turn, the extent of pyrene aggregation depends on the probe concentration and polarity of its environment. It was found that aggregates formed while adding pyrene ethanol solution to aqueous suspension of mitochondrial membranes do not dissociate on the probe incorporation in the membrane interior. However, no pyrene aggregates were observed in the suspension of egg PC liposomes [23]. Pyrene dimerization in PC/CL membranes seems to be hardly probable for the following reasons. Firstly, dimer formation should manifest itself in a strong hypochromism and decrease of monomer fluorescence [23,30]. However, in the absence of lysozyme, no marked changes in the pyrene fluorescence intensity were observed on increasing CL content from 0 to 40 mol% (Fig. 1). Secondly, according to the estimates of Blackwell et al., equilibrium constant for dimer formation (K_D) in the PC bilayer is ca. $1.4\text{--}2.8\text{ M}^{-1}$ [4]. Pyrene concentration used in our experiments, was $13\text{ }\mu\text{M}$ in solution, the value, corresponding to ca. 0.8 mol% of bilayer incorporated probe, or ca. 0.016 M in the lipid phase (C_{PL}), assuming that pyrene is distributed in nonpolar membrane region. Denoting dimer concentration by c_D and given that $K_D = c_D/c_M^2$, $C_{PL} = c_M + 2c_D$, one can estimate the extent of pyrene

dimerization provided that pyrene distribution in the lipid phase is homogeneous. Taking $K_D = 2.8\text{ M}^{-1}$, $C_{PL} = 0.016\text{ M}$, c_D was found to be about 4% of C_{PL} , suggesting that contribution of static excimers to the total fluorescence is insignificant. In this regard, it is also noteworthy that no ground-state dimers or other molecular aggregates were detected with up to 4 mol% of pyrene incorporated into PC liposomes [30].

Based on the above reasoning, we considered pyrene excimerization in the model membranes under study as a diffusion-controlled process. Diffusion in membranes is commonly described in terms of the free volume model. 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 membrane interior. A local free volume arises from the lateral displacement of hydrocarbon chain following the kink formation. Free volume of a lipid bilayer depends on its composition, lipid lateral distribution, degree of acyl chain saturation, extent of hydration, temperature, etc. [31]. The free volume model considers diffusion of membrane constituents or guest molecules as a three-step process: (i) opening a cavity in a lipid monolayer due to formation of kinks in the hydrocarbon chains; (ii) jump of the diffusing molecule into a cavity leading to appearance of a void; (iii) closing the void by the movement of a defect in an adjacent chain.

As seen in Fig. 2B, I_E/I_M dependence on CL content exhibits a dip (decrease by ca. 11%) at 10 mol% CL. This finding suggests that free volume of PC/CL model membranes varies with CL mole fraction. One explanation for such variations involves the changes in conformational state and packing density of phospholipid molecules. Several lines of evidence are indicative of CL ability to modify PC bilayer structure. Specifically, significant decrease in water permeability of PC bilayer on incorporation of small amounts of CL was reported by Shibata et al. [32]. Likewise, based on FTIR data, these authors hypothesized that CL is capable of enhancing the hydration of ester C=O groups and inducing a cooperative conformational changes in PC head groups. These changes were assumed to involve movement of the N^+ end of P–N dipole toward orientation more parallel to the membrane surface, thereby resulting in the rearrangement of water bridges at the bilayer surface and stabilization of the intermolecular hydrogen-bonded network including hydration water. All the above phenomena may account for reduction of the free volume of PC/CL bilayer, deduced here from the decrease of pyrene excimerization extent observed on increasing CL content from 0 to 10 mol%. Further increase of I_E/I_M value at higher CL concentrations (Fig. 2B) can be attributed to the free volume increase resulted from repulsion of the negatively charged CL head groups.

However, it is important to note that there exists an alternate explanation for free volume variations in PC/CL bilayers. Such an explanation is provided by the lattice

model of lipid lateral distribution [27,33–35]. This model can be outlined as follows. Lipid molecules in binary mixtures are regarded as being either domain segregated, randomly distributed or regularly distributed on a lattice [36]. In the case of regular distribution, the guest molecules tend to be maximally separated because interaction energy between different lipids considerably exceeds that between similar lipids. Lipid separation is driven by the membrane elastic deformation stemming from the difference in the cross-sectional areas between the guest and matrix lipids. Thermal fluctuations, variations in bilayer curvature and impurities lead to coexistence of the regular and irregular lipid distributions. The extent of regular distribution varies periodically with mole fraction of the guest lipid, thereby giving rise to respective variations in the membrane free volume which is less abundant in regular regions than in irregular ones. This model was successfully employed to explain the origin of dips and kinks in the I_E/I_M dependence on Pyr-PC mole fraction in binary mixtures Pyr-PC/DPPC, Pyr-PC/egg PC, Pyr-PC/DMPC [33–35]. It is tempting to assume that CL molecules tend to distribute regularly in the PC matrix. In this case, membrane free volume variations accompanied by I_E/I_M dips and kinks might be expected to occur with varying CL contents. Unfortunately, the results reported here do not allow us to answer the question concerning lateral organization of PC/CL model membranes. This point requires further in-depth examination.

At the next step of the study, the process of pyrene excimerization was examined in the systems liposomes+lysozyme. As shown in Fig. 4, lysozyme binding to PC/CL model membranes resulted in the decrease of I_E/I_M ratio, suggesting reduction of the bilayer free volume on the formation of protein–lipid contacts. In view of the I_E/I_M dependence on CL content, it seems of importance to analyze how the differences in the initial free volume of liposomes could modulate lysozyme effect on the bilayer structure. At CL concentrations 0 and 40 mol%, initial I_E/I_M values were virtually identical, but the magnitude of the lysozyme-

induced decrease of this parameter was significantly greater for the charged liposomes. Specifically, at protein concentration 1.7 μM , I_E/I_M was found to reduce by 8% in PC liposomes and by 22% in liposomes containing 40 mol% CL. The lowest initial free volume was observed for membranes with CL content 10 mol%. In this case lysozyme addition led to further I_E/I_M decrease whose relative magnitude was about 17% at protein concentration 1.7 μM . Despite the differences in initial I_E/I_M values, one can assert that lysozyme condensing effect on membrane structure enhances with increasing CL content. Such a tendency is likely to reflect the superposition of at least two factors: i) increased extent of lysozyme binding to liposomes with increasing CL/PC ratio and ii) specific contribution of electrostatic protein–lipid interactions to the structural modification of a lipid bilayer. Lysozyme ability to reduce bilayer free volume may be a consequence of the decreased rate of the *trans-gauche* isomerization of the hydrocarbon chains. Another possible reason for bilayer condensation involving lowered degree of bilayer hydration seems to be less probable, because polarity-sensitive relative intensities of vibronic bands remained unchanged in the presence of lysozyme. Additional argument in favor of the protein condensing effect comes from the observation that pyrene fluorescence intensity increased on lysozyme addition to lipid vesicles (Fig. 1), suggesting restricted mobility of phospholipid groups in the probe microenvironment. The results presented here are in accordance with the ESR data pointing to condensation of PC/PS bilayer under the influence of lysozyme [37]. The finding that this protein is capable of lowering the efficiency of pyrene excimerization in PC liposomes agrees with the results of Posse et al. [14], indicating that lysozyme can interact not only with negatively charged, but also with neutral membranes. Our data suggest formation of both electrostatic and hydrophobic lysozyme–lipid contacts to bring about reduction of the bilayer free volume.

To summarize, analysis of pyrene fluorescence spectra in PC and PC/CL model membranes revealed that lysozyme does not exert influence on the monomer distribution along the bilayer thickness. However, the rate of pyrene lateral diffusion tends to decrease on the protein binding to liposomes, suggesting that lysozyme gives rise to the bilayer condensation. Significant enhancement of this effect on increasing CL content is indicative of predominant role of electrostatic protein–lipid interactions in the lysozyme-induced reduction of phospholipid packing density. Increase of membrane rigidity may be an important factor determining the mechanism of lysozyme action on bacterial cells.

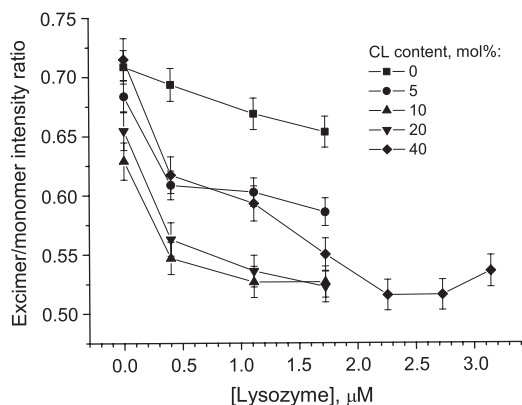


Fig. 4. Dependence of excimer-to-monomer fluorescence intensity ratio in PC and PC/CL liposomes on lysozyme concentration. Lipid (double acyl chain) concentration was 1.6 mM, pyrene concentration was 13 μM for all types of liposomes.

Acknowledgement

This work was supported in part by the Grant No. 2155 from the Science and Technology Center in Ukraine. The authors are indebted to referee for valuable remarks.

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