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ORIGINAL PAPER

Tracing Lysozyme-Lipid Interactions with Long-Wavelength Squaraine Dyes

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Abstract The applicability of the two newly commercial available squaraine labels Square-670-NHS and Seta-635-NHS to exploring protein-lipid interactions has been evaluated. The labels were conjugated to lysozyme (Lz) (squaraine-lysozyme conjugates below referred to as Square-670-Lz and Seta-635-Lz), a structurally well-characterized small globular protein displaying the ability to interact both, electrostatically and hydrophobically with lipids. The lipid component of the model systems was represented by lipid vesicles composed of zwitterionic lipids egg yolk phosphatidylcholine (PC) and 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC), and their mixtures with anionic lipids either beef heart cardiolipin (CL) or 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG), respectively. Fluorescence intensity of Square-670-Lz was found to decrease upon association with lipid bilayer, while the fluorescence intensity of Seta-635-Lz displayed more complex behavior depending on lipid-to-protein molar ratio. Covalent coupling of squaraine labels to lysozyme exerts different influence on the properties of dye-protein conjugate. It was suggested that Square-670-NHS covalent attachment to Lz molecule enhances protein propensity for self-association, while squaraine label Seta-635-NHS is sensitive to different modes of lysozyme-lipid interactions—within the L:P range 6–11, when hydrophobic protein-lipid interactions are predominant, an aggregation of membrane-bound protein molecules takes place, thereby decreasing the fluorescence intensity of Seta-635-Lz. At higher L:P values (from 22 to 148) when electrostatic interactions are enhanced fluorescence intensity of Seta-635-Lz increases with increasing lipid concentrations.

Keywords Squaraine label · Lysozyme · Liposomes · Protein-lipid interactions

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Introduction

Extrinsic labeling of biological macromolecules with tags is widely used in biological sciences for applications such as fluorescence microscopy, lifetime-based sensing, fluorescence resonance energy transfer studies, flow cytometry, DNA/RNA assays and microarrays, and in clinical analysis (blood and tissue screening, photodynamic therapy, immunohistochemical studies and medical applications) where these labels are used as *in vivo* and *in vitro* tracers [1]. Site-specific attachment of functionalized dye markers to reactive groups of proteins, nucleic acids, antibodies, etc. permits obtaining valuable information about the structure, dynamics and functioning of biomolecules, cells or even the whole organism. Despite the broad selection of commercially available markers, there is still a need for new and improved labels. Some

of the main issues associated with the fluorescent labelling with dyes are fading upon coupling to biomolecules, autoabsorbance and autofluorescence of biological samples, and nonspecific fluorescence caused by nonspecific interactions between, for example, antibody and antigen which may affect the label spectral properties [2, 3]. Some of these problems can be circumvented using long-wavelength amine-reactive labels for fluorescence assays which absorb and emit in the red and near-infrared (NIR) region. Reactive squaraines belong to one of the promising classes of dyes for covalent labeling of biomolecules for several reasons:

(i) they contain cyanine-type chromophore, and a central squarate bridge. The central squaraine bridge stabilizes the cyanine chain and helps to increase photostability;
(ii) their absorbance and emission are in the so-called optical window of biological tissues and cells where the autofluorescence is greatly reduced;
(iii) they exhibit a significant increase in quantum yield on coupling to biomolecules;
(iv) lack of nonspecific binding;
(v) pH – insensitivity in a wide pH range [4, 5].

An important area for the use of labeled biomolecules involves tracing of membrane processes. Biological membranes are complex and well-organized multicomponent assemblies and play a pivotal role in a variety of cellular processes. Membrane organization and function are largely determined by the interactions between two major membrane constituents, lipids and proteins [6–8]. Fluorescence represents a powerful tool to gain further insight into the mechanisms of protein-lipid interactions.

Motivated by the above rationales, we investigated the spectral behavior of the two newly commercially available reactive squaraine labels Square-670-NHS and Seta-635-NHS in our model protein-lipid systems. These labels were conjugated to lysozyme (Lz), a structurally well-characterized small globular protein with the ability to interact both, electrostatically and hydrophobically with lipids [9–11]. The lipid component in the model systems are lipid vesicles composed of zwitterionic lipids egg yolk phosphatidylcholine (PC) and 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC), and their mixtures with anionic lipids either beef heart cardiolipin (CL) or 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG), respectively. The present study is important because, firstly, there are no reports on the spectral properties of squaraine labels in organized media (liposomes), and, secondly, the obtained results may prove to be useful in interpreting the effects observed in more complex systems like biological membranes and cells.

Experimental procedures

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. SOPC and POPG were from Avanti Polar Lipids (Alabaster, AL, USA). The purity of the lipids was checked by thin layer chromatography on silicic acid-coated plates (Merck) developed with chloroform:methanol:water (65:25:4). Chicken egg white lysozyme was from Sigma (St. Louis, MO, USA). Square-670-NHS (K8-1320) and Seta-635-NHS (K8-1762) were purchased from SETA Biomedicals, LLC (Urbana, IL, USA).

Preparation of lipid vesicles

Unilamellar lipid vesicles composed of SOPC and 20 mol% POPG and PC mixtures with 10, 20 or 40 mol% CL were prepared using the extrusion technique [12]. Appropriate amounts of lipid stock solutions were mixed in chloroform (SOPC:POPG liposomes) or in ethanol (PC:CL liposomes), evaporated to dryness under a gentle nitrogen stream, and then left under reduced pressure for 1.5 h to remove any residual solvent. The dry SOPC and POPG lipids were subsequently hydrated with 20 mM HEPES (pH 7.4) while PC and CL were hydrated with 1.2 ml of 5 mM Na-phosphate buffer (pH 7.4). The resulting dispersions were extruded through a 100 nm pore size polycarbonate filter (Nucleopore, Pleasanton, CA). The lipid concentration determined according to the procedure of Bartlett [13] was 5 mM for SOPC:POPG and 10 mM for PC:CL liposomes.

Protein labeling procedure

For the preparation of the stock solution of the squaraine label 1 mg of Square-670-NHS or Seta-635-NHS was dissolved in 100 μl of anhydrous DMF. Then 15.3 mg of Lz were dissolved in 10 ml of 100 mM borate buffer, pH 9.1. Thereafter 20 μl of Square-670-NHS or Seta-635-NHS stock solution was added dropwise to 3 ml of Lz solution under continuous stirring and the sample was further incubated for 3 h at 25°C in the dark. After completion of the conjugation reaction unbound label was removed by dialysis in an excess of 20 mM HEPES buffer (pH 7.4) at 4°C. The degree of labeling was determined spectrophotometrically using the extinction coefficients for squaraines $\boldsymbol{\varepsilon}_{667}^{\mathrm{Sq1-NHS}} = 1.88 \times 10^5\ \mathrm{M^{-1}\ cm^{-1}}$, $\boldsymbol{\varepsilon}_{634}^{\mathrm{Sq2-NHS}} = 1.31 \times 10^5\ \mathrm{M^{-1}\ cm^{-1}}$, $\boldsymbol{\varepsilon}_{280}^{\mathrm{Sq1-NHS}} = 1.07 \times 10^4\ \mathrm{M^{-1}\ cm^{-1}}$, $\boldsymbol{\varepsilon}_{280}^{\mathrm{Sq2-NHS}} = 1.09 \times 10^4\ \mathrm{M^{-1}\ cm^{-1}}$. The lysozyme

concentration was calculated using an extinction coefficient $\varepsilon_{280} = 3.78 \times 10^4$ M^{-1} cm^{-1}, after subtracting the label absorbance at 280 nm. The dye-to-protein molar ratio was found to be ca. 0.1 for both squaraine labels.

Steady-state fluorescence measurements

Fluorescence measurements were performed at 20°C with a PerkinElmer Life Sciences LS50B spectrofluorimeter (experiments with SOPC:POPG liposomes) and Varian Cary Eclipse spectrofluorometer (experiments with PC:CL liposomes), equipped with magnetically stirred thermostated cuvette holders. The excitation wavelength for both squaraine labels was 635 nm. Excitation and emission band passes were set at 5 nm.

Results and discussion

Presented in Fig. 1 are absorption and emission spectra of squaraine-lysozyme conjugates (Square-670-Lz and Seta-635-Lz) in buffer solution (20 mM HEPES, pH 7.4). Binding of Square-670-Lz to SOPC:POPG liposomes resulted in a shift of the emission maximum (λ_{max}) from 685 to 691 nm, while in the case of Seta-635-Lz the λ_{max} shift was much larger—from 676 to 692 nm (Fig. 2). The long-wavelength shift of the emission maximum observed upon association of Square-670-Lz and Seta-635-Lz with liposomes is indicative of a less polar microenvironment. Changes of the emission maximum upon binding of the lysozyme conjugates to lipid vesicles is a valuable fluorescent property of squaraine labels which make them suitable for monitoring protein binding to lipid bilayers.

The fluorescence intensity of Square-670-Lz was found to decrease upon membrane association (Fig. 2A), while the fluorescence intensity of Seta-635-Lz exhibited more complex changes depending on lipid-to-protein molar ratio (L:P). The observed differences in the spectral responses of Square-670-Lz and Seta-635-Lz are related to the distinct ability of these labels to trace protein conformation, and, as a consequence, on the ability of Lz to associate with lipids. However, this assumption is in conflict with the results of light scattering measurements indicating that Square-670-Lz and Seta-635-Lz have similar affinity for liposomes (Fig. 3). Light-scattering profiles reflect the changes in size distribution and the degree of aggregation of lipid vesicles with increasing Lz concentration. The above parameters depend on the coverage of the liposome surface with the protein, i.e. on its affinity for the lipid bilayer. Neutralization of the membrane's negative charge upon Lz binding, and the protein ability to bridge adjacent liposomes through its multiple binding sites may facilitate aggregation of lipid vesicles. Our data indicate that fluorescent labeling leads to a

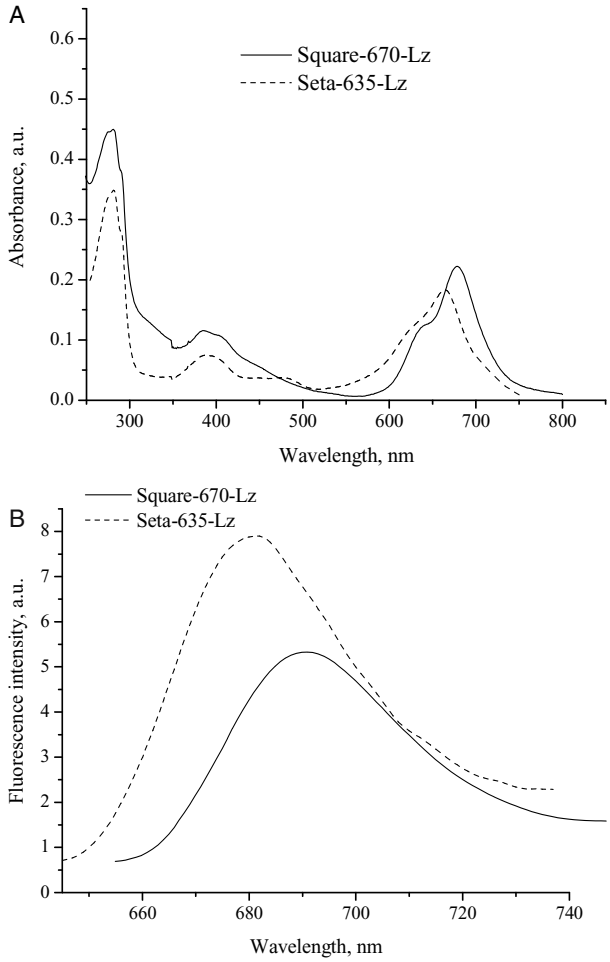


Fig. 1 Absorption (A) and emission (B) spectra of squaraine-lysozyme conjugates in buffer solution

reduction of the Lz affinity for the membrane, as can be evidenced, in particular, from the lower L:P ratio at which the light scattering intensity starts to decrease ($L:P_{crit}$) (Fig. 3B and C). Such an intensity decrease can be attributed to the fact that the size of vesicle aggregates is becoming larger than the light's wavelength, so that interference phenomena reduce the intensity of the scattered light [14]. As can be seen in Fig. 3B and C, for the squaraine-labeled Lz the value of $L:P_{crit}$ is about 80, while that of unlabeled Lz is about 140 (Fig. 3A). This indicates that the Lz concentration at which the extent of membrane binding proves sufficient for charge neutralization and vesicle cross-linking through multiple protein-protein bridges is lower in case of the unlabeled protein. The changes in the lipid-association ability of Square-670-Lz and Seta-635-Lz are most likely due to the reduction of the net positive surface charge of the protein upon covalent attachment of the labels. Based on the above rationale, the altered conformation of the labeled Lz cannot be considered as a main reason for the differences in spectral behavior of Square-670-Lz and Seta-635-Lz. The reactive

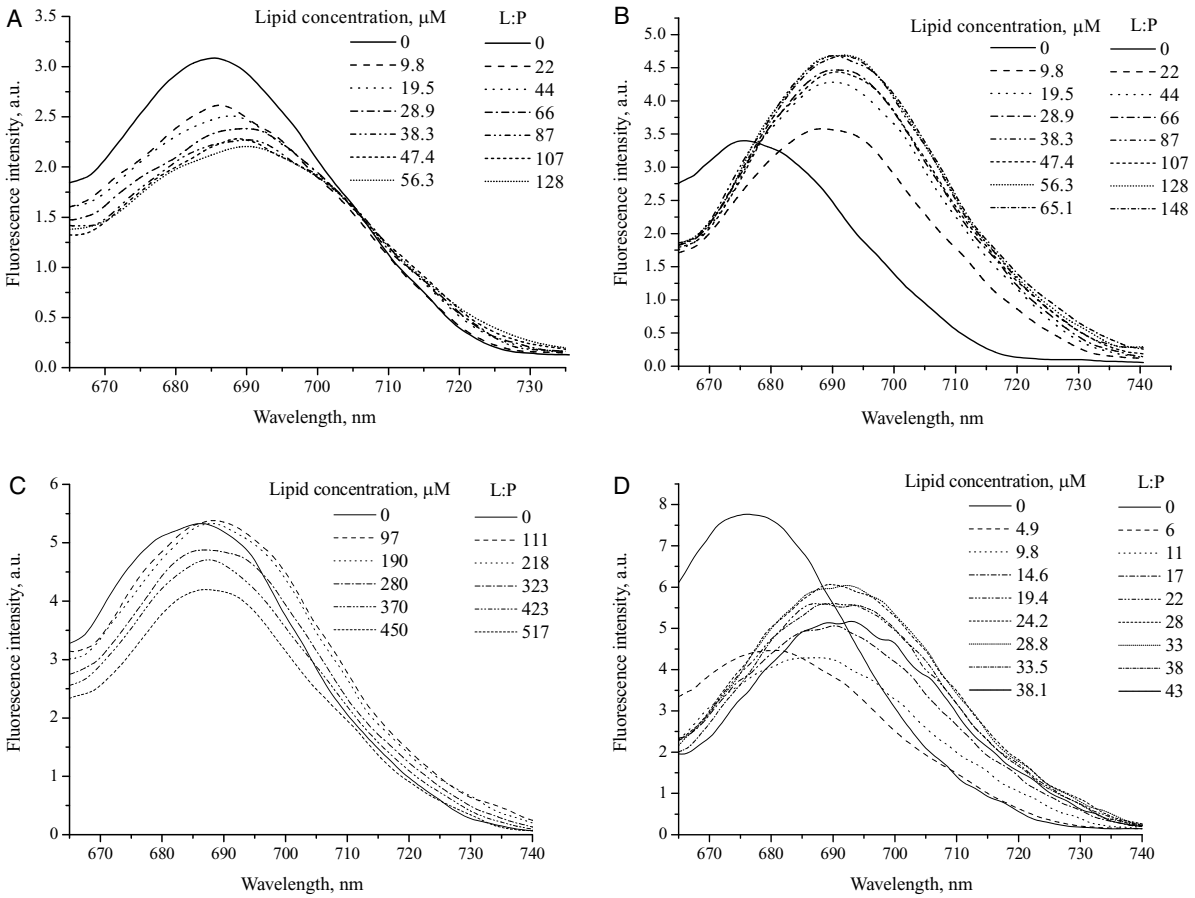


Fig. 2 Emission spectra of Square-670-Lz (A) and Seta-635-Lz (B) in SOPC:POPG liposomes at moderate L:P values (the concentration of the dye-protein conjugate was 0.44 μM), emission spectrum of Square-670-Lz at high lipid:protein ratios (C) and Seta-635-Lz (D) at low lipid:protein ratios (conjugate concentration was 0.87 μM)

group of the employed tags is *N*-hydroxy-succinimide ester (NHS-ester) which attaches to Lys amino acid residues of the protein molecules upon labeling [4]. The reactive group of the employed tags is *N*-hydroxy-succinimide ester (NHS-ester) which tends to attach to Lys amino acid residues of the protein molecules upon labeling [4]. As judged from the analysis of Lz structure by means of WebLab ViewerPro Trial37 software using the Protein Data Bank file [PDB entry 1HEW] the protein contains 6 Lys residues—Lys1, Lys13, Lys33, Lys96, Lys97 and Lys116. Furthermore, according to the hypothesis of Ibrahim *et al*. [15] three of these residues, namely, Lys96, Lys97 and Lys116, belong to the helix-loop-helix domain of Lz which is thought to be responsible for the protein-membrane binding. Electrostatic Lz-lipid attraction occurs mainly via these Lys residues. Thus, suggesting the homogeneous distribution of the label over entire Lz molecule, it can be assumed that coupling of the tags to Lz molecule partially neutralizes the positive charge of Lys residues which reduces the net positive charge of the whole protein which, in turn, decreases the bilayer binding ability of the labeled protein.

Notably, according to our observations, affinity of Square-670-Lz and Seta-635-Lz for the model membranes appeared comparable to that of fluorescein-labeled Lz (FITC-Lz) as judged from $L:P_{crit}$ for FITC-Lz which was found to be ca. 77.

Alternatively, one can assume that spectral response of these labels is controlled by the extent of aggregation of the protein and lipid vesicles, as well as the lysozyme-membrane interactions, which, in turn, depends on the amount of surface coverage or, in other words, on the L:P ratio [16]. At high L:P values (>150), in excess of lipid many binding sites for the protein are available, and in this case the electrostatic association of lysozyme with the membrane surface seems to be predominant. Lowering the L:P ratio increases the surface coverage and weakens the electrostatic forces due to the neutralization of the protein and membrane charges because of the formation of protein-lipid complexes. At relatively low L:P values (<30) mostly hydrophobic lysozyme-lipid interactions are expected to occur. Within the L:P range from 22 to 148 the hydrophobic binding of Lz conjugates to the model membranes competes with electrostatic

binding and liposome aggregation takes place. With increasing L:P ratio electrostatic binding increases while the membrane-membrane interactions become weaker. These processes lead to the decrease of Square-670-Lz emission and to an increase of the Seta-635-Lz emission (Fig. 2). A further increase of the L:P ratio up to 517, i.e. to the range where the electrostatic protein-lipid interactions are expected to be predominant, did not lead to any spectral changes of Square-670-Lz (Fig. 2C). This suggests that Square-670-NHS is insensitive to the different modes of protein-lipid interactions. In contrast to Square-670-Lz, the emission spectrum of Seta-635-Lz showed clear dependence on the L:P ratio. The fluorescence intensity decreases at L:P values between 6–11 and subsequently increases at higher L:P ratios (Fig. 2D). The decrease in brightness of squaraine emission upon interactions with lipids could be the consequence of at least three processes:

(i) conjugate self-association;
(ii) change in the environments pH due to dye transfer into lipid-water interface where the pH could be reduced due

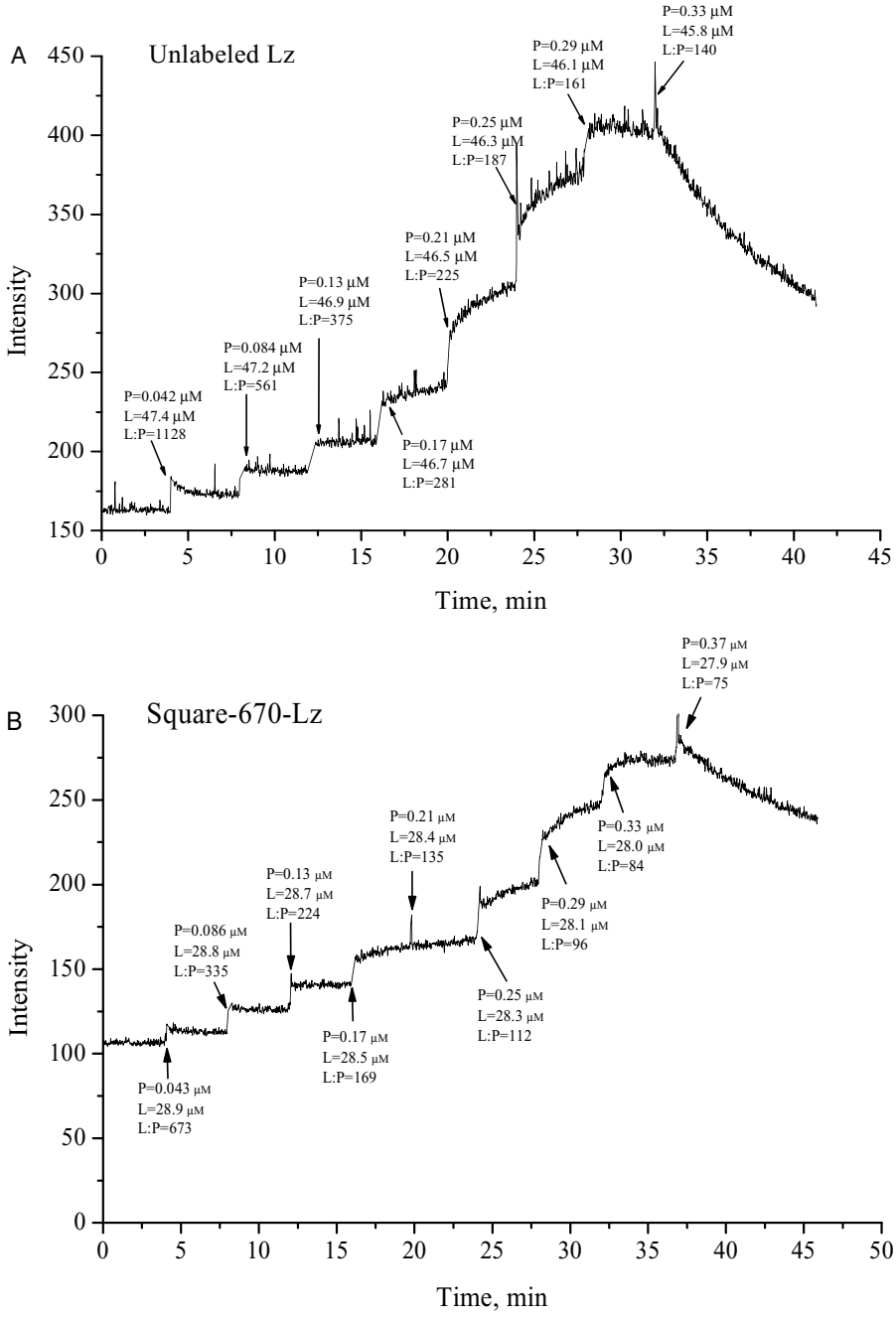
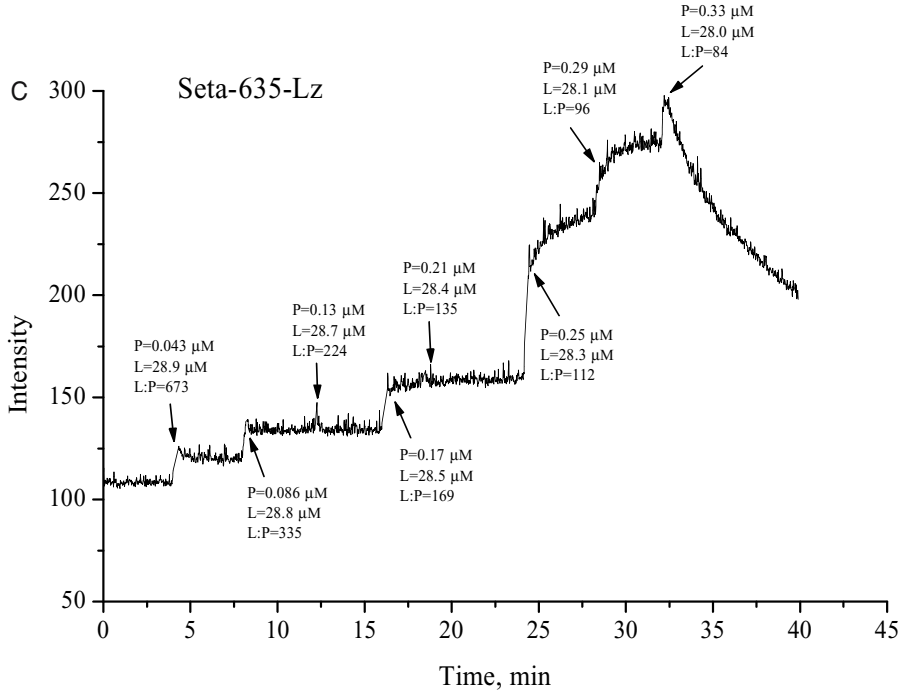


Fig. 3 Time-dependent right-angle light scattering of unlabeled Lz (A), Square-670-Lz (B) and Seta-635-Lz (C) measured at 500 nm

Fig. 3 Continued



to proton accumulation near a negatively charged surface on the model membranes under study;

(iii) fluorescence quenching of the dye by certain lipid or protein moieties.

In separate experiment we examined the pH-sensitivity of the studied squaraine labels. As seen in Fig. 4, upon lowering the pH from 7.4 to 2.92 the fluorescence intensity of both Square-670-Lz and Seta-635-Lz decreases by 40%. In order to make sure that the pH-dependent effects are not responsible for the observed fluorescence decrease that is observed upon the binding of Seta-635-Lz to liposomes we studied its association with several PC:CL model membranes differing in the amount of anionic lipid (CL). According to our estimates that were obtained using Gouy-Chapman double layer theory proton accumulation near the surface of PC:CL membranes results in a pH decrease to 5.8 (10 mol% CL), 5.4 (20 mol% CL) or 5.2 (40 mol% CL). Hence, a decrease in the Seta-635-Lz fluorescence is expected on binding to PC:CL liposomes, the effect becoming more pronounced upon increasing the CL content from 10 to 40 mol%. However, as seen in Fig. 5, Seta-635-Lz exhibits the opposite behavior—the fluorescence increased with lipid concentration, and the intensity changes (ΔI) became smaller with increasing the CL proportion. These observations led us to conclude that pH effect do not play a dominant role in the intensity changes of squaraine-conjugates observed upon protein interaction with liposomes.

One cannot rule out the possibility that after fluorescence labeling the self-association tendency on membranes of the

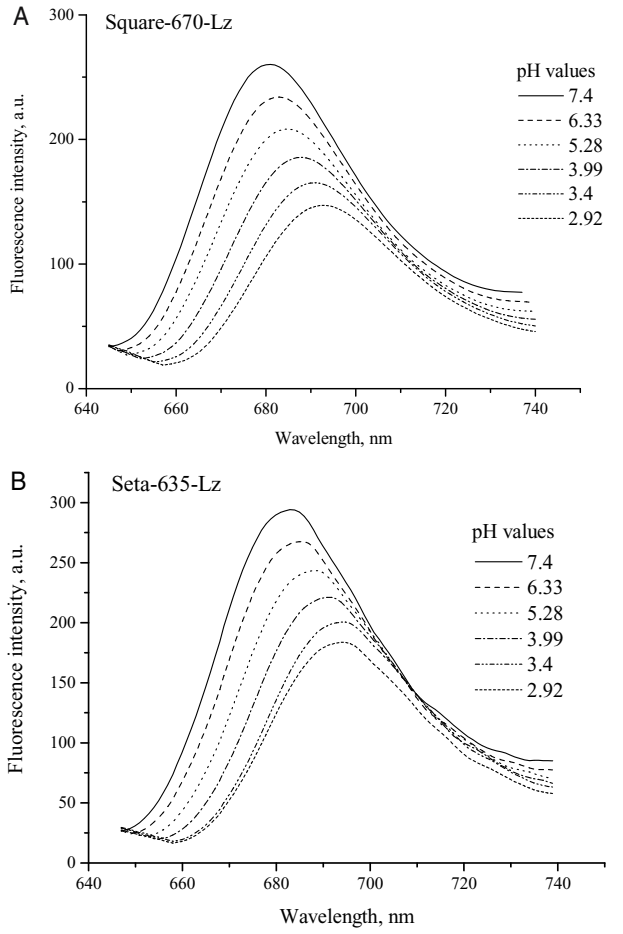


Fig. 4 Emission spectra of Square-670-Lz (A) and Seta-635-Lz (B) in buffer solution at different pH values

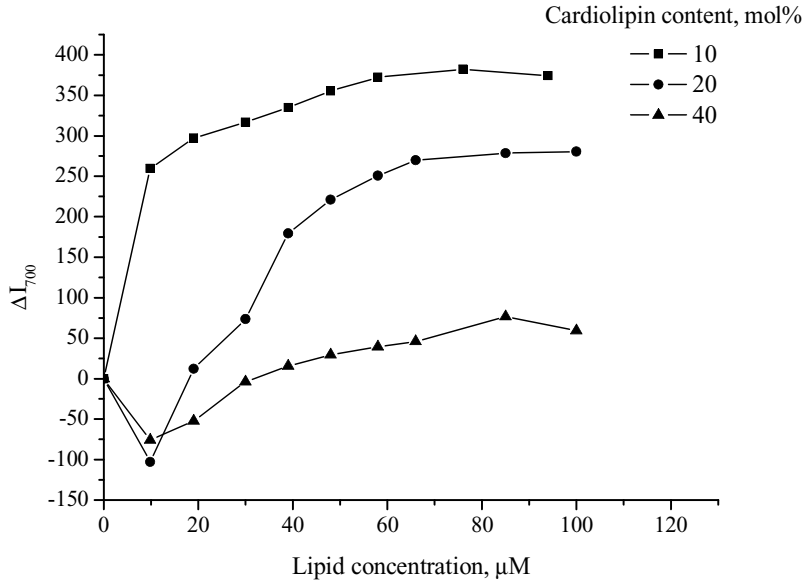


Fig. 5 Changes in Seta-635-Lz fluorescence upon increasing PC:CL membrane surface potential. ΔI was taken as the difference between conjugate fluorescence in lipid and aqueous phases. The concentration of the dye-protein conjugate was 0.88 μM

Lz-conjugates increases which may lead to conjugate self-association accompanied by fluorescence quenching.

The observed changes of the Seta-635-Lz fluorescence may originate from a complex interplay between protein-lipid, protein-squaraine, protein-protein and squaraine-membrane interactions whose relative contributions vary with the lipid concentration. It seems probable that within the L:P range 6–11, which is below saturation coverage, an aggregation of membrane-bound protein molecules takes place, thereby decreasing the fluorescence intensity of Seta-635-Lz. At higher L:P values both hydrophobic and electrostatic protein-lipid interactions seem to have an influence on the changes of the Seta-635-Lz fluorescence. As shown in Fig. 5, at low CL content (10 mol%), when the electrostatic lysozyme-membrane interactions compete with the hydrophobic ones, the spectral responce of Seta-635-Lz is similar to that observed in SOPC:POPG lipid vesicles at moderate L:P (from 22 to 148)—namely the fluorescence intensity increases with increasing lipid concentrations. Increasing the CL concentration up to 40 mol% leads to an increase in surface coverage, formation of hydrophobic Lz-lipid contacts and an increase of the protein self-association. All these processes are likely to account for the observed trends in fluorescence changes (Fig. 5).

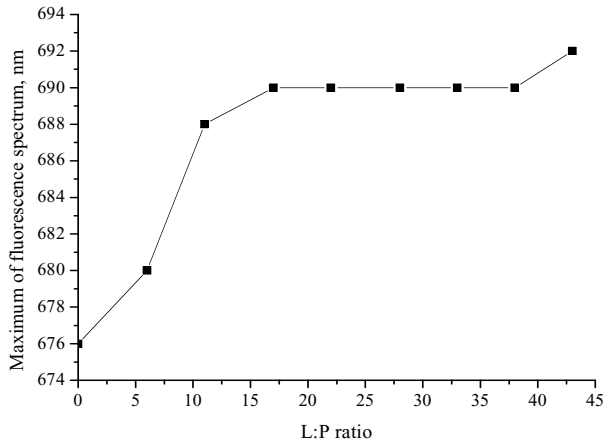


Fig. 6 The position of fluorescence spectrum maximum of Seta-635-Lz in the presence of SOPC:POPG model membranes at high L:P

The observed shifts in λ_{max} (Fig. 6) indicate that at L:P ratio of approximately 20 the membrane surface is fully saturated with the protein. This finding is consistent with the fact the cross-section (ca. 13.5 nm^2) of one Lz molecule covers about 21 lipid headgroups.

In conclusion, the present study demonstrated that covalent coupling of two squaraine labels to lysozyme exerts different influence on the properties of dye-protein conjugate. Based on steady-state fluorescence measurements, it was assumed that covalent attachment of Square-670-NHS to Lz molecule increases the protein tendency for self-association, while squaraine label Seta-635-NHS is sensitive to different modes of lysozyme-lipid interactions. This valuable fluorescent property of Seta-635-NHS makes it a good choice for monitoring protein association with membranes. The obtained results suggest that these new squaraine lysine-conjugates are promising reporter molecules for the investigation of protein-protein and lipid-protein interactions.

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