J Fluoresc (2009) 19:1017–1023
DOI 10.1007/s10895-009-0501-z

ORIGINAL PAPER

A Novel Squarylium Dye for Monitoring Oxidative Processes in Lipid Membranes

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Received: 24 December 2008 / Accepted: 5 June 2009 / Published online: 10 June 2009

Abstract A novel squaraine probe SQ-1 has been found to be appropriate for monitoring the peroxidation processes in membrane systems. Formation of free radicals was triggered by methemoglobin (metHb) or cytochrome *c* (cyt *c*) binding to the model lipid membranes composed of zwitterionic lipid phosphatidylcholine (PC) and anionic lipid cardiolipin (CL). Protein association with the lipid vesicles was followed by drastic quenching of SQ-1 fluorescence. The observed spectral changes were suppressed in the presence of free radical scavengers, butylated hydroxytoluene (BHT) and thiourea (TM) suggesting that SQ-1 decolorization can be attributed to its reactions with lipid radicals.

Keywords Squarylium dye · Methemoglobin · Cytochrome *c* · Lipid peroxidation

Introduction

The unique photophysical properties of squaraines have resulted in their extensive use in xenography, optical recording media, nonlinear optics, organic solar cells, to name only a few [1-5]. Despite the technological utilization of these compounds is continuously growing, their applicability as long-wavelength fluorescent probes in biological assays, so far remain scantily evaluated. Meanwhile, spectral characteristics of squaraines satisfy all the requirements for an ideal fluorescent tracer. Long-wavelength absorption and fluorescence, high extinction coefficients and quantum yield, photo- and chemical stability, and reduced background signal [6] make squarylium dyes particularly attractive as bioimaging agents. Our previous studies revealed one prospective squaraine probe, SQ-1 (Fig. 1a, inset), highly sensitive to the protein-induced alterations in structural state and physicochemical properties of a lipid bilayer [7]. Cumulative data from the binding, anisotropy, quenching and kinetic experiments, led us to conclude that cationic globular proteins lysozyme and ribonuclease A provoke membrane dehydration, reduction in bilayer free volume, and lipid lateral redistribution demixing. Inspired by such unrivalled potential of SQ-1 to trace the alterations in lipid bilayer structure and dynamics, in the present work we extended the investigation of SQ-1 performance in biological media and concentrated our efforts on evaluating the ability of this dye to respond to another process that may substantially affect membrane architecture and properties — lipid peroxidation (LPO). Oxidation of lipids is a free radical — mediated chain reaction, initiating the cascade of events which finally results in a loss of cellular functions and cell death. To detect reactive oxygen species (ROS) several powerful methods have been employed, including fluorescent and chemiluminescent spectroscopy [8, 9], ESR [10], NMR [11], etc. Of these, fluorescence techniques have obvious superiority due to their extraordinary sensitivity, relative simplicity, high selectivity, and experimental convenience.

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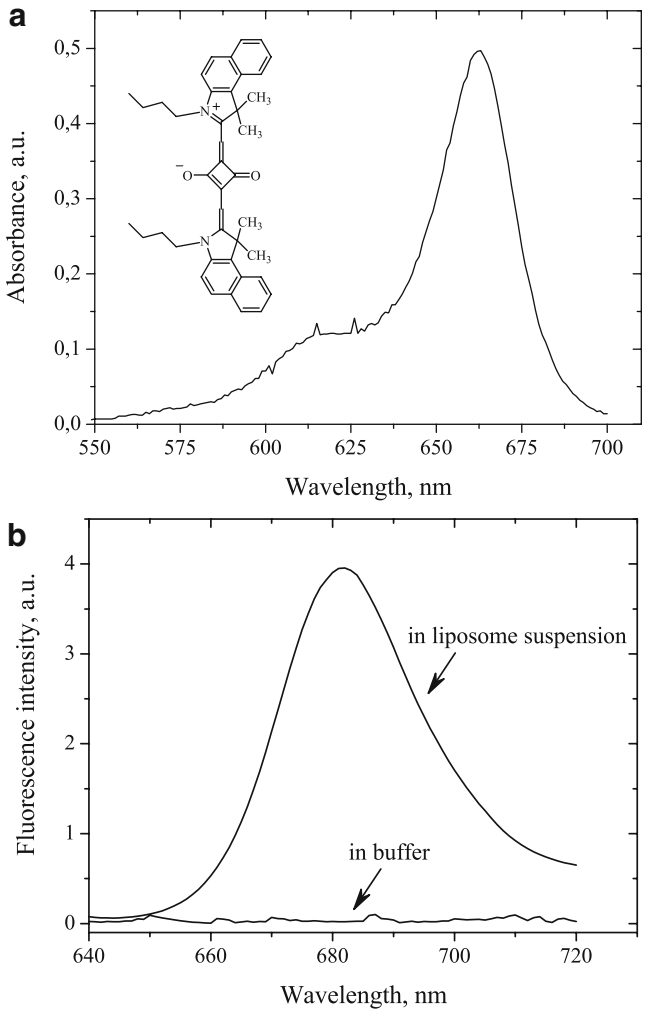

Fig. 1 Absorption **a** and emission **b** spectra of SQ-1. Shown in the inset is SQ-1 chemical structure

A wide range of fluorescent probes involving fluorescein and rhodamine derivatives [12], phosphine-based fluorophores [13], and lanthanide coordination complexes [14], have been employed to identify the lipid free radicals. Although being prized for high specificity to different kinds of ROS, these reporter molecules suffer from one severe drawback — their absorption maxima are well below 600 nm. Owing to this pitfall, sensitivity of the above probes may be seriously compromised by background signals (i.e. light scattering, autoabsorption and autofluorescence) resulting from the major membrane constituents — proteins and lipids. Following this reasoning it might be anticipated that advantageous photophysical properties of SQ-1 coupled with its high lipid-binding ability would render this probe a promising candidate for sensing the oxidative reactions in membrane systems. In the present study, the applicability of SQ-1 to monitoring lipid peroxidation has been evaluated using the model protein-lipid systems containing heme-proteins — methemoglobin (metHb) and cytochrome *c* (cyt *c*), and lipid vesicles composed of zwitterionic lipid phosphatidylcholine (PC) and its mixtures with anionic lipid cardiolipin (CL).

Experimental

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. Horse heart cytochrome *c* (ferric form) was from Fluka (Buchs, Switzerland). Horse blood methemoglobin, thiourea (TM) and butylated hydroxytoluene (BHT) were purchased from Reanal (Hungary). Iron sulfate ($FeSO_4$) and ascorbate were from Sigma Aldrich, Germany. SQ-1 was synthesized as described previously [7]. All other chemicals were of analytical grade and used without further purification.

Preparation of lipid vesicles

Unilamellar lipid vesicles composed of neat PC and its mixtures with 5 (CL5), 10 (CL10) or 20 (CL20) mol% CL were prepared by the extrusion method [15]. 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 NaP_i buffer (pH 7.4) at room temperature. Thereafter lipid suspensions were extruded through a 100 nm pore size polycarbonate filter (Nucleopore, Pleasanton, CA). The phospholipid concentration was determined according to the procedure of Bartlett [16].

Fluorescence measurements

Steady-state fluorescence measurements were performed at 20 °C with CM 2203 spectrofluorimeter equipped with magnetically stirred, thermostated cuvette holder (SOLAR, Belarus). SQ-1 absorption spectrum was recorded in ethanol, and the concentration of probe ethanolic stock solution was determined using the molar absorptivity $\varepsilon_{663} = 2.3 \times 10^5 M^{-1}\ cm^{-1}$. SQ-1 was used in micromolar concentrations to prevent dye aggregation. Emission spectra of SQ-1 were recorded in buffer and liposomal suspension with excitation wavelength at 660 nm. In kinetic experiments emission wavelength was set at 683 nm. Excitation and emission slit widths were 5 nm. To incorporate SQ-1

into lipid phase of the model membranes the dye-lipid mixtures were incubated for 30 min at room temperature.

MetHb and cyt *c* concentrations were determined spectrophotometrically, using extinction coefficients $\varepsilon_{406} = 1.415 \times 10^5 M^{-1}\ cm^{-1}$ and $\varepsilon_{406} = 1.05 \times 10^5 M^{-1}\ cm^{-1}$, per heme, respectively.

Results and discussion

SQ-1 is a zwitterionic symmetric squarylium dye with two butyl substituents connected to nitrogen atoms in the hetercycles. Due to its electronic symmetry, SQ-1 exhibits intense and sharp absorption in the range 550–700 nm with a major peak at 662 nm (Fig. 1a). Being non-fluorescent in buffer, SQ-1 displays strong emission when bound to the lipid vesicles (Fig. 1b). It has been proposed that deactivation of the fluorescent state of squarylium dyes in polar solvents results from intramolecular charge transfer and twisting around C-C bond between the heterocyclic ring and the C_4O_2 unit [17]. Significant enhancement of SQ-1 fluorescence on its transfer from aqueous to lipid phase can be ascribed to the decreased dielectric constant and polarity of the probe microenvironment coupled with its restricted rotation.

Association of methemoglobin and cytochrome *c* with the model lipid membranes was accompanied by a drastic time-dependent quenching of SQ-1 fluorescence with the magnitude of this effect being increased with the molar fraction of CL (Figs. 2 and 3, Table 1). Cyt *c* and metHb are heme-containing metalloproteins abundantly found in living organisms. Apart from their main physiological function (oxygen transport for haemoglobin, and electron transfer for cytochrome *c*), these proteins are known to act as effective catalysts of lipid peroxidation. To verify whether the disappearance of SQ-1 emission spectra arises from the dye interactions with protein-induced lipid free radicals, a series of experiments was carried out in the presence of well-known LPO inhibitors, BHT and TM. Addition of antioxidants resulted in significant suppression of the protein influence on SQ-1 emission with the magnitude of this effect being much more pronounced for metHb (Figs. 2 and 3, Table 1). These findings suggest that lipid peroxidation, initiated by metHb and cyt *c*, manifests itself in SQ-1 decolourization. To obtain additional arguments in favor of this assumption, we activated LPO by metal-catalyzed oxidation system (MCO) $FeSO_4$ + ascorbate (FA). Figure 4 represents the time course of SQ-1 emission following FA addition to the lipid vesicles. The observation that SQ-1 fluorescence steeply decreases in time can be considered as another for squaraine sensitivity to lipid peroxidation.

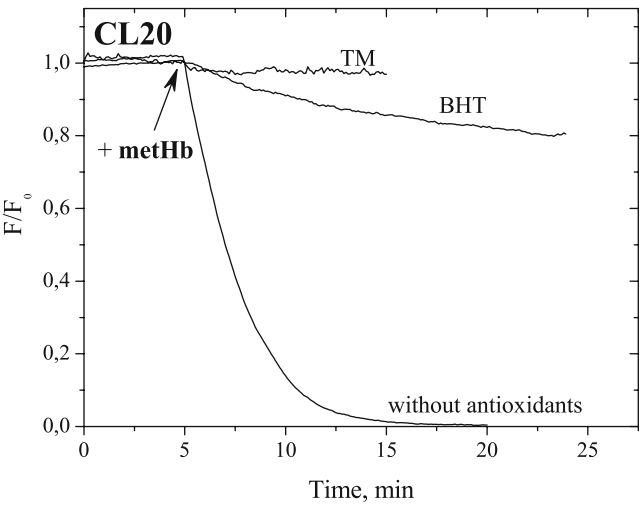

Fig. 2 Time course of SQ-1 fluorescence decrease induced by metHb association with PC/CL (20 mol%) model lipid membranes. F and F_0 denote SQ-1 fluorescence intensity at 683 nm after and prior metHb addition. Lipid concentration was 0.08 mM. The dye and protein concentrations were 0.1 and 0.05 μM, respectively

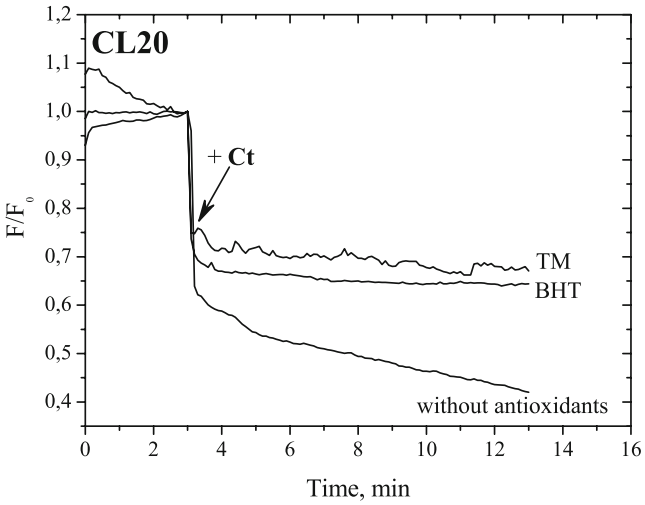

Fig. 3 Time-dependent changes in SQ-1 emission upon cyt *c* binding to PC/CL (20 mol%) liposomes. Lipid concentration was 0.08 mM. The dye and protein concentrations were 0.1 and 0.6 μM, respectively

Table 1 Decrease in SQ-1 fluorescence intensity upon formation of protein-lipid complexes, %

System	Control		+ BHT		+ TM	
	metHb	cyt *c*	metHb	cyt *c*	metHb	cyt *c*
PC	0.8	0.1	0.3	0.2	0.3	0.1
CL5	33	14	6	13	5	12
CL10	86	30	7	17	7	16
CL20	97	55	13	32	8	30

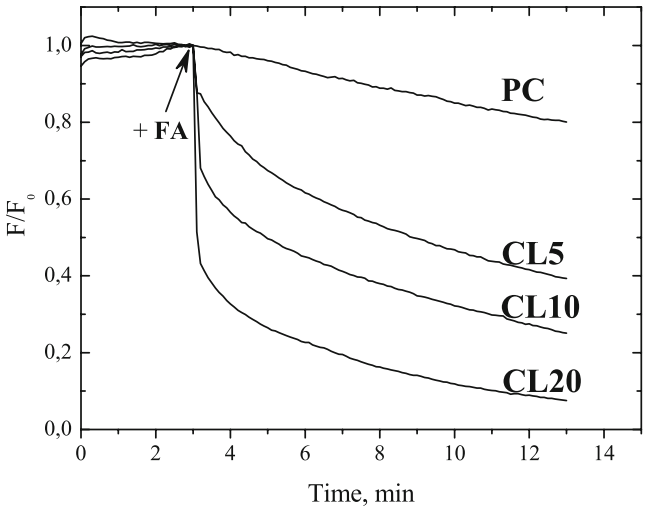

Fig. 4 SQ-1 fluorescence response to FA-induced lipid peroxidation. Lipid and ascorbate concentrations were 0.08 and 0.5 mM. The dye and $FeSO_4$ concentrations were 0.1 and 12 μM, respectively

Several lines of evidence indicate that protein-driven LPO activation is a complex process proceeding through the following steps: protein membrane binding → destabilization of the protein structure which involves opening of the heme pocket and increasing iron accessibility to the lipid species → iron interactions with polyunsaturated fatty acids (PUFA) [18-20]. Further events may occur via two pathways. The former starts with the iron-triggered decomposition of lipid hydroperoxides (LOOH), prominent intermediates of oxidative reactions inevitably present in membranes. This results in generation of reactive radical species, *i.e.* alkoxyl ($LO^{\bullet}$) and peroxyl radicals ($LOO^{\bullet}$) [21], which stimulate the chain reaction of lipid oxidation by further abstraction of hydrogen atoms from PUFA (Figs. 5, 6 and 7, Eqs. a,b). Second possible route is Fenton-like reaction, also known as Haber-Weiss cycle, implying the formation of hydroxyl radical ($OH^{\bullet}$) through the oxidation of iron by hydrogen peroxide (H_2O_2) [22] (Fig. 7, Eqs. c-e). Since BHT, a potent deactivator of $LOO^{\bullet}$, and TM, a well-known $OH^{\bullet}$ scavenger, inhibited metHb- and cyt *c*-induced LPO, we supposed that both of these mechanisms (LOOH-related and Fenton-like) may take place in our systems. Notably, the proposed pathways fairly explain the intensification of peroxidative reactions with CL content, which may be a consequence of both i) enhanced protein membrane binding due to the rise in bilayer surface charge or ii) increased initial level of LOOH in PC/CL liposomes resulting from the higher oxidability index of CL compared to PC.

While analyzing the data, it seems reasonable to account for the well-documented ability of the transition metals to quench the fluorescence of NIR dyes [23]. This property has been interpreted in terms of electron-transfer mecha-

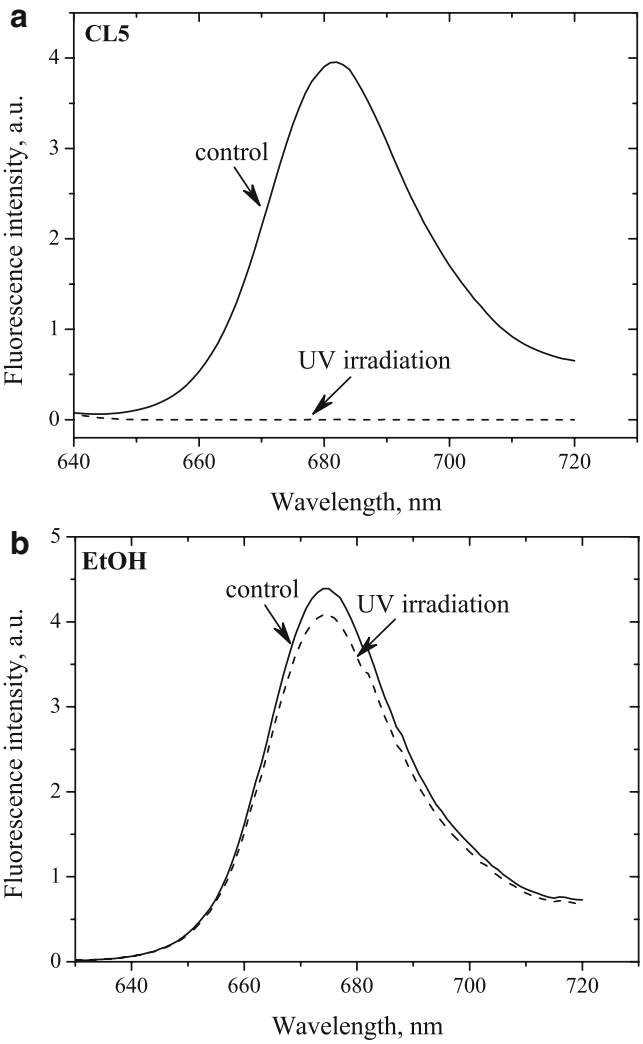

Fig. 5 Effect of UV irradiation on emission of SQ-1 bound to CL5 lipid vesicles **a** or free in ethanol **b**

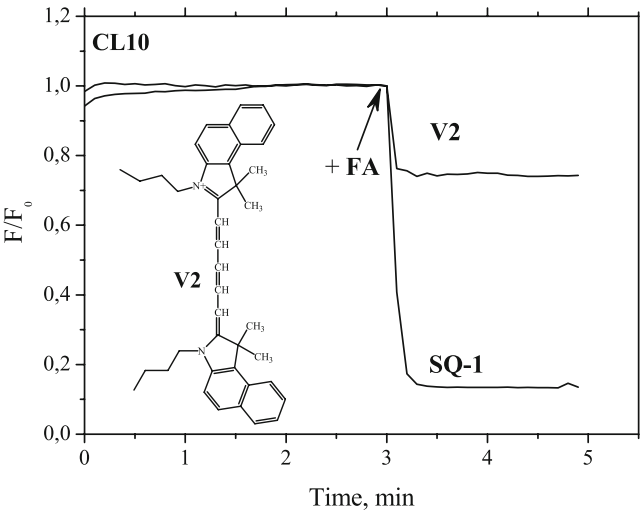

Fig. 6 Comparison of SQ-1 and V2 fluorescence changes arising from the dyes interactions with lipid free radicals

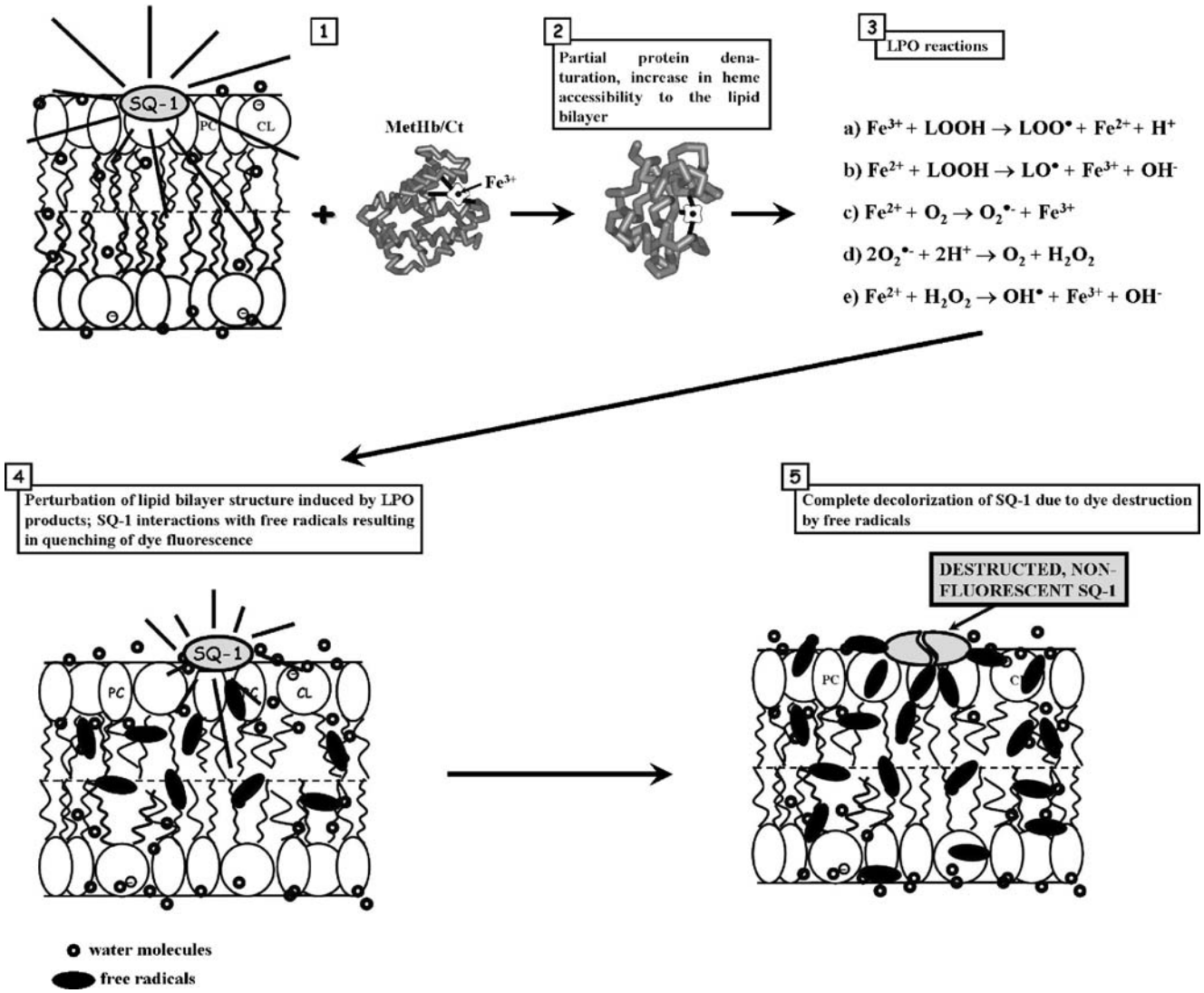

Fig. 7 Schematic representation of free radical-promoted SQ-1 bleaching

nism whereby the dye serves as π-donor ligand forming the charge-transfer complexes with the metal. Formation of such complex perturbs the π-conjugated system of the squaraines, and subsequently leads to the drop in fluorescence. In view of this, charge transfer can be assumed to contribute to the observed SQ-1 bleaching. However, this mechanism is unlikely to play the predominant role for the following considerations. First, inhibitory effect of BHT and TM strongly challenges this possibility. Second, according to the data of Tarazi et al. [23], two-fold quenching of squaraine emission by Fe^{3+} ions corresponds to the metal:dye molar ratio 10:1. In our case, more pronounced decrease in SQ-1 fluorescence is observed at substantially lower Fe^{3+}/SQ-1 molar ratios (2:1 for metHb and 5:1 for cyt *c* assuming that concentration of ferric ions is proportional to the concentration of heme in the sample). Third, SQ-1 was found to be sensitive not only to iron-catalyzed but also to UV irradiation-induced lipid peroxidation. More specifically, we didn't observe any SQ-1 emission in UV irradiated liposomes (Fig. 5a). This finding cannot be explained by the destructive effect of UV on squaraine since irradiation of SQ-1 ethanolic solution caused only a slight fluorescence decrease (Fig. 5b). Hence, disappearance of SQ-1 emission, most probably, stems from the dye interactions with lipid free radicals produced by UV. These considerations lend support to the above hypothesis that the main reason for SQ-1 decolorization upon complexation of heme-proteins with model membranes is iron-triggered LPO but not static fluorescence quenching by Fe^{3+} ions.

In the following, it is tempting to analyze, what molecular events are behind the uncovered bleaching of SQ-1. Formation of lipid free radicals may exert an influence on squaraine emission via (i) indirect effects

originating from LPO-induced changes in probe microenvironment, and (ii) direct interactions of ROS with SQ-1. Accumulating evidence indicates that lipid peroxidation produces considerable alterations in the structure and physicochemical properties of a lipid bilayer [24-27]. The processes accompanying membrane oxidation are reported to include: (i) increase in water permeability, (ii) disordering of lipid tails, (iii) interdigitation of lipid terminal methyl segments, (iv) increase of dielectric constant of membrane core, (v) rise in bilayer viscosity associated with the free radicals cross-linking. These processes may significantly contribute to the observed spectral effects. More specifically, since the dye under study is nonfluorescent in aqueous solutions, the reduction of its emission is likely to reflect increased bilayer hydration evoked by LPO. Parallel to this, the changes in SQ-1 spectral parameters may originate from modification of the probe structure upon radical attack on the dye. In this context it is worthy of mentioning that dramatic loss of dye emission has been also observed by Nicolescu et al. while investigating the response of BODIPY fluorophore towards the lipid peroxyl radicals [28]. Using the LC/MS analysis in combination with the spectroscopic measurements, the authors proved that BODIPY decolorization arises from the oxidative cleavage of olefinic bond adjacent to the pyrrole ring by $LOO^{\bullet}$. This conclusion is in a good harmony with HPLC results obtained by Guivarch and co-workers [29] who postulated that interactions of free radicals with organic compounds (such as fluorescent probes) may occur via four types of reactions: a) hydrogen atom abstraction, b) electrophilic addition on a double bond, c) oxidation of a double bond, d) electron transfer. Although it is difficult to assess what reaction is predominant in our case, we tried to identify which structural features of SQ-1 may account for its sensitivity to the ROS. In approaching this goal, we investigated the behavior of polymethine dye V2, a structural analog of SQ-1 lacking the central squaric moiety. As seen in Fig. 6, under the same conditions the generation of lipid free radicals gives rise to 70%-quenching of SQ-1 fluorescence, and only to 30%-quenching of V2 emission. This finding allowed us to hypothesize that reaction between ROS and SQ-1 occurs at the squaric moiety or in its vicinity.

Cumulatively, the results presented here are suggestive of the following scenario of free radical-promoted SQ-1 bleaching (Fig. 7). Initial step of this process is metHb and cyt *c* membrane adsorption which is followed by partial denaturation of proteins, destabilization of heme-globin linkage, and iron transition in LPO-initiating form. Once formed, free radicals attack SQ-1 thereby causing the dye degradation and eventually decolorization.

In conclusion, the present study has been undertaken to evaluate the potential of SQ-1 as a novel long-wavelength probe for detection of reactive oxygen species. This squaraine derivative was found to be extremely sensitive to the lipid peroxidation processes, thus opening the way for the use of this class of dyes in a diversity of free radical sensing systems.

Acknowledgment This work was supported by the grant № 4534 from the Science and Technology Center in Ukraine.

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