*БІОФІЗИКА КЛІТИНИ*

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# SPECTRAL BEHAVIOR OF AMYLOID – SPECIFIC DYES IN PROTEIN – LIPID SYSTEMS. I. CONGO RED BINDING TO MODEL LIPID MEMBRANES

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The phenomenon of abnormal protein aggregation currently attracts ever growing attention due to its involvement in etiology of a number of so-called conformational diseases, including neurological disorders, type II diabetes, prion diseases, etc. In vivo, transformation of polypeptide chain into partially folded aggregation-prone conformation can be initiated by protein-lipid interactions. Lipid bilayer, a basic structural element of biological membranes, may act as an effective catalyst of fibrillogenesis, providing an environment where protein molecules adopt conformation and orientation promoting their assembly into protofibrillar and fibrillar structures. Identification of amyloid fibrils in protein-lipid systems with widely employed spectroscopic criteria involving amyloid-specific dyes Congo Red (CR) or Thioflavin T (ThT) may be complicated by interferences of spectral responses from protein- and lipid-bound dye species. To circumvent this problem, all optical amyloid markers must be thoroughly characterized with respect of their lipid-associating abilities. In the present study, the interactions between CR and model lipid membranes composed of phosphatidylcholine (PC) and its mixtures with anionic lipid cardiolipin (CL), cationic detergent cetyltrimethylammoniumbromide (CTAB) and cholesterol (Chol) have been examined using absorption spectroscopy technique. It was found that CR can effectively interact with PC, PC:Chol and PC:CTAB bilayers. The observed shifts of absorption maxima suggest that the dye is capable of penetrating into interfacial region of uncharged model membranes, while remaining at the bilayer surface in positively charged membranes. No CR binding to negatively charged bilayers has been detected. Differential absorption spectra of the lipid-bound dye exhibited maximum at 524 nm, the value different from that characteristic of amyloid-bound dye (545 nm). These findings suggest that CR can be used for detection of amyloid growth in protein-lipid systems, especially for identification of amyloid fibrils induced by anionic lipids.

**KEY WORDS:** Congo Red, liposomes, dye-lipid interactions

Aggregation of soluble proteins into highly ordered beta-sheet fibrillar structures (amyloids) is a key factor in etiology of a number of so-called conformational diseases, including neurological disorders, type II diabetes, prion diseases, etc. [1,2] The phenomenon of abnormal protein aggregation extends far beyond pathophysiology, encompassing both fundamental and applied problems associated with protein misfolding. Accumulating evidence lends support to the hypothesis that structural transformation of a polypeptide chain into partially folded conformation is a critical prerequisite for fibril formation [3,4]. One factor that can initiate such a transformation in vivo involves protein adsorption at interface. It is the presence of large amount of interface, formed by cellular membranes, that determines the principal difference between in vitro and in vivo amyloid growth. Lipid bilayer, a basic structural element of biological membranes, may act as an effective catalyst of fibrillogenesis, providing an environment where protein molecules adopt conformation and orientation promoting their assembly into protofibrillar and fibrillar structures. Moreover, membrane represents a direct target for the toxic pre-fibrillar aggregates of misfolded proteins, which cause membrane damage via formation of non-specific ion channels and/or uptake of lipids into the forming fiber [5,6].

To classify a protein aggregate as an amyloid fibril a combination of the following criteria has been proposed: i) β-sheet secondary structure; ii) specific binding of the dyes such as Congo Red, Thioflavin T or Thioflavin S; iii) gel formation; iv) seeded kinetics of fibrillization; v) low solubility in denaturants and protease resistance [7]. Despite widespread use of these criteria, their applicability to each particular system must be thoroughly evaluated. Specifically, being applied to protein-lipid systems, spectroscopic criteria involving amyloid-specific dyes CR or ThT may suffer from the contribution of lipid-bound dye to the observed spectral response. For this reason, it seems of importance to characterize lipid-associating properties of the chromo- and fluorophores currently used for identification of amyloid fibrils. In the present study our efforts were concentrated on elucidating the nature of CR interaction with the model lipid membranes whose components were chosen in a way providing variations of the main physical characteristics of a lipid bilayer – surface charge and acyl chain mobility. More specifically, we examined CR binding to four types of lipid vesicles prepared from zwitterionic lipid phosphatidylcholine, and its mixtures with anionic lipid cardiolipin, cationic detergent cetyltrimethylammoniumbromide and cholesterol.

## MATERIALS AND METHODS

Egg yolk PC and beef heart CL were purchased from Biolek (Kharkiv, 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 cholesterol were from Sigma (St. Louis, MO, USA). Congo red was from Aldrich (Milwaukee WI). All other chemicals were of analytical grade.

Large unilamellar lipid vesicles composed of PC and its mixture with 10 mol% CL, 5 mol% CTAB or 30 mol% Chol were prepared by the extrusion technique. The thin lipid films were obtained by evaporation of lipids' ethanol solutions and then hydrated with 1.2 ml of 5 mM sodium-phosphate buffer (pH 7.3). Lipid suspension was extruded through a 100 nm pore size polycarbonate filter. Phospholipid concentration was determined according to the procedure of Bartlett [8].

Absorption measurements were performed with SF-46 spectrophotometer. CR concentration was determined spectrophotometrically using the extinction coefficients $\varepsilon_{CR}^{498} = 3.7 \times 10^4$ M$^{-1}$cm$^{-1}$.

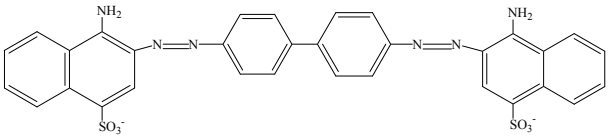


Fig. 1. Structure of CR molecule.

The absorption spectroscopy methodology was employed to determine quantitative parameters of CR binding to liposomes of different composition. The dye partition coefficient is defined as:

$$K_P = \frac{N_L V_W}{N_W V_L} \tag{1}$$

where $N_L$ and $N_W$ are the moles of the dye in the lipid and aqueous phases, respectively, $V_L$ and $V_W$ are the volumes of these phases. The volume of lipid phase was calculated as*:*

$$V_L = N_A C_L \sum \nu_i x_i \tag{2}$$

here $C_L$ is the molar lipid concentration, $x_i$ is mole fraction of the i-th bilayer constituent, $\nu_i$ 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 [9]. Since cholesterol exerts a condensing effect on the lipid bilayer, in PC:Chol model membranes *v* value was reduced by the factor 1.3. Given that under the employed experimental conditions $V_L$ is much less than the total volume of the system, $V_W \approx V_t = 1$ dm$^3$.

By examining the absorbance change ( $\Delta A_{\text{exp}}$ ) upon the dye association with the liposomes as a function of lipid concentration, the values of $K_P$ can be derived from the following relationship:

$$\Delta A_{\text{exp}} = A_L - A_W = \frac{K_P V_L \left(A_{\max} - A_W\right)}{1 + K_P V_L} \tag{3}$$

where $A_L$ is the absorbance observed in liposome suspension at certain lipid concentration $C_L$, $A_W$ is the dye absorbance in buffer, $A_{\max}$ is the limit absorbance in the lipidic environment. The partition coefficient was derived by the fitting procedure involving minimization of the function:

$$f = \frac{1}{N} \sum_{i=1}^{N} \left(\Delta A_{\text{exp}} - \Delta A_t\right)^2 \tag{4}$$

where $\Delta A_t$ is $\Delta A$ calculated according to Eq. (3), *N* is the number of experimental points in $\Delta A$ dependency on lipid concentration.

## RESULTS AND DISCUSSION

Shown in Fig.1 are CR absorption spectra in buffer solution and suspensions of PC, PC:Chol, PC:CL and PC:CTAB lipid vesicles. The observed differences between the dye spectra in aqueous and liposome media indicate that CR is capable of partitioning into PC, PC:Chol and PC:CTAB bilayers. Such a capability manifests itself in the shifts of absorption maximum from 496 nm (free dye) to 502 nm (PC bilayer), 498 nm (PC:Chol bilayer) and 485 nm (PC:CTAB bilayer). In the meantime, in the suspension of PC:CL liposomes the dye adsorption spectra remain virtually unchanged (Fig. 1, D), suggesting that CR binding to negatively charged bilayer, if any, is very weak. The opposite spectral effects – shift of the maximum position to longer wavelengths in PC and PC:Chol systems, and to shorter wavelengths in PC:CTAB system can be rationalized in terms of distinct mechanisms underlying CR association with uncharged and positively charged lipid bilayers. The long-

wavelength shift of adsorption maximum is most probably to arise from the chromophore transfer to lipid phase whose polarity is lower than that of water. Since CR molecule contains two negatively charged sulfonic groups, the dye is unlikely to penetrate deeply into nonpolar membrane core, preferring to reside in the interfacial bilayer region. In this region, comprising phosphorylcholine, glycerol, carbonyl moieties and upper acyl chain carbons, the value of dielectric constant drastically changes from 80 in water to ca. 4 in the hydrocarbon core. The observed differences between CR spectral properties in PC and PC:Chol systems can be explained by cholesterol-induced structural reorganization of the lipid-water interface. In the lipid bilayer amphiphilic cholesterol molecule is thought to adopt quasi-perpendicular to the membrane surface orientation with 3$\beta$ -hydroxyl group being located in the interfacial region and apolar moiety composed of tetracyclic ring and isooctyl side chain embedded in the hydrophobic core. To date, the implications of cholesterol inclusion in the lipid bilayer are rather well characterized. For the liquid-crystalline lipid phase these include: i) increase of the separation of the phospholipid headgroups [10,11]; ii) increased freedom of motion of the phosphorylcholine moiety [12]; iii) enhanced headgroup hydration [13]; iv) reduced content of the acyl chain gauche conformations [14]; v) tighter lateral packing of lipid molecules (condensing effect) [15]. In view of this the observation that long-wavelength shift of absorption maximum becomes less pronounced on Chol inclusion into PC bilayer can be interpreted as arising from the increased hydration and enhanced interfacial mobility of PC:Chol membranes.

To obtain quantitative information on CR-membrane binding, we analyzed the dependencies of absorbance changes (at wavelength 520 nm) on lipid concentration (Fig. 2, C-D) in terms of the above partition model (Eqs. (1)-(4)). The CR partition coefficients were found to be $1.2 \cdot 10^3$ (PC), $3.8 \cdot 10^3$ (PC:Chol), $9.7 \cdot 10^4$ (PC:CTAB).

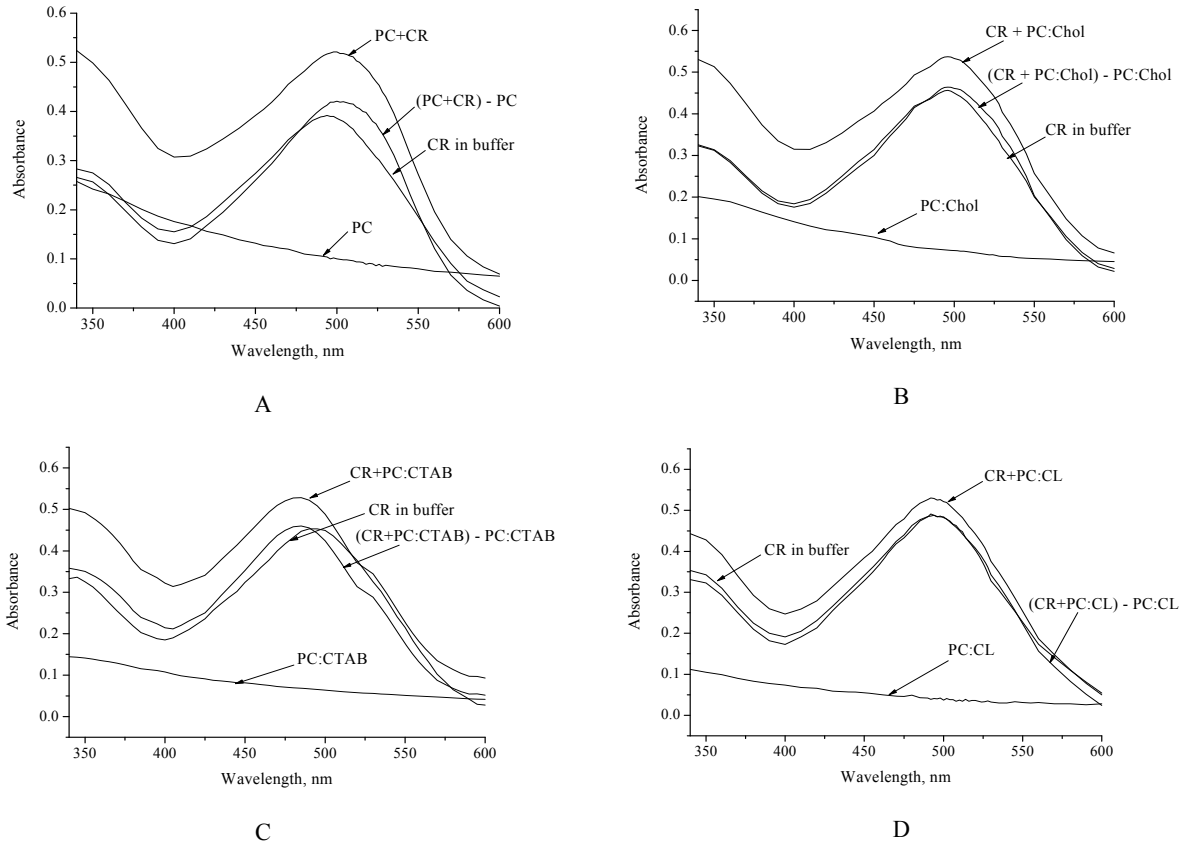


Fig. 1. CR absorption spectra in buffer and suspensions of liposomes: A – PC, B – PC:Chol (30 mol%), C – PC:CTAB (5 mol%), D – PC:CL (10 mol %). CR concentration was 10 μM

Contrary to the neutral PC and PC:Chol bilayers, CR seems to adopt surface location in the PC:CTAB model membranes, forming ionic contacts with positively charged nitrogen atoms of the detergent. The validity of this assumption is corroborated by the short-wavelength shift of CR adsorption maximum in PC:CTAB system (Fig. 1, C). Interestingly, differential absorption spectra of the lipid-bound dye exhibited similar maxima at ca. 524 nm, but different signs for uncharged and positively charged membranes (Fig. 2, A). Notably, CR specific binding to amyloid fibers is followed by the appearance of characteristic peak at 545 nm in the

differential absorption spectra [7]. The fact that maximum positions of lipid-bound (524 nm) and amyloid-bound dye (545 nm) are different may facilitate CR-based detection of the lipid-induced amyloid fibrils.

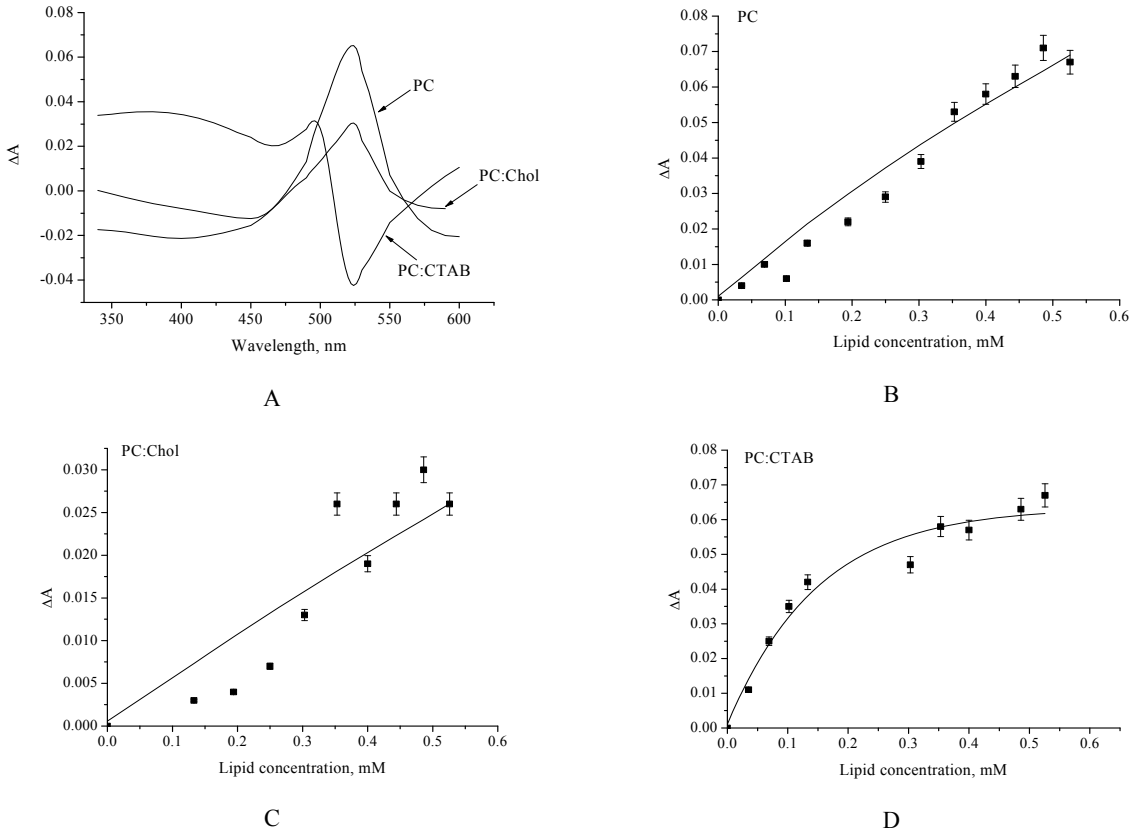


Fig. 2. A – CR differential absorption spectra in liposomal suspensions; B, C, D – CR absorbance changes at 520 nm as a function of lipid concentration for liposomes of different types

## CONCLUSIONS

Amyloid-specific dye Congo Red exhibits pronounced lipid-associating abilities, as evidenced by the shift of the dye adsorption maximum to longer wavelengths in the neutral (PC and PC:Chol) bilayers and to shorter wavelengths in the positively charged (PC:CTAB) bilayers. These spectral effects were interpreted in terms of CR penetration into interfacial region of PC and PC:Chol membranes, and electrostatically-controlled dye location at the surface of PC:CTAB membranes. The finding that CR absorption spectra are insensitive to the presence of negatively charged liposomes facilitates the use of this dye in detecting the amyloid aggregates induced by anionic lipids. Likewise, CR differential absorption spectra in liposomal suspensions are featured by extremum positions distinct from that characteristic of amyloid fibrils. Accordingly, CR-based spectroscopic criterion can be effectively employed to monitor amyloid growth in protein-lipid systems.

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