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STUDY OF CATIONIC PORPHYRINS BINDING TO BOVINE SERUM ALBUMIN BY METHOD OF FLUORESCENT SPECTROSCOPY

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It is shown that methods of the fluorescent spectra analysis allow determining the binding constants of porphyrins with the bovine serum albumin (BSA) and their localization on the protein globule correctly enough. Low values of binding constants of cationic porphyrins prove sorption of porphyrins on the surface of proteins.

Cationic porphyrins – serum albumin – fluorescent spectroscopy

Ցույց է տրված, որ ֆլուորեսցենտային սպեկտրերի վերլուծության մեթոդները թույլ են տալիս բավականին ճշգրիտ որոշել պորֆիրինների կապման հաստատունները ցուլի շիճուկային ալբումինի (ՑՇԱ) հետ և նրանց տեղայնացումը սպիտակուցի վրա: Կատիոնային պորֆիրինների կապման հաստատունների փոքր արժեքները վկայում են սպիտակուցի մակերևույթի վրա պորֆիրինների սորբցիայի մասին:

Կատիոնային պորֆիրիններ (շիճուկային ալբումին (ֆլուորեսցենտային սպեկտրոսկոպիա

Показано, что методы флуоресцентного анализа позволяют достаточно корректно определять константы связывания порфиринов с бычьим сывороточным альбумином (БСА) и их локализацию на белковой глобуле. Низкие значения констант связывания катионных порфиринов свидетельствуют о сорбции порфиринов на поверхности белка.

Катионные порфирины - сывороточный альбумин - флуоресцентная спектроскопия

The use of visible light in combination with administration of the photosensitizing drug is the key feature of photodynamic therapy of tumors (PDT) [3, 7]. Photosensitizers (PSs) which show high selectivity of accumulation in relation to tumor cells and tissues are introduced into the organism of the patient and then the tumor area is being illuminated by light. PSs excited by light reacting with surrounding molecules generate radicals and reactive oxygen species which destroy tumors due to cytotoxicity [3, 7]. The photosensitizers of cationic nature (basically porphyrin-related compounds) show especially high selectivity *in vivo* [18]. The main carriers of porphyrins are large proteins of blood (serum albumin and hemoglobin) [6, 20] and also high and low density

lipoproteins [12, 14]. Serum albumin plays an important role in the system of blood plasma proteins in the context of its relatively large quantities (about 60 % of plasma proteins) as well as of its functions (binding and transport of fatty acids, steroids, porphyrins, bilirubin, thyroxin, etc.) [19]. The aim of the present work was a study of binding of new cationic porphyrins [17, 10] to bovine serum albumin in vitro and determination of binding constants of porphyrin-protein complex by a method of fluorescent spectroscopy.

Material and Methods. BSA was purchased from Sigma-Aldrich Chemical Co. (USA). New water-soluble cationic porphyrins and metalloporphyrins with different central atoms of metal (Zn, Ag, Co, Fe, Mn, Cu, etc.) and different peripheral functional groups (oxyethyl, butyl, allyl, and methallyl) were synthesized at the Yerevan State Medical University according to the methods described in works [17, 10]. The following 8 new cationic porphyrins and metalloporphyrins were used in the present work: meso-tetra-[4-N-(2`-oxyethyl) pyridyl]porphyrin (TOE4PyP), Zn-TOE4PyP; meso-tetra-[4-N-(2`-butyl) pyridyl]porphyrin (TBut4PyP), Zn-TBut4PyP; meso-tetra-[4-N-(2`-allyl)pyridyl] porphyrin (TAll4PyP), Zn-TAll4PyP, Co-TAll4PyP; Co-meso-tetra-[4-N-(2`-methallyl) pyridyl]porphyrin (Co-TMetAll4PyP). The binding ability of these preparations was compared with the well-known photosensitizer chlorin e_6 (Chl, "The enterprise on manufacture of diagnostic and medicinal preparations", Minsk, Belorussia). All other reagents were of analytical grade

The concentration of BSA in a solution was regulated by absorption (A) on 279 nm considering the absorption of its 1 % solution to be $A_{279} = 6.67$ [21].

Fluorescent emission spectra of BSA solution and BSA-porphyrin complex were measured with ETC-273 FP-6500 spectrofluorometer (JASCO, Japan) in 1 cm quartz cell and recorded in a region from 300 to 450 nm (excitation at 295 nm). The binding of the porphyrins to BSA was studied by titration of the protein with increasing concentrations of the porphyrins and monitoring the changes in the florescence spectra of protein-bound versus free porphyrin. For the quantitative analysis of the interaction between porphyrins with BSA, the Stern–Volmer equation was used. For static quenching, fluorescence quenching can be analyzed using modified Stern–Volmer relation [16]:

$$Io / Io-I = 1 / fK[Cp] + 1 / f \qquad (1)$$

where I and I_o are the fluorescence intensities of BSA in the presence and absence of quencher (porphyrin), respectively; K is the Stern–Volmer quenching constant and $[C_p]$ is quencher concentration; f is the fraction of the initial fluorescence accessible to quencher. The plots of I_o / I_o-I versus $1/[C_p]$ yields 1/f as the intercept, and 1/fK as the slope. In a number of works it was shown that the binding constant in the reaction of complex formation was the constant of static quenching which arose from the formation of a dark complex between fluorophore and quencher [2, 8]. Proceeding from these results we will consider that determining Stern-Volmer constant K we determine protein-porphyrin binding constants.

When determining the values I and Io of the fluorescent intensity we used the software of spectra processing "Spectra Manager for Spectra Analysis, Version 1.53.00, JASCO Corporation, 2000 ". This program corrects the value of fluorescent intensity I in view of spectra asymmetry and deviation from a base (zero) line. The value of K is determined according to relation of Stern–Volmer (1). The results of experiments have been processed statistically with use of Student criterion.

Results and Discussion. It is proven that the photosensitizers upon administration distribute and bind to serum proteins [12.] There are two complementary spectral methods for research of the protein binding to ligand *in vitro*: absorption and fluorescent spectroscopy. The method of absorption spectroscopy showed that study of protein-ligand binding should be carried out at sufficiently low concentration of proteins because of their possible dimerization and polymerization at larger concentrations. In this case formation of isosbestic point (the common point of spectral curves intersection) is a good criterion of two-component system (the protein and ligand monomers) presence in the solution [1]. The investigations carried out earlier by the method of absorption spectroscopy demonstrated the competence of such an approach; the binding constants

of new cationic porphyrins with some blood proteins (serum albumin and hemoglobin) were obtained [13, 20]. However the investigations of this kind by the method of absorption spectroscopy at low concentration of proteins are limited by the factor of sensitivity of measurements. The method of fluorescent spectroscopy is considerably more sensitive than absorption spectroscopy and allows registering a signal from 10^{-17} M of a fluorescent substance solution [5]. To preserve linear dependence of fluorescence signal strength (I) and concentration of substance (c), the optical density of solution A = εcL should be less than 0.1 (ε is a molecular factor of extinction, L is a thickness of a solution layer) [5]. The concentration of BSA was equal to 1.5×10^{-6} M in all experiments with studying of BSA solution titration by solutions of various porphyrins which corresponds to absorption value of A = 0.0667 < 0.1. A typical series of fluorescence spectra of BSA solution (in 0.01 M the phosphate buffer pH 7.2) is presented in Fig. 1 at titration by increasing concentrations of porphyrin Zn-TOE4PyP (ratio C_{porph} / C_{prot} = 0.88; 1.31; 2.36; 3.77; 5.17; 9.61; 17.93).

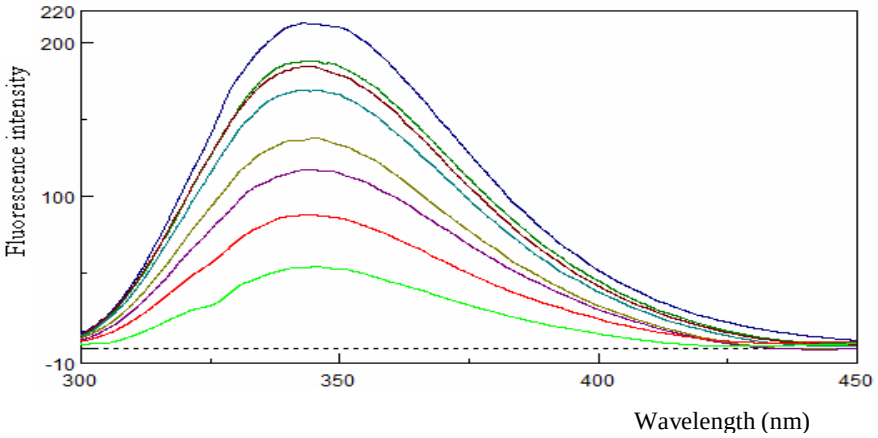

Figure 1. Fluorescence spectra (λ_{exc} = 295 nm) obtained via titration of BSA solution with porphyrin Zn-TOE4PyP (the top curve is a spectrum of BSA fluorescence without addition of porphyrin, decreasing curves are the fluorescence of BSA-porphyrin complex upon addition of increasing concentration of porphyrin).

For determination of binding constants *K* according to relation of Stern–Volmer (1) the adsorption isotherms in coordinates I_o/I_o-I vs $1/[C_{porph}]$ have been plotted. In Fig. 2 experimentally obtained values I_o/I_o-I for increasing concentrations of porphyrin Zn-TOE4PyP at the wave length which corresponds to the point of BSA fluorescence maximum (λ_{emiss} = 345 nm) are presented as points. The points have been obtained via the program of spectra processing "Spectra Manager for Spectra Analysis, ver. 1.53.00, JASCO Corporation, 2000" which also considered the fact of small dilution of a porphyrin solution at each step of the protein next portion addition.

According to the above-stated, the binding constant (K) to BSA has been determined for each investigated porphyrin. The results of determination of binding constants for 8 new porphyrins and metalloporphyrins as well as for the known photosensitizer chlorin e_6 (Chl) to the BSA are presented in Table 1.

From the results of the Table 1 it follows, that the metalloporphyrins bind a little bit better than porphyrins without metal (except for Zn-TOE4PyP). The high binding constant of Chl with BSA is close to results of work [15] in which the binding constant of BSA with Chl was obtained via two independent methods of the fluorescent analysis.

The constant of static fluorescence quenching ($K_{st} = 4.1 \cdot 10^6$ M^{-1}) determined from the data on quenching of protein fluorescence, practically coincides with the binding constant
($K_{bind} = 3.6 \cdot 10^6$ M^{-1}), found from the polarizing measurements of Chl luminescence [15]. Such coincidence of results testifies to practically full quenching of protein fluorescence at incorporation of Chl in the protein globule. Our data together with the results of work [15], and also with present-day ideas about binding of heme in subdomain I B of serum albumin [9] allow drawing the conclusion on incorporation of chlorin e6 in nonpolar (hydrophobic/waterproof) sites of a protein matrix.

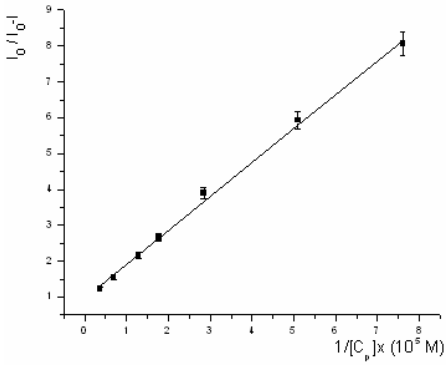

Fig. 2. Absorption isotherm of metalloporphyrin Zn-TOE4PyP to BSA

Table 1. The binding constants of porphyrins and metalloporphyrins to BSA

NN	Porphyrins and metalloporphyrins	BSA K x 10^{-5}, (M^{-1})
1.	TOEt4PyP	3.48 ± 0.21
2.	Zn-TOEt4PyP	1.01 ± 0.05
3.	TBut4PyP	0.95 ± 0.05
4.	Zn-TBut4PyP	2.29 ± 0.14
5.	TAll4PyP	1.01 ± 0.05
6.	Zn-TAll4PyP	1.02 ± 0.05
7.	Co-TAll4PyP	2.25 ± 0.13
8.	Co-TMetAll4PyP	1.95 ± 0.12
9.	Chlorin e_6	6.44 ± 0.32

Relatively lower values of cationic porphyrins binding constants (0.95-3.5x10^5 M^{-1}) prove absorption of porphyrins on the surface of proteins and also binding electrostatic character of porphyrins positively charged groups with negatively charged amino acids residues on a surface of BSA(at pH 7.4 negative charge of SA is equal to 18 [4]). These results are also in agreement with conclusions of work [13] that being positively charged the cationic porphyrins bind on surface of BSA and are not capable of getting into hydrophobic subdomains of BSA.

Thus, methods of the fluorescent analysis allow determining the binding constants of porphyrins with protein and the porphyrins localization on protein globule correctly enough. Electrostatic character of porphyrins binding to SA, probably, allows them to compete with fatty acids for binding places on albumin; the method of the fluorescent analysis is perspective for studying the processes of competitive binding of these substances on serum albumin.

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