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BINDING OF SOME CATIONIC PORPHYRINS WITH SERUM ALBUMIN

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It is shown, that the methods of absorption and fluorescence spectroscopy allow determining the binding constants of porphyrins with various types of serum albumin (SA) and their localization on the protein globule correctly enough. Researches testify about good binding of porphyrins with protein at low ionic strength of solution whereas at 0,1 M buffer solution the interaction of bovine serum albumin with porphyrins is considerably weak. Values of binding constants of cationic porphyrins prove their sorption not only on the surface of proteins but also in the globule of SA, which is confirmed also by computer simulation of SA-porphyrin interaction.

Bovine serum albumin – cationic porphyrins – binding constants

Ցույց է տրված, որ կլանման և ֆլուորեսցենտային սպեկտրասկոպիայի մեթոդները թույլ են տալիս բավականին ճշգրիտ որոշել պորֆիրինների կապման հաստատունները շիճուկային ալբումինի (ՇԱ) տարբեր տեսակների հետ և նրանց տեղայնացումը սպիտակուցի վրա: Հետազոտությունները վկայում են որ ցածր իոնային ուժի դեպքում պորֆիրինները լավ են կապվում սպիտակուցի հետ, մինչդեռ 0,1 M բուֆերի լուծույթում ցուլի շիճուկային ալբումինի համագործակցությունը պորֆիրինների հետ զգալիորեն թույլ է: Կատիոնային պորֆիրինների կապման հաստատունների մեծությունները վկայում են նրանց սորբցիայի մասին սպիտակուցի ինչպես մակերևույթի վրա, այնպես էլ գլոբուլի մեջ, ինչը նույնպես հաստատվում է համակարգչային մոդելավորումով ՇԱ-պորֆիրին փոխազդեցության ուսումնասիրությամբ:

Ցուլի շիճուկային ալբումին - կատիոնային պորֆիրիններ - կապման հաստատուններ

Показано, что методы абсорбционной и флуоресцентной спектроскопии позволяют достаточно корректно определять константы связывания порфиринов с различными типами сывороточного альбумина (СА) и их локализацию на белковой глобуле. Исследования свидетельствуют о хорошем связывании порфиринов с белком при низкой ионной силе, тогда как в 0,1 М растворе буфера взаимодействие бычьего сывороточного альбумина с порфиринами значительно слабее. Константы связывания катионных порфиринов свидетельствуют об их сорбции как на поверхности белка, так и внутри глобулы СА, что подтверждается также компьютерным моделированием взаимодействия СА □ порфирин.

Бычий сывороточный альбумин – катионные порфирины – константы связывания

The cationic photosensitizers (porphyrins) are the main form of compounds, which are widely used in photodynamic therapy of tumors (PDT) and photodynamic inactivation (PDI) of microorganisms. Photosensitizers (PS) are selectively accumulated by malignant cells and via photoirradiation provide a means of highly specific cell killing [19]. The high selectivity of cationic porphyrins has been used for tumor destruction in vivo [24]. Due to their positive charge and lipophilicity, these compounds are localized in mitochondria in response to a negative-inside membrane potential [6], and there is evidence to suggest that the mitochondrion may be a primary subcellular site of damage. Cationic photosensitizers also show high activity in photodynamic inactivation of microorganisms, where they are successfully used for both Gram (+) and Gram (-) microorganisms [21] and fungi [3].

The study of cationic photosensitizers binding and carrier is very important for PDT. On the organism level the PS transport to tumors is done by blood carrier proteins such as serum albumin (SA) [5, 22], low and high density lipoproteins [14], and hemoglobin [23]. The study of porphyrins binding to proteins on molecular level will clarify binding mechanisms and determine medium impact. In the blood plasma protein system the serum albumin takes leading place, considering its large amount (about 60% of plasma proteins) and its functions [20]. It posses a wide range of physiological functions involving the binding, transport, and delivery of fatty acids, porphyrins, bilirubin, tryptophane, thyroxin, and steroids [2, 8]. In the present work, bovine serum albumin (BSA) is selected as protein model, because it has wider application than human serum albumin (HSA) and is suitable not only for humans, but also for other animals, and there is lack of information on the porphyrin-BSA binding mode [13, 15, 18]. At the same time there are not enough studies on pH and solution ionic strength, as well as cationic porphyrins lateral radicals' impact on their binding to BSA. The main purpose of present study is investigation of above mentioned factors on cationic porphyrins-BSA binding via spectral methods.

Material and Methods. BSA (Product Number A2153), BSA Fatty Acid Free (Product Number A6003), HSA Fatty Acid Free (Product Number A1887) were obtained from Sigma-Aldrich Chemical Co. (USA) and used without further purification. The new water-soluble symmetrically and asymmetrically meso-substituted cationic N-quarternized 3- and 4-pyridyl-porphyrins and metalloporphyrins (Zn, Ag, Co, Fe, Mn, Cu, et al.) with different lateral functional groups (oxyethyl, butyl, allyl, and methallyl) were synthesized according to our previously reported methods [9]. The structure of the synthesized compounds was confirmed by nuclear magnetic resonance (NMR), infrared and absorption spectroscopy methods. The following porphyrins used in this 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;
- meso-tetra-[4-N-(2`- methallyl) pyridyl] porphyrin (TMetAll4PyP), Zn-TMetAll4PyP.

The binding ability of these preparations was compared with the well-known photosensitizer chlorine e6 [16] (Chl e6, "The enterprise on manufacture of diagnostic and medicinal preparations", Minsk, Belorussia). All other materials were of analytical grade.

Measurements and methods. Fluorescence emission spectra were measured with ETC-273 FP-6500 spectrofluorometer (JASCO). Fluorescence spectra of BSA and porphyrins-BSA complex were recorded from 300 to 450 nm (excitation at 295 nm). In fluorescence investigations binding of the porphyrins to BSA were 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 [17]:

$$Io / Io\text{-}I = 1 / fK[Cp] + 1 / f \quad (1)$$

where I and Io are the fluorescence intensities of BSA in the presence and absence of quencher (porphyrin), respectively; K is the Stern–Volmer quenching constant and [Cp] is quencher concentration; f is the fraction of the initial fluorescence accessible to quencher. In the linear plots Io / Io-I versus 1 / [Cp] yields 1 / f as the intercept, and 1 / fK as the slope. The static quenching interaction between cinnamic acid and BSA indicates [2], that the quenching constant (K) decreased with the temperatures increasing. It was found that the quenching constant (K) decreased with the temperatures increasing, which indicates the static quenching interaction between cinnamic acid and BSA. The results interpreted as the binding constant of the complexation reaction because static quenching arises from the formation of a dark complex between fluorophore and quencher [2, 7]. Experiment results on BSA-chlorine e6 binding show that Stern-Volmer static quenching constants K determined from data on protein fluorescence quenching closely match to binding constants determined from polarizing measures of chlorine e6 luminescence [15]. According to these results we will consider that determining Stern-Volmer constant K we define protein-porphyrin binding constants.

UV–Vis absorbance spectra were measured with a Shimadzu UV–VISIBLE Recording Spectrophotometer UV-2100 (Japan). In absorption spectroscopy investigations binding of the porphyrins to BSA were studied by titration of the porphyrins with increasing concentrations of the BSA and monitoring the changes in the absorption spectra of porphyrins-bound versus free BSA. The binding constant K, for the interactions porphyrins-BSA was determined by analyzing the reduced absorbances at Soret band (for example 440 nm for Zn-TOE4PyP) due to addition of BSA to the porphyrin solution following the modified relation of Kapp et al. [12]:

$$Ao / Ao\text{-}A = 1 / fK[Cprot] + 1 / f \quad (2)$$

where Ao and A are the porphyrin absorbances in the absence and presence of the protein (BSA), respectively. In the linear plots Ao / Ao-A vs. 1 / [Cprot], 1 / f is obtained from the intercept on the Ao / Ao-A axis, corresponding to 1 / [Cprot] = 0.

By defining values of intensity Io (Ao) and I (A) we used “Spectra Manager for Spectra Analysis, Version 1.53.00 JASCO Corporation, 2000” software. This software corrects fluorescence intensity I or absorbance A value considering asymmetric spectra and shift from baseline (zero line). The value of K defined according to relations of Stern–Volmer (1) or Kapp (2).

The results of experiments have been processed statistically using the criterion of Student.

Results and discussion. Numerous studies on fatty acids, porphyrins and other ligands binding done with methods of equal dialysis, fluorescent and absorption spectroscopy as well as electron spine resonance (ESR) and X-ray analysis show that serum albumin molecule has several types of ligands binding sites [8, 15]. From the analysis of its primary structure, HSA appears to be formed by three homologous domains (named I, II, and III) with two separate helical sub domains (named A and B) for each domains [8]. According to the data obtained by X-ray analysis, nuclear magnetic resonance, ESR, spectral and other methods, number of fatty acid binding sites on SA molecule varies between 2 to 8 with binding constants from 10^4 to $5x10^6$ M^{-1}. HSA is able to bind seven equivalents of long-chain fatty acids (FAs) at multiple binding sites with different affinities. In sites FA1-FA5 carboxylate moiety of fatty acids is anchored by electrostatic/polar interactions. On the contrary, sites FA6 and FA7 do not display a clear evidence of polar interactions that keep in place the carboxylate head of the fatty acid, thus suggesting that sites FA6 and FA7 are low-affinity FA binding sites [8, 10]. These studies show that five sub domains of SA apparently, except the sub domain IA, bind fatty acids. Naturally, here emerges the question: where and how bind multiple other ligands, particularly porphyrins? Studies of heme-SA binding show the allosteric type of heme binding regulation with medicine (warfarin). In fact, the affinity of HSA for heme decreases by about one order of magnitude upon warfarin (anticoagulant) binding; likewise, heme binding to HSA decreases the warfarin affinity by the same extent [1]. Studies on ligand binding constants definition explain this kind of SA behavior. Spectral studies are able to show rather exact quantitative characteristics,

comparison of which can explain mechanisms of SA interaction with such important molecules as fatty acids, porphyrins, medicines and etc. Herein the studies with two independent spectral methods such as fluorescence and absorption spectroscopy will complete each other.

The interaction of water-soluble cationic porphyrins with bovine serum albumin was studied at variable ionic strength and pH of phosphate buffer solution (PBS).

Fluorescence study on interaction of porphyrins with BSA. The interaction of porphyrins with bovine serum albumin was examined by fluorescence emission spectra of BSA at the excitation wavelength 295 nm. We have studied BSA molecule binding with 8 cationic porphyrins and well known photosensitizer chlorine e_6 used in clinics. In a typical experiment BSA $1{,}5\text{x}10^{-6}$ M solution (0.01 M PBS, pH 7,2) titrated with increasing concentrations of porphyrin ($1{,}3\text{x}10^{-6}$ M – $2{,}7\text{x}10^{-5}$ M). As an example, in Fig. 1 shows the fluorescence emission spectra of BSA with the addition of different concentrations of porphyrin TAll4PyP (ratio $C_{porph}/C_{protein}$ = 0,88; 1,31; 2,36; 3,77; 5,17; 9,61; 17,93).

In all experiments it can be found, that the fluorescence intensity of BSA decreased and the maximum of fluorescence emission wavelength had a slight blue shift (so, for porphyrin TAll4PyP from 343,5 nm to 338 nm, Fig. 1) with the increasing in porphyrin concentration. The results showed that the binding of porphyrins to BSA, as well as cinnamic acid [2], is associated with conformation changes in BSA, suggesting, that after adding the solution of porphyrins the chromophore of BSA was placed in more hydrophobic environment [25].

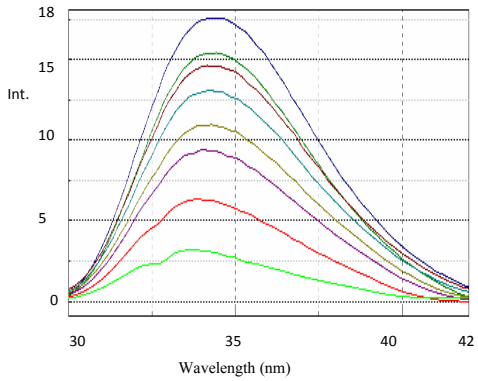

Fig. 1. Typical emission spectra of the titration of BSA by different concentrations of porphyrin (λ_{ex}=295 nm, λ_{em}=343.5 nm).

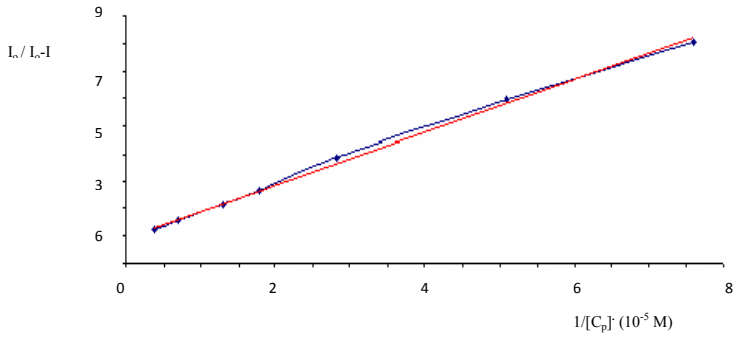

Fig. 2. Stern–Volmer curve for quenching of BSA with porphyrin TAll4PyP. The points are experimental data and a straight line is the theoretical curve according to equation (1).

The typical Stern–Volmer graph in Fig. 2 is shown (the titration of BSA by porphyrin TAll4PyP). Here **I** and $\mathbf{I_o}$ are the fluorescence intensities of BSA in the presence and absence of porphyrin, respectively; **K** is the Stern–Volmer binding constant (quenching constant) and $\mathbf{[C_p]}$ is porphyrin concentration. It can be found that plot is linear. According to formula (1) and "Spectra Manager for Spectra Analysis" we have estimated binding constants **K** for all 9 studied porphyrins for 3 different types of serum albumin: BSA, BSA Fatty Acids Free and Human SA Fatty Acids Free (Table 1). For BSA binding constant **K** values obtained for cationic porphyrins were approximately from 2 to 7 times less than for chlorine e_6 (Table 1, column 1). It was determined before [15], that chlorine e_6 resides in inner, hydrophobic parts of BSA molecule, and has high binding constant, more than 10^6 M^{-1}. The results determined by us can be interpreted as cationic porphyrins binding on the surface of BSA molecule. BSA which has negative charged amino acid residues on the surface and negative charge in general (neutral pH) [4], binds positive charged porphyrins on the surface of globule, preventing them from entering hydrophobic (inner) parts of molecule. As the result binding constants generally are compatibly smaller. However the value of a binding constant some of porphyrins (TOE4PyP, Zn-TOE4PyP, TBut4PyP, Zn-TBut4PyP) not much more differs from a binding constant for chlorine e_6, that specifies their possible binding inside of serum albumin globule.

Table 1. Estimated values of porphyrin – SA binding constants (K) (in 0,01 M PBS, pH 7,2)

Spectrophotometric study on interaction of porphyrins with BSA. In literature there are a lot of data on binding constants values of different porphyrins with serum albumin (human and bovine), obtained by UV-VID spectroscopy and fluorescence methods. These values were obtained in different mediums and experiment conditions, and as a matter of fact the values differ a lot [5, 13, 15, 18]. We have made an attempt to

NN	Porphyrins	BSA, K (10^5 M^{-1})	BSA Fatty Acids Free, K (10^5 M^{-1})	HSA Fatty Acids Free, K (10^5 M^{-1})
		1	2	3
1	TOE4PyP	3,48	2,83	1,94
2	Zn-TOE4PyP	2,01	1,23	3,13
3	TBut4PyP	2,35	2,16	1,51
4	Zn-TBut4PyP	2,29	2,51	2,79
5	TAll4PyP	1,01	1,84	1,42
6	Zn-TAll4PyP	1,02	0,69	1,47
7	TMetAll4PyP	1,89	1,83	1,16
8	Zn-TMetAll4PyP	1,77	1,92	1,45
9	Chlorine e_6	6,44	-	-

study *in vitro* medium factor (pH and ionic strength of solution) impact on porphyrin Zn-TOE4PyP binding to bovine serum albumin.

In the first group of experiments we have studied the influence of pH (from 4,0 to 9,5) on porphyrin Zn-TOE4PyP binding with bovine serum albumin. In all expe-riments the ionic strength of solution was constant and quite low (measurements were taken in 0,01 M PBS, ionic strength 0,04). Proportion of protein – porphyrin was chan-ged from 0,1 to 40. The study was taken under 20^0. A typical example of the absorption spectra obtained by titration of Zn-TOE4PyP with increasing concentration of BSA is shown in Fig. 3 (pH = 6,15). Fig. 3 shows the shift of Soret band into the area of more long waves (batochromic effect) and also a decrease of absorption intensity (hypochromic effect). Observed hypochromic change can be explained with appearance of complexes BSA–porphyrin in solution where chromophore exists in more ordered form compared

to its free state. Intensifying titration leaded to the increase of the portion of bound porphyrin in the solution. Therefore, the hypochromic change increases until further increase of BSA concentration does not influence on spectra. Existence of isosbestic point (point of intersection of curves) on absorption spectra indicates that porphyrin in solution can be only in two states - free and bound with BSA. pH changes towards alkaline values bring to more crucial shifts of hypochromic and batochromic effects.

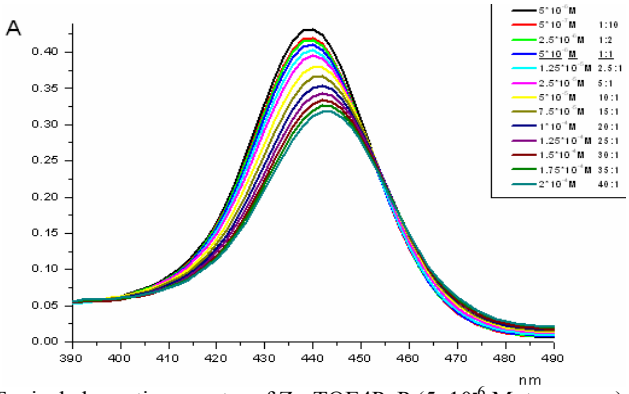

Fig. 3. Typical absorption spectra of Zn-TOE4PyP ($5x10^{-6}$ M, top curve) titration with increasing concentration of BSA (ratio protein/porphyrin presented at right).

Similarly to fluorescent studies, graphics according to modified relation of Kapp (2) were built. Similar graphic dependencies were obtained like in Fig. 2, however on the axis y instead of $\mathbf{I_0 / I_0\text{-}I}$ was entered $\mathbf{A_0 / A_0\text{-}A}$, and on the axis x a va-lues $\mathbf{1 / [C_{prot}]}$. Here **A** and $\mathbf{A_o}$ are the absorbance intensities of porphyrin Zn-TOE4PyP in the presence and absence of BSA, respectively; **K** is the binding constant and $\mathbf{[C_{prot}]}$ is the BSA concentration. It can be found that plot also is linear. According to relation (2) and by running "Spectra Manager for Spectra Analysis" software we have determined binding constants **K** for porphyrin Zn-TOE4PyP under various pH (Table 2) and ionic strength of solution (Table 3). Study results of porphyrin Zn-TOE4PyP binding with bovine serum albumin under various values pH of solution are presented in Table 2.

Table 2. Studies of porphyrin Zn-TOE4PyP binding with bovine serum albumin under various pH of solution

NN	Molarity of buffer, M	pH	Binding constant, M^{-1}
1.	0,01	4,0	$5{,}95 \times 10^4$
2.	0,01	6,15	$1{,}38 \times 10^5$
3.	0,01	7,2	$1{,}78 \times 10^5$
4.	0,01	8,25	$2{,}59 \times 10^5$
5.	0,01	9,0	$3{,}42 \times 10^5$
6.	0,01	9,5	$1{,}98 \times 10^6$

As we can see from Table 2, the binding constant increases by 33 times along with increase of pH from acid (4.0) to alkaline (9.5) medium. Under medium pH changes at pH 7,2 compared to pH 4,0 takes place rapid decrease of binding constant 3 times, which shows BSA conformation changes from globular to extended ellipsoid [11] and porphyrin outgo from SA molecule in the medium. Under pH 9,5 takes place rapid increase of binding constant, more than 11 times compared to pH 7,2. This reflects in grows of batochromic effect: big shift of absorption peak for 10 nm (from 440 to 450 nm).

In the second part of study we have investigated the influence of ionic strength on porphyrin Zn-TOE4PyP binding to bovine serum albumin. During the experiments pH

of solution was kept constant 7,2. Experiment results of porphyrin Zn-TOE4PyP binding to bovine serum albumin under constant pH (7,2) and different ionic strength of solutions are presented in Table 3. As we can see from Table 3, with increase of molarity (ionic strength) a decrease of binding constant takes place, which shows probable conformation changes of molecule BSA under medium changes [11]. This can be also explained by partial neutralization of albumin molecule charge (under pH 7.4 negative charge of albumin is 18 [4]) under increase of phosphate buffer positive ions in the medium (increase of solution molarity from 0,01 to 0,1 M), and negative ions of buffer probably partially deprotonize of porphyrin molecules. Under this conditions electrostatic interaction of cationic porphyrins with negative groups of amino acid residues weakens, which reflects in decrease of binding constant. Optimum conditions *in vitro* experiments is a molarity corresponding 0,01 M phosphate buffer.

Table 3. Porphyrin Zn-TOE4PyP – bovine serum albumin binding studies under solution different molarities

NN	Molarity of buffer, M	pH	Binding constant, M^{-1}
1	0,01	7,2	$1{,}78 \times 10^5$
2	0,03	7,2	$1{,}39 \times 10^5$
3	0,05	7,2	$1{,}05 \times 10^5$
4	0,1	7,2	$1{,}06 \times 10^5$

Thus, studies of Zn-TOE4PyP and serum albumin interaction showed not specific and reversible nature of this interaction. This kind of interaction mechanism makes possible the equality between porphyrin bound with albumin and porphyrin bound with tissue elements. As a result, the porphyrin has an ability to accumulate in tissues with high affinity to it, have a therapeutic action, and then wash away from the tissue. At the same time electrostatic type of binding lets porphyrin to compete with other substances, which can be transported by albumin, and particularly with fatty acids. The data obtained can be used for further study of competitive binding of fatty acids and porphyrins with binding sites of serum albumin macromolecule. The importance of the studies on porphyrin binding with biological structures in the aspect of synthesis porphyrin based of medicines for PDT should be noted.

Computer simulation of SA-porphyrin interaction. Above-mentioned results were used for construction of computer models of SA-porphyrins (TOE4PyP, TBut4PyP and TAll4PyP) interaction. Probable conformations of their binding have been done via 50-step-by-step docking by means of the program AutoGrid 4, AutoDock 4 software. It is shown, that these porphyrins are located in a "pocket" of subdomain I B of SA globule.

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