Photonics and Optoelectronics (P&O) Volume 3, 2014 www.jpo-journal.org
doi: 10.14355/jpo.2014.0301.03

The Relationship between the Structure and the Binding to Bovine Serum Albumin of Some Styrylcyanine Dyes

Eldar N. Kurtaliev[1], Negmat Nizomov[1], Gairat Khodjayev[2*]

[1] Physic department, Samarkand state university, University Blvd., 15, 140104 Samarkand, Uzbekistan

[2] Samarkand agriculture institute, M. Ulugbek str., 77, 140103 Samarkand, Uzbekistan.

[1]kurtaliev@rambler.ru; [1]nnizamov@yandex.ru; *[2]gayrat_kh@mail.ru

Received 7 June 2013; Revised 24 September 2013; Accepted 27 September 2014; Published January 2014

Abstract

Spectral-fluorescent characteristics of styrylcyanine dye Sbt ((E)-2-(4-(dimethylamino) styryl)-3-methylbenzo [d] thiazol-3-ium iodide) and its derivatives in aqueous solutions with and without the presence of bovine serum albumin (BSA) were studied. In the presence of BSA for all studied dyes in the absorption spectra, we observed an increase in the absorption intensity, a small broadening, and a new band of the shorter wavelengths with λ_{max}=412 nm. The intensity of the fluorescence emission of the baseband of the studied dyes in the presence of BSA increased from 1.1 to 4.6 times. The binding constant (K) and the number of binding sites (N) of the studied dyes with BSA were determined. The dependence of the binding constant with BSA on the dipole moment of the dye molecules was determined, which indicated that besides electrostatic attraction forces, hydrophobic interactions are significant between styrylcyanine dyes with BSA molecules. It was demonstrated that the aggregation of dyes influences the interactions of dyes with BSA.

Keywords

Styrylcyanine Dyes; Fluorescence; Electronic Absorption Spectra; Bovine Serum Albumin; the Binding Constant

Introduction

Organic dyes are used in quantum electronics (Svetlichnyi, *et al.,* 2009; Bezrodnyi, *et al.,* 2009), analytical chemistry (Tian, *et al.,* 2012), photodynamic therapy (Yukruk, et al., 2005), tissue optics (Smith, *et al.,* 2007) and in biomedical research for the detection of nucleic acids (Timtcheva, *et al.,* 2000). In order to expand the area of their application, research and synthesis of new organic dyes is necessary. In particular, the search for new fluorescent probes and labels is stimulated by the growing volume of biomedical research and diagnosis of diseases (Khodjaev, *et al.,* 2007; Matiukas, *et al.,*2007).

Various photophysical and photochemical properties of the fluorophores, such as quantum yield (Al-Shamiri, *et al.,* 2009), the fluorescence lifetime (Lee, *et al.,* 2008), photostability (Song, *et al.,* 2009) and pH dependence (Mazieres, *et al.,* 2009) define their applicability area. For *in vivo* measurements, the organic dyes of different classes are used-triphenylmethane (Guo, *et al.,* 2009), xanthene (Delgadillo, *et al.,* 2010) and polymethine (Tatikolov, 2012). Recently, styrylcyanine dyes gained popularity and are widely used as fluorescent probes and labels (Krieg, *et al.,* 2011; Wuskell, *et al.,* 2006; Kurtaliev, *et al.,* 2009). Fluorescent probes that are used in medicine and biology vary in structure and have specific characteristics during the interaction and binding with various biomacromolecules which then manifest themselves differently in the absorption and fluorescence spectra and subsequently affect the photophysical properties of the probes. Therefore, the study of interaction of new synthetic styrylcyanine dyes with biomacromolecules is of great scientific and practical interest, with priority placed on their further application as probes in biomedical research as well as for targeted synthesis of organic dyes with specified spectral fluorescent properties.

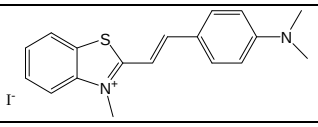
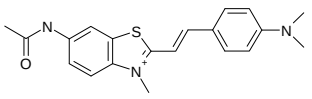
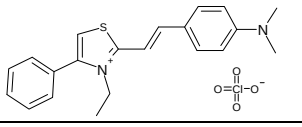
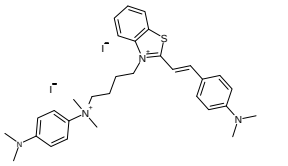
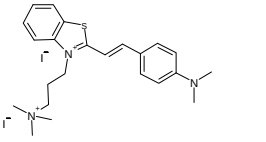
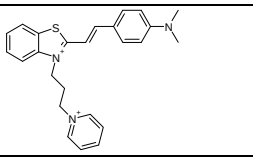
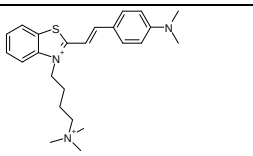
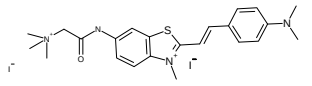
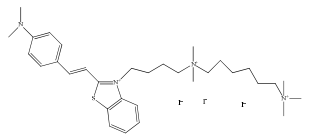
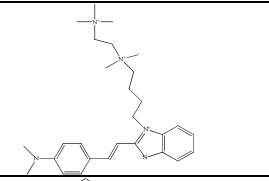
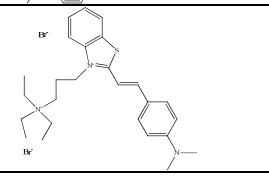
In this paper, we present the results of a study on the relationship between the structure and bonding of styrylcyanine dye Sbt and its derivatives with BSA.

Methods and Experiment

The structural formulas of the studied compounds are

displayed in Table 1. The synthesis of dyes and test of their purity were carried out at the Institute of Molecular Biology of the National Academy of Science of Ukraine according to the methods described in (Kovalska, *et al.*, 2005; Tokar, *et al.*, 2006; Kovalska, *et al.*, 2004). The electronic absorption spectra were measured with the help of a Specord 50 SA (Analytikjena, Germany) spectrophotometer which allows measurements with an accuracy of (+/- 0.003 D), resolution of (0.3 nm) in the range of 190-1100 nm and automatic registration of light scattering. Fluorescence spectra were measured with the help of an experimental fluorescence measurement setup assembled on the basis of the monochromator MDR-12 (LOMO, Russia) with automatic correction and PC output, and a photomultiplier tube FEU-100 (Russia). Excitation light sources were high-brightness LEDs of blue glow with light filters SZS22 and SZS23 (λ=450 nm), SZS8 and SZS20 (λ=485 nm). BSA ("Medpreparat" Konotop, Ukraine) was used as a protein. As a solvent we used double distilled water (pH=7.1). The pH values were measured on the pH-meter "Seven Easy"(Mettler Toledo, Switzerland). Preparation of stock dye solutions was performed by volume-weight method. To obtain stock solutions with a specified concentration, dye samples were weighed on microanalytical scales AB-135-S/FACT (Mettler Toledo, Switzerland) with an accuracy of 0.01 mg. Operational concentrations 10^{-5}-10^{-6} M of solutions were prepared by diluting the stock solution. To avoid re-absorption, fluorescence measurements were carried out with thin layers of the solutions, in which the absorption of the excitation light did not exceed ~5%. Depending on the concentration of the solution, special quartz cells with thickness of the layer in the range of 0.1-50 mm were used. The values of concentrations of aqueous solutions of BSA were determined spectrophotometrically by the absorption intensity. The concentration of BSA (p) is defined by the formula: p=1.45×D280–0.74×D260 (in mg/ml), where D_{280} and D_{260}-optical density of BSA solution at the wavelengths of the absorption 280 nm and 260 nm (Gerhard, 1981). To determine the values of the binding constant (K) and the number of binding sites (N) dyes with BSA, the titration was performed by the method of Scatchard (Dobretsov, 1989). Titration of dye with BSA was conducted at a constant concentration of dye by serial dilution of the stock concentration of BSA. Reverse titration of BSA by dye solution was conducted at a constant concentration of BSA by adding of 20, 40, 60, 80 and 100 μl of dye aqueous solution to 3 ml of the BSA solution.

TABLE 1 STRUCTURAL FORMULAS OF THE STUDIED DYES.

Dye	Structural formulas	Chemical name
Sbt		(E)-2-(4-(dimethyl amino)styryl)-3-methylbenzo[d] thiazol-3-ium iodide
S-5		(E)-6-acetamido-2-(4-(dimethylamino)styryl)-3-methylbenzo [d]thiazol-3-ium
S-6		(E)-2-(4-(dimethyl amino)styryl)-3-ethyl-4-phenylthiazol-3-ium perchlorate
S-12		(E)-3-(4-((4-(dimethyl amino)phenyl) dimethylammonio)butyl)-2-(4-(dimethylamino) styryl)benzo[d]thiazol-3-ium iodide
S-20		(E)-2-(4-(dimethylamino) styryl)-3-(3-(trimethyl ammonio)propyl) benzo[d]thiazol-3-ium iodide
S-34		(E)-2-(4-(dimethylamino) styryl)-3-(3-(pyridinium-1-yl)propyl)benzo[d] thiazol-3-ium
S-36		(E)-2-(4-(dimethylamino) styryl)-3-(4-(trimethyl ammonio) butyl) benzo [d]thiazol-3-ium
S-38		(E)-2-(4-(dimethylamino) styryl)-3-methyl-6-(2(trimethylammonio) acetamido) benzo[d] thiazol-3-ium iodide
D-165		(E)-3-(4-(dimethyl(6-(trimethylammonio) hexyl)ammonio)butyl) -2-(4-(dimethylamino) styryl) benzo [d] thiazol-3-ium iodide
D-166		(E)-3-(4-(dimethyl(2-(trimethylammonio)ethyl) ammonio)butyl)-2-(4-(dimethylamino)styryl) benzo [d]thiazol-3-ium
D-174		(E)-2-(4-(dimethylamino) styryl)-3-(3-(triethyl ammonio)propyl) benzo [d] thiazole -3-ium bromide

Fluorescence intensities of dye in a solution of BSA (I_1) and water (I_0) were measured at a wavelength of maximum. I_1 was determined by subtracting of I_0

from I_1. The values of $I_{un}=1/y_0C$ and $K_{CI}=x_0$ were determined on the graph of dependency of $1/\Delta I_1$ from 1/p as intersections with 1/p(x) and $1/\Delta I_1$(y) axises. Fluorescence intensity of dye in solutions of BSA (I_2) was determined in maximum. Based on I_2 and I_0, their difference ΔI_2 was calculated. Then the concentration of bound probe $r=\Delta I_2/I_{sp}$ and the relative concentration of the bound probe $r/c= \Delta I_2/(cI_{sp}-\Delta I_2)$ were calculated - where *c* is the total concentration of the dye and *r* is the concentration of bound probe. The binding constant of the probe $Kc=y_0/x_0$ and the quantity of binding sites to the BSA $N=x_0/p$ were identified from the graphical dependence of r/c against *r*, where y_0 is the ordinate of intersection with r/c axis and x_0 is the abscissa of intersection with the *r* axis. The value of the possible systematic error does not exceed ~1-2%, and it is related to the inaccuracy of graduation counting marks and different wetting of walls of the measuring utensils, as well as identifying the main spectral-luminescent properties and parameters of the binding. All measurements were performed at a room temperature (297K). For ease of comparison, the presented absorption and fluorescence spectra were normalized to unity. The calculation of the dipole moment and the charge distribution on the dye atoms was performed during the quantum-chemical calculation of the structure of the dye molecules by semi-empirical method AM1 with the help of the MOPAC 2009 software package (http://www.openmopac.net) with a standard set of parameters (Stewart, 1990). All calculations were performed for isolated molecules in a vacuum. Previously, we made optimization of the geometry of the molecules using a restricted Hartree-Fock method and Polak-Ribier algorithm with accuracy 0.001 kcal/(Å×mole) and by taking into account the different variants of initial conformations. Optimization was to be finished when the rms energy gradient was less than 1 kcal/mol/Å. Among the resulting outcomes, we selected only those with complete final energies that are higher than the most minimal to no more than 1 kkal/mol, representing approximately 1.7 kT at the room temperature.

Results and Discussion

The concentration dependence of the absorption and fluorescence spectra of the selected dyes in water was studied. It was found that in 10^{-6}-10^{-5}M concentration range for Sbt, S-5, S-20, S-34, S-36, S-38, D-165, D-166 and D-174 dyes, and in 10^{-6}-10^{-3}M concentration range for S-6, S-12 dyes, the shape of absorption and fluorescence spectra remain constant and they belong to the monomer form of dye molecules. The following main spectral-fluorescent characteristics of dyes in monomeric form were determined based on the experimental measurement data for aqueous solutions of the studied dyes in accordance to calculation procedures described in (Nizomov, *et al.*, 2006): extinction coefficient (ε), oscillator strength (f_e), radiative lifetime of the excited state (τ), the frequency of purely electronic transition ($\nu_{0\text{-}0}$) and Stokes shift (SS) (displayed in Table 2). The quantum yields of aqueous solutions of the studied dyes are very low, approximately in the range of 0.01-0.02% .

TABLE 2 SPECTRAL-FLUORESTSENT CHARACTERISTICS OF AQUEOUS SOLUTIONS OF THE STUDIED DYES.

Dye	$\lambda_{max}^{abs.}$, (nm)	$\lambda_{max}^{fl.}$, (nm)	ε, l×mole^{-1} × cm^{-1})	f_e	τ, (ns)	$\nu_{0\text{-}0}$ (cm^{-1})	SS (cm^{-1})
Sbt	511	596	23500	0.27	0.1	17480	2790
Sbt+BSA	512	598	19200	0.33	0.2	17500	2808
S-5	513	589	47700	0.90	4.7	17820	2515
S-5+BSA	508	582	40800	0.84	4.8	17850	2502
S-6	469	584	6100	0.15	0.2	18350	4198
S-6+BSA	461	572	9600	0.40	0.04	18870	4209
S-12	529	598	9000	0.15	0.3	17450	2181
S-12+BSA	528	594	9500	0.18	0.24	17640	2104
S-20	531	600	48400	0.80	4.6	17730	2165
S-20+BSA	533	591	51800	0.97	3.4	17820	1841
S-34	533	599	50500	0.87	5.3	17360	2067
S-34+BSA	533	595	50500	0.95	4.8	17390	1955
S-36	522	597	38600	0.69	6.5	17540	2406
S-36+BSA	522	593	35100	0.77	5.9	17600	2293
S-38	523	598	32400	0.62	7.2	17510	2398
S-38+BSA	509	591	27900	0.65	6.4	17640	2725
D-165	523	604	49300	0.95	4.6	17330	2564
D-165+BSA	520	602	41800	0.99	4.4	17290	2619
D-166	524	598	51900	0.93	4.6	17530	2361
D-166+BSA	519	593	47600	0.98	3.8	17590	2404
D-174	527	598	59000	0.94	3.7	17470	2252
D-174+BSA	531	602	49200	0.99	3.1	17710	2221

The Sbt dye is a monomer, and S-5, S-20, S-34, S-36, S-38, D-165, D-166 and D-174 dyes are its derivatives, distinguished from Sbt by different substituents in their structures. For example, S-5 and S-38 dyes differ from the Sbt dye as their structure contains methylacetamide (S-5) and trimethyl-2-(methylamino) -2-oxoethan-1-aminium (S-38) groups, which can lead to a bathochromic shift of the absorption maximum of 2 and 12 nm, and an increase of the extinction coefficient of 1.4 and 2 times, respectively. Introduction of toluidine and the methyl group (S-6 dye) to the cyclopentane fragment, and the replacement of anion of the iodine atom by the anion of perchlorate leads to hypsochromic shift of the maximum of absorption and fluorescence at 42 nm and 12 nm, respectively, and four-time decrease in the extinction coefficient as

compared with the Sbt dye.

Hypsochromic shift in the absorption spectra of dye S-6 is explained by the difference in the basicity of heterocycles on the end and the fact that a rotation, decreasing steric hindrance, is around the bond between thiazole ring and benzene ring. Formation of a non-planar molecule leads to a reduction of the dipole moment as will be shown below, and hence reduces the intensity of absorption. Introduction of polymethylene chains of different lengths to the structure of the dye molecules, and presence of variety of substituents and effector groups (S-12, S-20, S-34, S-36, D-165, D-166 and D-174 dyes) results in a bathochromic shift of the absorption maximum on 11-20 nm and an increase in the extinction coefficient of 1.6-2.5 times. The presence of dimethylanyline group in the structure of S-12 dye leads to a bathochromic shift of the absorption spectra of 7 nm and a decrease in the extinction coefficient of 4-time as compared with the S-36 dye. Comparison of S-20, S-34 and D-174 dyes shows that the replacement of trimethylamine group (S-20) with pyridinium (S-34) leads to a slight bathochromic shift and an increase in the extinction coefficient. The introduction of additional methyl groups and the replacement of the anion iodine atom with atom of bromine (D-174) result in a hypsochromic shift of 4 nm and an increase in the extinction factor correction by 20% as compared with the S-20 dye. Comparison of D-166 dye with S-36 dye shows that the introduction of trimethylamine group to the structure of the chromophore dye leads to an increase in the extinction coefficient of an aqueous solution of D-166 dye to 1.5 times as compared with the S-36 dye. Replacing pyridinium group in the S-34 dye with the trimethylammonium (S-36 dye) leads to a hypsochromic shift of 11 nm and a decrease in the molar extinction coefficient. However, for the S-20 dye, shortening of the polymethylene chain connecting trimethylammonium group with the nitrogen atom to one link increases the molar extinction coefficient of 25% as compared with the S-36 dye.

Study of the interaction of the presented compounds to BSA demonstrated that with the increase concentration of BSA, the increase in the intensity of the absorption and fluorescence is observed (Fig. 1). Figure 1 shows that as the concentration of BSA in aqueous solution S-6 dye increases, a two-time increase of the intensity of the absorption and fluorescence spectra is observed. In addition, with the increasing the content of BSA, a new band with λ_{max}=412 nm is observed in short wavelength side, and hypsochromic shift of about 10-12 nm is observed in the fluorescence spectra. The broadening and increase of the intensity of the main absorption band with λ_{max}=461 nm and the appearance of a band with λ_{max}=412 nm in the dye S-6, is possibly due to the formation of aggregates of the dye molecules. By increasing the amount of protein, the concentration of binding sites increases (for dye S-6, it has a maximum value), thereby the dye molecule are dispersed by binding sites. A similar pattern is observed with the increasing concentration of BSA in the solution in the absorption and fluorescence spectra for the remaining of the studied dyes (Table 2).

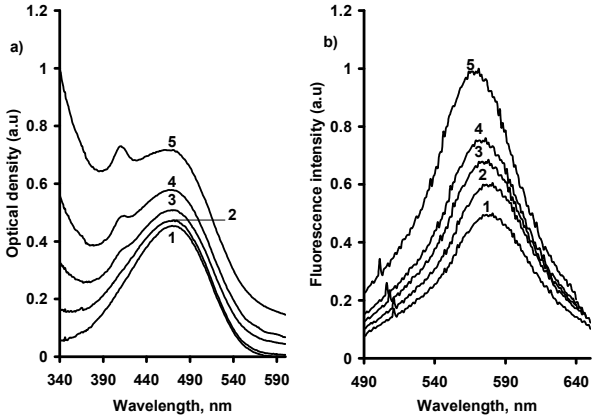


FIG. 1 THE SPECTRA OF ABSORPTION (a) AND FLUORESCENCE (B) OF AQUEOUS SOLUTIONS OF DYE S-6 (c=10^{-5}M) WITH THE ADDITION OF BSA: 1-0, 2-1.6×10^{-6}, 3-3.2×10^{-6}, 4-6.5×10^{-6}, 5-1.3×10^{-5}M.

The increase in fluorescence intensity can be explained by the interaction of the dye molecules to BSA in the excited state increases, and leads to a strengthening of a flat configuration of monomer dye molecules bonded with the BSA, as well as increasing of the rigidity of the dye molecules in complex formation with BSA. Transport of low molecular weight substances is one of the main functions of BSA (Lakovicz, 2002). Regulation of this process, depending on the content of modulating substances, occurs due to the changes in the binding properties of albumin. Availability of free binding sites on the BSA molecules in relation to a particular connection, and the extent of their binding capacity determine the functional properties of albumin.

Binding parameters of the dye with bioobject serve as a quantitative measure of the interaction of dyes with biological objects: the binding constant (K) and the concentration of binding sites (N). Binding parameters of the studied dye molecule with BSA were determined by titration method of the dye solutions with BSA solutions, and, conversely, BSA solutions with dye solutions (Table 3). Table 3 shows that S-12,

D-174, Sbt, S-5 and S-38 dyes have the highest binding constants, and S-34 and S-6 dyes have the lowest binding constants. S-12 dye has the strongest binding, while the S-34 and S-6 dyes have 1379 times and 727 times lower binding, respectively, compared with the S-12.

TABLE 3 BINDING PARAMETERS OF THE STUDIED DYES WITH BSA.

Dye	Concentration, M		K, M^{-1}	N	I_{BSA}/I_{water}
	Dye	BSA			
Sbt	2.5×10^{-5}	4.6×10^{-6}	4.8×10^{5}	6.0	3.5
S-5	1.0×10^{-5}	2.8×10^{-5}	3.5×10^{5}	0.5	1.1
S-6	1.0×10^{-5}	1.3×10^{-5}	5.8×10^{2}	14.0	2.1
S-12	1.0×10^{-5}	1.3×10^{-5}	8.0×10^{5}	1.5	4.1
S-20	5.0×10^{-6}	1.2×10^{-5}	8.6×10^{4}	0.58	3.6
S-34	1.0×10^{-5}	3.0×10^{-5}	1.1×10^{3}	4.7	2.5
S-36	1.0×10^{-5}	3.2×10^{-4}	1.5×10^{4}	0.6	4.2
S-38	1.0×10^{-5}	1.3×10^{-5}	2.4×10^{5}	3.8	3.1
D-165	1.0×10^{-5}	2.7×10^{-6}	3.9×10^{4}	1.3	1.4
D-166	5.0×10^{-6}	2.4×10^{-5}	5.6×10^{4}	0.7	4.6
D-174	1.0×10^{-5}	5.1×10^{-6}	7.6×10^{5}	2.7	2.3

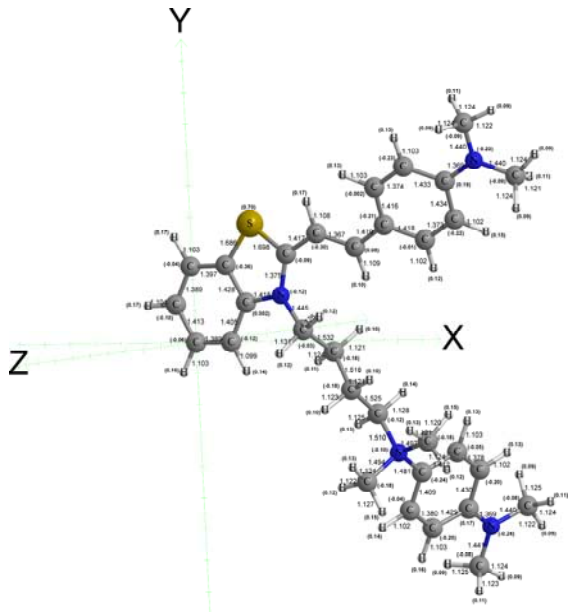


FIG. 2 THE ORIENTATION OF THE AXES OF COORDINATES, BOND LENGTHS AND THE DISTRIBUTION OF CHARGES ON THE ATOMS OF THE DYE S-12.

Fluorescence of S-12 and S-20 dye solutions with BSA increased approximately 4 times. Inclusion of the benzene ring and an effector group in structure of the molecule S-12 dye apparently increases the negative charge of the trimethylamine part of the dye, thus increasing the attraction of the dye molecules to the positively charged areas of BSA. The presence of the two groups of trimethylamine in the dye structure also enhances the interaction of the dye molecule and the positively charged areas of BSA. This assumption is confirmed by the performed calculation of the charge distribution, which shows that the value of a charge on the nitrogen atoms in the trimethylamine part of the dye, equals to -0.24. The low value of the binding constants of dye S-6 can be explained by the fact that the introduction of toluidine into the structure of the dye creates a strong obstacle in the interaction with BSA. Availability spatial complications upon binding to protein leads to that dye S-6 is unsuitable as fluorescent probe proteins. Introduction of pyridinium group into the structure S-34 dye leads to increased fluorescence, with simultaneous weakening of binding of the dye molecules to BSA. Conversely, replacement of pyridinium group by trimethylamonium in the dye S-36 increases the binding constants by one order of magnitude, with simultaneous 4.2 fold increase in fluorescence. Increasing the length of the poly-methylene chain in the structure of the molecules of D-165 and D-166 dyes rises the binding constant at 35 times and 51 times, respectively, compared with the S-34 dye.

In S-20 dye, the presence of the shorter connecting polymethylene chain than in S-36 dye leads to a decrease of fluorescence intensity in BSA solution and to an increase in the binding constants of 5.7-fold. Introduction of an oxygen atom (dyes S-38 and S-5) to the structure of molecules enhances the interaction of dyes with BSA and increase of the binding constant in 218 times and 318 times, respectively, compared to the S-34 dye. In addition, the calculated distribution of the charge on the oxygen atoms has negative values and equal to -0.32 and -0.33, respectively. A similar pattern is observed with the introduction of the methyl groups into the structure of the D-174 dye and replacement of the anion-atom of iodine with the atom of bromine, which leads to an increase of the binding constant in 690 times in comparison with the dye S-34. The concentration of binding sites is a measure of N limiting the concentration of bound probe in the protein. The number of binding sites for dye S-5, S-20, S-36 and D-166 is less than unity. This may be due to the fact that large protein concentration chosen for these dyes on the other side of positive cooperative binding (K increases with decreasing N).

The structure of the dye molecules and the peculiarity of the binding sites of the protein are determining factors of the interaction of protein and dye. It is known that negatively charged groups of the first and second domains of BSA are found inside the globules, while the positively charged are found on the surface of the globule (Luik, *et al.*, 1984). Binding between dyes and proteins can occur due to both hydrophobic interactions, and electrostatic interactions. It should be

noted that during the non-covalent binding of the dye with the protein, the dye chooses binding sites can respond to changes not only in a certain fragment of the protein, but also in other places where the binding sites may occur, and it can be redistributed. Active groups BSA surrounded by hydrophobic regions form the binding sites capable of interacting with dye molecules. The presence of the various effector groups in the structure of molecules of S-20, D-166, D-165, S-36, S-34, and S-6 dyes can spatially hinder interaction of dyes with BSA. Therefore, it can be assumed that the binding to BSA is due to the electrostatic attraction between the negatively charged oxygen atoms (dyes S-5 and S-38), the nitrogen atoms in the trimethylamine group (S-12, D-174, Sbt, S-20, S-38, D-166, D-165 and S-36) and positively charged globules located on the surface of the protein.

Moreover, the relatively small values of the binding constants can be explained by the fact that at different ratios of concentration of BSA and the dye, molecules of dye tend to form non-fluorescent complexes between dye and BSA. Ability to bind dyes depends on the concentration of the protein. Decline of the fluorescence intensity during the interaction of BSA with dyes is due not only to the formation of non-fluorescent dye complexes, but possibly due to change of the conformation of albumin.

For the successful use of dyes as a probe it is necessary that they do not damage the structure and function of proteins during binding. Protein conformational changes inevitably occur at the point of the probe location. The higher the concentration of the dye, the more is the damaging activity. Therefore, during higher degree binding, dyes can significantly change the conformation of albumin. Hence, optimal choice of the dye concentration and protein is important. As an example, Figure 3 shows a plot of the dependence of the fluorescence intensity (F/F_0) on the ratio of the concentration of the dye to the concentration of BSA (C/P). Sbt, S-12, S-36, D-165, and D-166 dyes when bound to BSA increase the intensity of fluorescence (F/F_0) in 3.5, 4.1, 4.2, 3.1, 1.4 and 4.6 times, respectively, with equal ratio of the concentration of the dye/ protein concentration (C/P) 1:5 (Fig. 3, curves 1-3, 5,6).

A similar pattern is observed for the S-38 dye in binding to BSA: fluorescence intensity (F/F_0) of which is increased by 3.1 times with equal ratio of 1:2.5 (Fig.3, curve 4). A further increase in the amount of dye leads to a decrease in fluorescence intensity. Such pattern can be explained by static fluorescence quenching, which is related to the formation of non-fluorescent complexes in the ground state between the dye molecules and BSA, or between the dye molecules directly with each other, by means of the solvent molecules (dimers or larger aggregates) with increasing concentration of the dye.

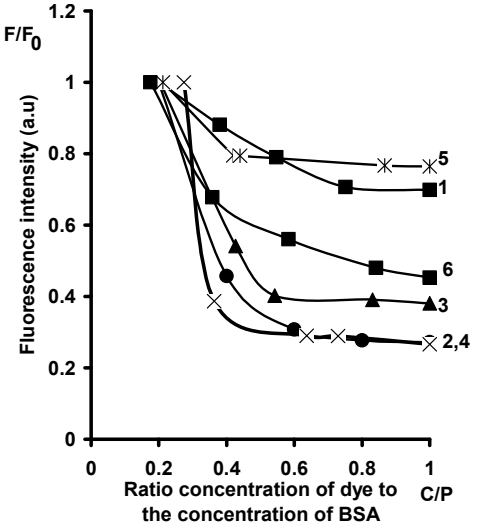


FIG. 3 DEPENDENCE OF RELATIVE FLUORESCENCE INTENSITY RATIOS OF DYE ON THE CONCENTRATION OF BSA IN WATER: 1-Sbt (9.3×10^{-6}M), 2-S-12 (8.9×10^{-6}M), 3-S-36 (6.5×10^{-6}M), 4-S-38 (8.6×10^{-6}M), 5-165 (5.1×10^{-6}M), 6-D-166 (4.2×10^{-6}M).

Large dye concentration increases the degree of complexation of the dye molecules, and this in turn results in a decrease to the binding capacity of BSA. In other words, the proteins in solutions with dyes is a competition: binding processes bind dye molecules to each other on one side and bind the dye molecules with another molecule of BSA. Such a pattern is detected for derivatives rhodamine dyes upon binding to serum albumin (Nizomov, *et al.,* 2006). Since the formation of the complex occurs in the ground state, it can lead to changes in the absorption spectrum of the dye, which is observed in the experiment (Figure 3 and Table 3). Electrostatic forces play essential role in the formation of molecular aggregates, as well as in the interaction of the dye with the proteins (Patonay, *et al*., 2004; Nizamov, *et al.,* 2011). To test this assumption, we calculated the dipole moment of the studied dyes. Presented dependence of the binding constant on the dipole moment of the dye molecules (Fig. 4) shows that there is a correlation between the binding constant of dyes with BSA and their molecular structure, in particular, with its electrostatic field. The growth in row of dyes S-6, S-20 and S-38 (arrow 1) and the growth of the binding constant in the series S-6, S-5, Sbt, D-174 and S-12 (arrow 2) indicates that between dye and BSA molecules, in addition of electrostatic attraction, there are significant hydrophobic interac-

tions. Increasing fluorescence intensity and the fluorescence hypsochromic shift suggest that the binding of a protein fluorophore is flagged for hydrophobic entourage. For S-6, S-20 and S-38 dyes increased protein binding correlates with increasing charge and dipole moment of the ground state. Increase of binding protein with S-5 and Sbt dyes leads to more dependence on hydrophobic interactions, whereas for the dye D-174 and S-12, the dependence is on the total charge of the molecule. Reduction of chain thrimetylamonium group D-166 dye leads to a more rigid conformation and increased fluorescence is more than 3 times compared to D-165. Thus, it can be argued that electrostatic attraction, combined with a hydrophobic interaction, underlies the interaction of the dye molecules with BSA. In addition, it is suggested that there is a hydrophobic cavity close to the positively charged site of the BSA molecule, which can be penetrated by the dye molecules.

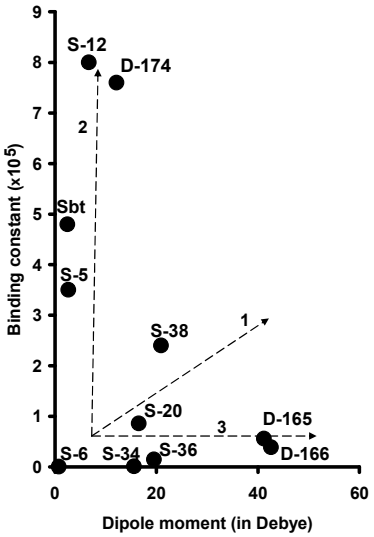


FIG. 4 DEPENDENCE OF BINDING CONSTANT WITH BSA ON THE DIPOLE MOMENT OF DYE MOLECULES.

The same conclusion was reached by authors (Welder, *et al*., 2003; Sophianopoulos, *et al*., 1997) who studied non-covalent interaction between symmetric and asymmetric squaraine dyes with BSA, which demonstrated that the hydrophobicity of the dye molecules strongly affects their ability to bind to the protein, and the binding occurs in the hydrophobic cavity located close to the positively charged site of the BSA. The electrostatic and hydrophobic nature of the binding protein molecules is indicated by the fact that surface-active compounds (surfactants) have an influence on this interaction. This detergent nonpolar moiety will bind to hydrophobic protein adsorption centers and thus inhibit their subsequent interaction with the dye molecules (Nizomov, *et al*., 2006). However, the BSA molecule has other binding sites, such as uncharged hydrophobic. In fact, with which BSA binds the dye center, it is strongly influenced by the number of deputies, and particularly their location. Apparently, this is connected with such a significant difference between the values of the binding constant. Arrow 3 (Fig. 4) shows that there is an increase of the electrostatic field of the dye molecules in S-6, S-34, S-36, D-165 and D-166 series of dyes, where the values of binding constant of S-34, S -36, D-165 and D-166 dyes are similar. Such dependence may indicate that the molecules of these dyes tend to form aggregates or dimers. The detected dependence of the dimerization and aggregation processes of studied dyes on the molecule's electrostatic field, represented by integrally characterized dipole moment projections on the coordinate axes (Fig. 5) serves as the confirmation of these findings.

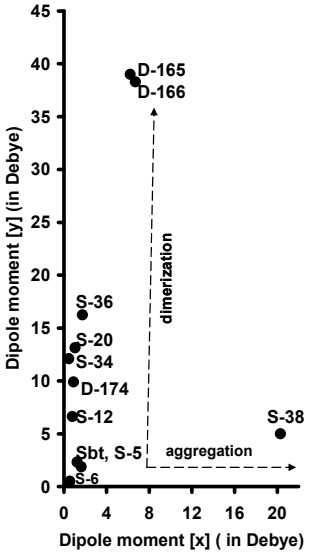


FIG. 5 THE DEGREE OF FORMATION AGGREGATE OF THE DIPOLE MOMENT OF THE MOLECULES.

Average values of the absolute values of the dipole moment and its projections along the coordinate axes for the molecules of studied compounds are displayed in Table 4.

TABLE 4 THE CALCULATED DIPOLE MOMENTS OF STUDIED DYES.

Dye	Charge	[Dx]	[Dy]	[Dz]	D
Sbt	1	1.6	1.8	0.2	2.4
S-5	1	1.2	2.3	0.1	2.6
S-6	1	0.5	0.4	0.1	0.7
S-12	2	0.7	6.6	0.03	6.6
S-20	2	1.0	13.1	9.9	16.5
S-34	1	0.4	12.1	9.8	15.6
S-36	1	1.7	16.2	10.7	19.5
S-38	2	20.3	5.0	0.1	20.9
D-165	3	6.2	39.0	16.1	42.6
D-166	3	6.7	38.2	13.7	41.2
D-174	2	0.9	9.9	6.8	12.1

Charges on the atoms of the substituents make a fundamental contribution to the dipole moment of the molecules so that the dipole moment is oriented along the Y-axis and natural growth of the dipole moment in S-6, S-5, Sbt, S-12, D-174, S-34, S-20, S-36, D-166 and D-165 dyes is observed. As seen from Figure 5, the dye S-38 oriented dipole moment along the axis X has a tendency to form aggregates, while dyes S-6, S-5, D-174, S-34 and D-165 practically do not exhibit predisposition aggregation, and are inclined to dimerization.The ability to form different aggregates and dimmers is explained by the structure of their chromophores, namely significant dipole moments, the alternation of opposite charges, and planar structure of the chromophore, which causes the high energy of the intermolecular interactions (Ishchenko, 1994).This assumption is confirmed by the study of the concentration dependence of the absorption and fluorescence spectra present compounds in the water, as well as a binary mixture of water+dioxane. It should be noted that the preparation of solutions in binary solvent was carried out so that the concentration of the dye remained constant, while the ratio of binary solvent change. Found that increasing the concentration of Sbt, S-5, S-20, S-34, S-36, S-38, D-165, D-166 and D-174 dyes in aqueous solution increases, a new band located on the side of short wavelength is observed, starting from the concentration of 10^{-4} M (Fig. 6). Observed maximum in the absorption spectrum of a dilute solution with λ_{max}=523 nm (Fig. 6, curve 1) refers to the monomeric form of the dye D-165. As the concentration of the dye in the absorption spectrum increases, a slight drop in the intensity of the band with λ_{max}=523 nm, and a new band with λ_{max}=504 nm are observed in the short wavelengths side (Fig. 6, curve 3). At the same time, with the increase of concentration of the dye, the decline in the intensity of light, that is, the concentration quenching of fluorescence is observed in the fluorescence spectra. A similar pattern in the absorption spectra and fluorescence of S-5, S-20, S-34, S-36, S-38, D-165, D-166 and D-174 dyes are observed with the increasing concentration of dye in aqueous solution. For example, for S-36, S-5, D-174, D-166 and S-34 dyes, with the increasing concentration of dyes, position of the absorption spectrum maximum hypsochromically shifts to 15 nm, 31 nm, 11 nm and 17 nm, respectively. Observed phenomena in the absorption and fluorescence spectra in aqueous solutions can be explained by formation of non-luminescent aggregates of dye molecules with solvent molecules (water), where the solvent molecules act as a connecting bridge between dye molecules through hydrogen bonding. The processes of aggregates formation with the increase in concentration of non-fluorescent dyes are widespread among series of organic dyes, in particular for squaraine (Nizamov, *et al.,* 2009), cyanine (Viteva, *et al.,* 2007), rhodamine (Nizomov, 1997) and styryl (Kurtaliev, Nizomov and Rahimov, 2011; Kurtaliev, 2011) dyes.

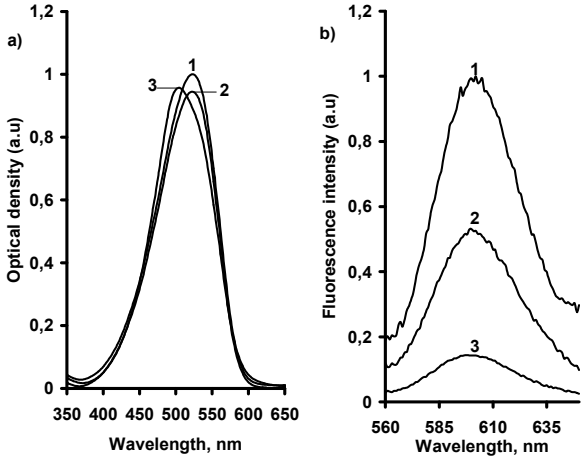


FIG. 6 CONCENTRATION DEPENDENCE OF ABSORPTION (A) AND FLUORESCENCE (B) SPECTRA OF DYE D-165 IN WATER:1-10^{-5}, 2-10^{-4}, 10^{-3} M

In the absorption and fluorescence spectra S-38 dye (c=10^{-5} M) at the transition from water to water-dioxane solution of bathochromic shift on 16 nm and increasing the absorption and fluorescence intensity is observed. (Fig. 7, curve 2). At the same time, the intensity of fluorescence increases up to a certain volume fractions (13% water+87%dioxane). The further increase of the content dioxane (7%water+93%dioxane) leads to the emergence of short-wavelength band with λ_{max}= 457nm; simultaneously, there is a decline in the intensity of the band λ_{max}=542 nm. At the same time, the intensity of fluorescence decreases proportional to the increase amount of dioxane. At the volume fraction, 1% water adding with 99% dioxane in the absorption spectra is observed with λ_{max}=441 nm, and the solution has practically no fluorescence ability (Fig. 7, curve 7). The observed phenomena in the absorption and fluorescence spectra of water-dioxane solution can be explained by the fact that at a small fraction of dioxane content in the solution solvation processes are observed that lead to batahromic shifts of the absorption and increase the intensity of fluorescence. Solvation may in turn result in the formation of aggregates ionic separated solvent or outer-sphere complexes (Shakhverdov, *et al.,* 1999). In this case, the cation is actually surrounded by solvent molecules

and visibly is separated from the anion. Further increase in dioxane solution leads to the fact that formed ionic aggregates unite scheme $K^+A^-…K^+A^-…K^+A^-$ and form H-aggregates, do not have fluorescent ability.

Thus, for the synthesis of fluorescent probes with higher protein sensitivity on the basis of styryl dyes, the presence of the methyl and effector groups is essential. This conclusion has been confirmed by the fact that the binding constant of the S-34 dye with a benzene ring attached by polymethylene chain is significantly lower, compared to dyes with methyl (D-174, S-38) and effector groups (S-12) which facilitate the binding of these dyes to the surface of the protein.

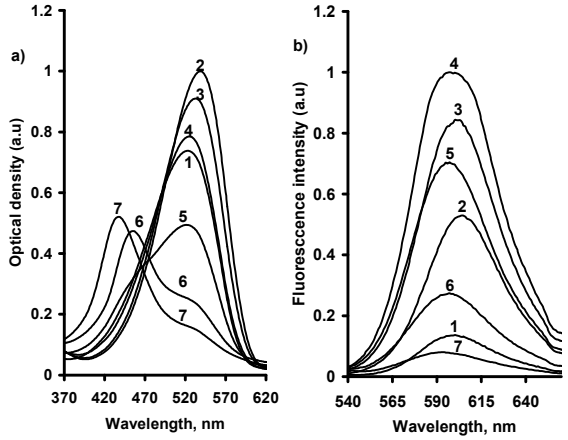


FIG. 7 THE ABSORPTION (a) AND FLUORESCENCE (b) SPECTRA OF S-38 DYE (c =10^{-5} M) AT MEASURE ADDITION DIOXANE: 1-AQUEOUS SOLUTION, 2-50% 3-74% 4-87% 5-90% , 6-93%, 7-99% DIOXANE.

Conclusion

It was established that for aqueous solutions of all studied dyes in the presence of BSA, an increase in the absorption capacity and a slight broadening and the appearance of a new band of the shorter wavelengths with λ_{max}=412 nm were observed. In the presence of BSA, the emission intensity of the main fluorescence band in all studied dyes increased from 1.1 to 4.6 times. Main spectral-fluorescent characteristics, the binding constant (K) and the number of binding sites (N) were identified for the dye molecules that are in monomeric form when bound to BSA. The dependence of the binding constant on the dipole moment of the dye molecules showed that in addition to the electrostatic attraction between the molecules of studied dyes with BSA, there are significant hydrophobic interactions. It was shown that processes of formation of dimers and aggregates of the dye molecules may contribute to the interaction of the latter with the BSA.

ACKNOWLEDGEMENTS

We greatfully acknowledge Prof. S.M. Yarmoluk of the Institute of Molecular Biology and Genetics, NAS Ukraine for the dye samples. This work was supported by grant U3104 (STCU, Ukraine).

REFERENCES

A.A. Ishchenko, Structure and spectral-luminescent properties of polymethine dyes, Naukova dumka, Kiev. 1994. (in Russian).

A.I. Luik, V.D. Lukyanchik, Serum albumin, and transportation of poisons, Medicine, Moskow. 1984. (in Russian).

A.J. Sophianopoulos, J.Lipowsky, N.Narayanan, G.Patonay, Association of Near-Infrared Dyes with Bovine Serum Albumin. Appl. Spectr. vol.51(10), pp.1511-1515, 1997.

A.Matiukas, B.G.Mitrea, M.Qin, A.M.Pertsov, A.G.Shvedko, M.D.Warren, A.V.Zaitsev, J.P.Wuskell, M.Wei, J.Watras, L.M.Loew, Near-infrared voltage-sensitive fluorescent dyes optimized for optical mapping in blood-perfused myocardium, Heart Rhythm, vol.4(11), pp.1441-1451, 2007.

A.S. Tatikolov. Polymethine dyes as spectral-fluorescent probes for biomacromolecules. J.Photochem. Photobiol. C: Photochemistry Reviews., vol.13(1), pp.55–90, 2012.

B. Song, Q. Zhang, W.H. Ma, X.J. Peng, X.M. Fu, B.Sh. Wang, The synthesis and photostability of novel squarylium indocyanine dyes. Dyes and Pigm., vol. 82(3), pp.396-400, 2009.

E.N.Kurtaliev, Spectral-luminescent and photochemical characteristics of homodimers of the styrylcyanine dye Sbt in solutions, Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy. vol.81. pp.449–457, 2011.

E.N.Kurtaliev, N.Nizomov, Sh.I.Rahimov, The spectral-luminescent and photochemical characteristics of several styrylcyanine dyes in solution, J. Mol. Liq. vol. 158, pp.43-49, 2011.

E.N. Kurtaliev, N. Nizomov, Sh.N. Nizamov, G. Khodjayev, Spectral-luminescent study of the interaction of some styrylcyanine dyes with bovine serum albumin and DNA in aqueous solution, J. Mol. Struc., vol. 936 pp.199-205, 2009.

F. Yukruk, A.L. Dogan, H. Canpinar, D. Guc, E.U. Akkaya, Water-soluble green perylenediimide (PDI) dyes as

potential sensitizers for photodynamic therapy, Org. Lett., vol.7 (14), pp.2885-2887, 2005.

F.Welder, B.Paul, H.Nakazumi, S.Yagi, C.L.Colyer, Symmetric and assymetric squarylium dyes as noncovalent protein labels: a study by fluorimetry and capillary electrophoresis, J. Chromatography B. vol.793 pp.93-105, 2003.

G.E. Dobretsov, Fluorescent probes in the study of cells, membranes and lipoproteins. Nauka. Moscow. 1989. (in Russian).

G.Khodjaev, E.N.Kurtaliev, Z.F.Ismailov, F.U. Khaydarova, Z.M.Hamidov, D.P.Khakimova, N.Nizomov, Possibility of using Rhodamine B dye in diagnosis of some mens diseases, Proc. SPIE, vol. 6653, 2007.

G.Patonay, J.Salon, J. Sowell, L.Strekowski, Noncovalent Labeling of Biomolecules with Red and Near-Infrared Dyes, Molecules. vol.9, pp.40-49, 2004.

H.A.S. Al-Shamiri, M.T.H. Abou Kana, I.M. Azzouz, Y.A. Badr, Photo-physical properties and quantum yield of some laser dyes in new polymer host, Optics & Laser Tech., vol. 41(4), pp.415-418, 2009.

H.J. Smith, B. Gray, K. Muirhead, B. Ohlsson-Wilhelm, B. Fritzsch, Long-Distance Three-Color Neuronal Tracing in Fixed Tissue Using NeuroVue Dyes, Immunol. Invest., vol.36 (5–6), pp.763–789, 2007.

H. Lee, M.Y. Berezin, M. Henary, L. Strekowski, S. Achilefu, Fluorescence lifetime properties of near-infrared cyanine dyes in relation to their structures. J.. Photochem. Photobiol A: Chemistry, vol.200 (2-3), pp.438-444, 2008.

Http://www.openmopac.net.

I. Timtcheva, V. Maximova, T.Deligeorgiev, N. Gadjev, K.H. Drexhaged, I. Petkova, Homodimeric monomethine cyanine dyes as fluorescent probes of biopolymers. J.. Photochem. Photobiol. B: Biology, vol.58 (2–3), pp.130–135, 2000.

J.H. Guo, L.N. Zhu, D.M. Kong, H.X. Shen. Triphenylmethane dyes as fluorescent probes for G-quadruplex recognition, Talanta, vol.80(2), pp.607-613. 2009.

J.J.P. Stewart, MOPAC: A semiempirical molecular orbital program, J. Computer Aided Mol. Desing. vol.4, pp.1-103, 1990.

J.P. Wuskell, D. Boudreau, M. Wei, L. Jin, R. Engl, R. Chebolu, A. Bullen, K. D. Hoffacker, J. Kerimo, L.B. Cohen, M.R. Zochowski, L.M. Loew. Synthesis, spectra, delivery and potentiometric responses of new styryl dyes with extended spectral ranges. J. Neuroscience Methods. vol.151 (2), pp.200-215, 2006.

J.R. Lakovicz, Bases fluorescent spectroscopy. Premium, New-York. 2002.

L.Viteva, T.Gospodova, J.Rashkova, I.Abrahams, I.Timitcheva, S.Simova, M.R.Mazieres, J.G.Wolf, Synthesis and Photophysical Properties of Some Rigidized Hepta- and Nonamethine Mono- and Bis(merocyanines): Ring-Opening of Quaternized 2-Methylbenzothiazole, Eur J.Org. Chem., vol.19, pp.3102-3114, 2007.

M.R. Mazieres, C. Duprat, J.G. Wolf, A.D. Roshal, pH dependent spectral properties and electronic structure of benzothiazol containing cyanine dyes. Dyes and Pigm., vol.80(3), pp.355-360, 2009.

M.Tian, X.Peng, J.Fan, J.Wang, Sh.Sun, A fluorescent sensor for pH based on rhodamine fluorophore, Dyes and Pigm., vol.95, pp.112-115, 2012.

N. Nizomov, Luminescence associated molecules of organic dyes in solutions and films, Zarafshon, Samarkand, 1997. (in Russian).

N.Nizomov, Z.F. Ismailov, Sh.N. Nizamov, M.K. Salakhitdinova, A.L.Tatarets, L.D.Patsenker, G.Khodjayev, Spectral-luminescent study of interaction between squaraine dyes and biological matters. Mol. struc. vol.788(1-3), pp.36-42, 2006.

N. Nizomov, Z. F. Ismailov, E. N. Kurtaliev, Sh. N. Nizamov, G. Khodzhaev, L. D. Patsenker, Luminescent spectral properties of rhodamine derivatives while binding to serum albumin, J. Appl. Spectr. vol.73(3), pp.432-436, 2006.

Ph. Gerhard, Manual of Methods for General Bacteriology, American Society of Microbiology, Washington.1981.

R.F. Delgadillo, L.J. Parkhurst. Spectroscopic properties of fluorescein and rhodamine dyes attached to DNA. Photochem. Photobiol., vol.86(2), pp.261-272, 2010.

R. Krieg, A. Eitner, K.J. Halbhuber, Tailoring and histochemical application of fluorescent homo-dimeric styryl dyes using frozen sections: From peroxidase substrates to new cytochemical probes for mast cells, keratin, cartilage and nucleic acids. Acta Histochem., vol.113 (7), pp. 682-702, 2011.

Sh.N. Nizamov, M.N. Barakaeva, E.N. Kurtaliev, A.L. Tatarets, L.D. Patsenker. Influence of molecular structure

of squaraine dyes on their aggregation in aqueous solutions. J. Appl. Spectr. vol.76(4), pp.464-469, 2009.

T.A. Shakhverdov, Z.N. Turaeva, N.Nizomov, V.L.Ermolaev, Luminescence study of the association of eosin with ions of rare-earth elements in water, Optics and spectroscopy. vol.87(2), pp.253-257, 1999 (in Russian).

V.A. Svetlichnyi, O.K. Bazyl', E.R. Kashapova, N.A. Derevyanko, A.A. Ishchenko, Influence of absorption from excited singlet states on the lasing parameters of polymethine dyes, Quantum electron, vol. 39 (8), pp. 739–744, 2009.

V.B. Kovalska, D.V. Kryvorotenko, A.O. Balanda, M.Yu.Losytskyy, V.P.Tokar, S.M. Yarmoluk, Fluorescent homodimer styrylcyanines: synthesis and spectral-luminescent studies in nucleic acids and protein complex, Dyes and Pigm. vol.67 pp.47-54, 2005.

V.B. Kovalska, M.Yu. Losytskyy, S.M. Yarmoluk. Luminescence spectroscopic studies of trimethinecyanines substituted in polymethine chain with nucleic acids and proteins.Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, vol.60(1–2), pp.129–136, 2004.

V.I. Bezrodnyi, N.A. Derevyanko, A.A. Ishchenko, A.V. Kropachev, Highly efficient passive Q switches for a neodymium laser based on thiopyrylotricarbocyanine dyes, Quantum electron, vol. 39 (1), pp.79–83, 2009.

V.P. Tokar, M.Yu. Losytskyy, V.B. Kovalska, D.V. Kryvorotenko, A.O. Balanda, V.M. Prokopets, M.P. Galak, I.M. Dmytruk, V.M. Yashchuk,S. M. Yarmoluk, Fluorescence of styryl dyes-DNA complexes induced by single and two-photon excitation, J.Fluorescence. vol.16(6), pp.783–791, 2006.