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Studies of Benzothiazole and Benzoselenazole Squaraines as Fluorescent Probes for Albumins Detection

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Abstract Series of squaraine benzothiazole and benzoselenazole dyes were studied as possible fluorescent probes for the detection of proteins, particularly albumins. It was shown that majority of the studied squaraines give significant fluorescent response on the human serum albumin (HSA) and bovine serum albumin presence. For squaraine dyes with *N*-hexyl pendent groups (P-1, P-2, P-3, P-5) about 100−540-fold fluorescence intensity increase upon albumins addition was observed. At the same time in presence of other proteins, namely insulin, avidin from hen egg white, immunoglobulin G (IgG), carbonic anhydrase fluorescence enhancement values were considerably lower —up to 43 times in IgG presence. It was noted that generally, squaraines with long *N*-hexyl pendent groups demonstrate higher emission increase values upon proteins addition comparing with their analogues with short *N*-ethyl tails. It was shown that fluorescence intensity enhancement for benzothiazole squaraine dye P-3, relates linearly to the HSA concentration over the wide range—from 0.2 to 500 μg/ml. Together with noticeable selectivity of this dye to albumins, existence of wide dynamic range gives possibility to propose P-3 dye as probe for HSA quantification.

Keywords Squaraines · Fluorescent probes · Proteins · Albumins detection

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Introduction

Determination of protein quantity in biological liquids is of great importance in many areas, such as biochemistry, biotechnology and immunodiagnostics [1].

Serum albumins are the major soluble protein constituents of circulatory system constituting 52% of the protein composition in this matrix and possess many physiological and pharmacological functions. Human serum albumin (HSA) is the most important and abundant constituent of blood plasma and serves as a protein storage component.

Changes in blood albumin levels can be caused by a number of disorders, including liver disease, nephrotic syndrome, neoplasia, severe dehydration etc. There also exists a connection between the content of HSA in urine and some diseases, such as diabetic nephropathy and chronic liver disease. Thus the quantitative determination of HSA in biological fluids is very important for clinical diagnosis.

The traditional methods of proteins detection are Lowry method [2] and Bradford method [3], however, these methods have some limitations, namely narrow linear range or slow reaction time, all these limit their practical applications. Up to date fluorometric methods are widely used for investigation and detection of proteins having the advantage over other methods for its high sensitivity, selectivity and convenience [4]. As a rule, commercially available fluorescent dyes ethidium bromide [5], eosin B and eosin Y [6], Coomassie Brilliant Blue (CBB) [7], Nano

Orange and Albumin Blue [8] are used for proteins determination in solution.

Studies conducted previously revealed squaraines to be efficient noncovalent label for albumins [9] and to exhibit high quantum yields when bound to these proteins [10]. As a continuation of these investigations Yan et al. [11] demonstrated applicability of squarylium dyes as HSA probes for capillary-electrophoresis-based assays.

According to Welder et al. [12] both symmetrical and unsymmetrical 3-oxo-substituted squarilium dyes also showed fluorescence intensity increase upon noncovalent interactions with proteins, however symmetrical dye interacted strongly with HSA, β-lactoglobulin A and trypsinogen whereas unsymmetrical one showed greater binding affinity to HSA and bovine serum albumin (BSA).

Previously we have studied series of novel unsymmetrical and symmetric squaraines for their sensitivity to various proteins. 3-Oxo- and 3-dicyanomethylene squaraines were shown to enhance emission up to 190 times in the presence of BSA and up to 24 times in HSA presence. It was also found out, that symmetrical *N*-methyl substituted indolenine squaraines in complexes with HSA show greater enhancement of fluorescence as compared to their homologues having substitution in heterocycle or containing *N*-carboxyalkyl group [13].

In present paper series of squaraine benzothiazole and benzoselenazole dyes are studied for their applicability as probes for detection of proteins, particularly albumins. For these purposes fluorescent properties of dyes in the presence of human serum albumin and bovine serum albumin are investigated. Also dyes are tested for their sensitivity to various type proteins, namely insulin, avidin (AVI) from hen egg white, immunoglobulin G (IgG), carbonic anhydrase (ANH). For the mostly sensitive to HSA dye lower detection limit and linear dynamic range for detection of this protein are determined. The dependence between dye molecule structure and its selectivity to certain protein is analyzed.

Materials and methods

Materials

0.05 M Tris-HCl buffer (pH 8.0), was used as solvent. Human serum albumin, bovine serum albumin, avidin from hen egg white, insulin, carbonic anhydrase, lysozyme and immunoglobulin G were purchased from Sigma-Aldrich (USA). Squaraines P-1, P-3, P-7, P-10 as well as aminosquaraines P-2, P-5, P-15, P-27 were synthesised as previously described [14] (Fig. 1).

Preparation of stock solutions of dyes and proteins

The 2×10^{-3} M dye stock solutions were prepared by dilution of the dye in dimethyl sulfoxide. Stock solutions of proteins (HSA, BSA, avidin, insulin, carbonic anhydrase, lysozyme and IgG) were prepared by their dissolving in 0.05 M Tris-HCl buffer (pH 8.0). Proteins concentrations in stock solutions were equal to 3 μM.

Preparation of working solutions

Working solutions of free dyes were prepared by dilution of the dye stock solution in buffer. Working solutions of dyes in presence of proteins were prepared by addition of the dye stock solution in proteins stock solution. The concentrations of dye and proteins in working solutions amounted to 5×10^{-6} M and 3 μM respectively. All working solutions were prepared immediately before the experiments.

With the aim to determine linear dynamic range and lower detection limit of HSA detection for benzothiazole squarilium dye P-3 the titration of the fixed 5×10^{-6} M concentration of the dye with the increasing amounts of HSA was performed.

Spectroscopic measurements

Fluorescence excitation and emission spectra were collected on a Cary Eclipse fluorescence spectrophotometer (Varian Inc., Australia; specifications could be found at: http://www.varianinc.com/image/vimage/docs/products/spectr/fluoro/brochure/87-1757.pdf). Fluorescence spectra were excited using pulse xenon lamp (pulsed at 80 Hz, peak power equivalent to 75 kW). Measurements were performed with excitation and emission slit widths set to 5 nm, and at a constant photomultiplier tube voltage. Fluorescence intensity was measured at the maximum wavelength of the fluorescence emission spectrum, the excitation wavelength being set at the maximum of the corresponding fluorescence excitation spectrum. The studied sample was contained in standard quartz cells (1×1 cm). All measurements were carried out at room temperature.

The quantum yield value for the dye P-10 in presence of HSA was determined using Nile Blue solution in methanol as the reference (quantum yield value 0.27 [15]).

The accuracy of the wavelength measurements with the Cary Eclipse fluorescence spectrophotometer is about 1.5 nm. At the same time, the possible error in intensity measurement is mainly determined with the accuracy of the dye concentration. The concentration error is mainly caused with the accuracy of the balance and pipettes and is estimated to be about 5%.

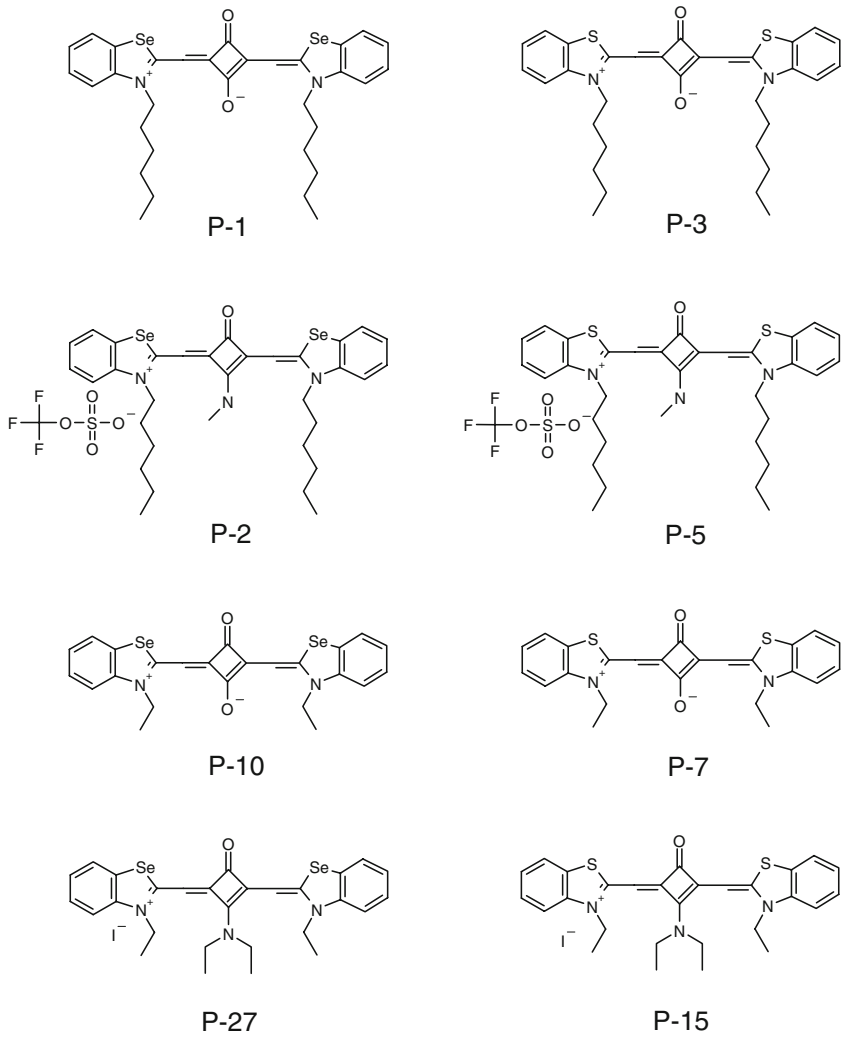

Fig. 1 Structures of studied squaraines

Results and discussion

Spectral-luminescent properties of free dyes in aqueous buffer

Fluorescent characteristics of series of symmetrical benzothiazole and benzoselenazole squaraine dyes in aqueous buffer are represented in Table 1.

The maxima of excitation spectra of the studied dyes in buffer are situated at 640−696 nm, while the fluorescence emission maxima lie between 651 and 703 (Table 1). All squaraines demonstrated quite low intrinsic fluorescence intensity (I_0)—up to 78 arbitrary units (a.u.). It should be admitted, that for the majority of benzothiazole squaraines I_0 values are higher than the corresponding values of their benzoselenazole analogues. For squaraines P-7 and P-10 having short *N*-ethyl pendent groups in the heteroaromatic nuclei emission intensity values in unbound state are few times higher than for their analogues with long *N*-hexyl tail group (P-3 and P-1 respectively).

Spectroscopic characterization of squaraine dyes in the presence of various proteins

Fluorescent properties of studied squaraines in the presence of proteins are represented in Tables 1 and 2. For the majority of dyes addition of the proteins resulted in the excitation maxima positions shift to the long-wavelength spectra region up to 34 nm. In case of benzothiazole dye P-3 and benzoselenazole squaraine P-27 excitation maxima are blue-shifted up to 22 nm.

Positions of emission spectra maxima of the studied dyes in proteins presence are red-shifted up to 26 nm as compared to the free dye buffer solution. For the squaraines P-3 and P-27 fluorescence maxima position are shifted to the short-wave region up to 26 nm.

Table 1 Spectral-luminescent properties of squaraines in free state in buffer, in presence of HSA and BSA

Dye	In buffer			In HSA presence				In BSA presence			
	λ_{ex}, nm	λ_{em}, nm	I_0, a.u.	λ_{ex}, nm	λ_{em}, nm	I^{HSA}, a.u.	I^{HSA}/I_0	λ_{ex}, nm	λ_{em}, nm	I^{BSA}, a.u.	I^{BSA}/I_0
P-1	663	686	1	682	693	306	306	680	687	540	540
P-2	690	700	2.2	691	710	232	105	702	709	520	236
P-3	671	690	3.6	663	670	502	139	663	671	1,241	345
P-5	648	668	2.5	681	691	261	104	682	692	943	377
P-7	640	651	78	664	672	1,409	18	663	671	1,068	13.5
P-10	656	666	43	680	692	1,773	41	681	688	1,641	38
P-15	661	675	20	686	699	405	20	686	698	170	8.5
P-27	696	703	3	674	684	303	101	676	684	196	65

λ_{ex} (λ_{em}) Maximum wavelengths of fluorescence excitation (emission) spectra, I_0 (I^{HSA}, I^{BSA}) fluorescence intensity of dye in buffer (and in presence of HSA, BSA)

HSA and BSA

Majority of the studied squaraines demonstrated bright fluorescence in both HSA and BSA presence (I^{HSA} and I^{BSA} values respectively; Table 1), however top emission intensity values (1,068–1,773 a.u.) were observed for the unsubstituted ones (O^-) P-7 and P-10 with *N*-ethyl pendent group. For the dye P-10 in presence of HSA the fluorescence quantum yield was measured, its value being equal to about 0.49.

For the studied squaraine dyes fluorescence intensity value enhances in presence of these proteins in up to 540 times (Fig. 2). The most pronounced emission increase was observed for the dyes P-1, P-2, P-3 and P-5. It should be noticed, that for squaraines P-7 and P-10, having *N*-ethyl pendent groups, emission intensity values in proteins presence were up to 5.8 times higher than for corresponding dyes with *N*-hexyl groups (P-3 and P-1 respectively).

It was shown that the length of the *N*-alkyl pendent group could effect on the binding specificity of the dyes. Generally, for squaraines with *N*-hexyl groups I^{BSA}/I_0 values were higher as compared to corresponding I^{HSA}/I_0 values. And vice versa, dyes having *N*-ethyl pendent group demonstrated fluorescence intensity increase in HSA presence higher, than corresponded I^{BSA}/I_0 values.

For squaraine dyes with *N*-hexyl groups about 100–540-fold fluorescence intensity increase upon albumins addition was observed, which makes them an attractive tool for selective albumins detection.

To estimate applicability of representatives of studied squaraine dyes as probes for determination of HSA we have chosen the benzothiazole dye P-3. This squaraine in the presence of albumins demonstrated high emission increasing (up to 140 times in HSA presence) and quite intensive fluorescence. At the same time for the dyes (P-7 and P-10) that have shown the brightest fluorescence in the presence of HSA emission intensity enhancement values were noticeably lower (18 and in 40 times correspondingly) than that for dye P-3. Since this experiment was aimed on the determination of dynamic range of the dye in HSA presence, using of the dye with the high emission increasing value was considered to be more preferable than ones with more significant value of fluorescence intensity.

Table 2 Spectral characteristics of squaraines in presence of IgG, avidin, insulin, and ANH

Dye	In IgG presence			In avidin presence			In insulin presence			In carbonic anhydrase presence		
	λ_{em}, nm	I^{IgG}, a.u.	I^{IgG}/I_0	λ_{em}, nm	I^{AVI}, a.u.	I^{AVI}/I_0	λ_{em}, nm	I^{INS}, a.u.	I^{INS}/I_0	λ_{em}, nm	I^{ANH}, a.u.	I^{ANH}/I_0
P-1	705	43	43	695	9.3	9.3	700	2.7	2.7	698	2.6	2.6
P-2	705	43	19.5	700	24	11	701	3	1.4	704	5.3	2.4
P-3	675	62	17.2	671	18	5	664	2.4	0.7	672	3.4	1
P-5	684	34	13.6	678	38	15.2	665	3.2	1.3	672	6.6	2.6
P-7	658	116	1.5	656	140	1.8	650	85	1.1	649	90	1.15
P-10	684	124	2.9	675	103	2.4	667	47	1.1	666	49	1.1
P-15	695	103	5.2	686	127	6.4	676	18	0.9	678	20	1
P-27	682	50	16.7	679	2.2	0.7	705	3.1	1	692	3.3	1.1

λ_{em} Maximum of fluorescence emission band, I_0 fluorescence intensity of dye in buffer, I^{IgG} (I^{AVI}, I^{INS}, I^{ANH}) fluorescence intensity of dye in presence of immunoglobulin G (avidin, insulin, carbonic anhydrase)

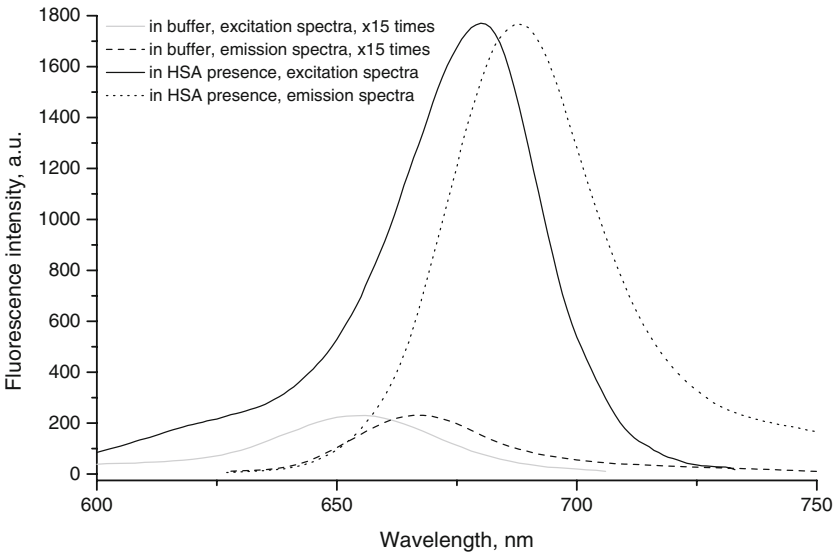

Fig. 2 Profiles of excitation and fluorescence spectra of dye P-10 in unbound state and in the presence of HSA. The low-intensive spectra of free dye are multiplied in 15 times (*a.u.*, arbitrary units)

The titration of the fixed 5×10^{-6} M concentration of the benzothiazole squarilium dye P-3 with the HSA has shown, that the fluorescence intensity enhancement relates linearly to the protein concentration over the wide range—from 0.2 to 500 μg/ml (Fig. 3). That gives possibility of quantification of HSA in this range. Detection limit for this dye was determined as 0.2 μg/ml that is comparable with commercially available dyes CBB and pyrogallol red protein [16].

Avidin, insulin, IgG, and carbonic anhydrase

Fluorescent characteristics of the squaraines in presence of avidin, insulin, IgG and ANH are shown in Table 2. Fluorescent response of the dyes on the presence of these proteins is considerably weaker than that for HSA or BSA. Fluorescence intensity of the dyes upon addition of mentioned proteins does not exceed 140 a.u. and emission intensity increases no more than in 43 times.

Upon determination of proteins in such biological liquids as blood serum we deal with a mixture of albumins and globulins in approximately same concentrations. For specific detection of albumins is biological liquids (the most frequent type of analysis) dyes should be non sensitive to presence of globulins. Thus we characterized fluorescent properties of studied dyes using IgG as model protein. It was shown, that addition of IgG, to the dyes solutions led to fluorescence intensity enhancement in 1.5–43 times that is noticeably lower than that for HSA. Also except insignificant fluores-

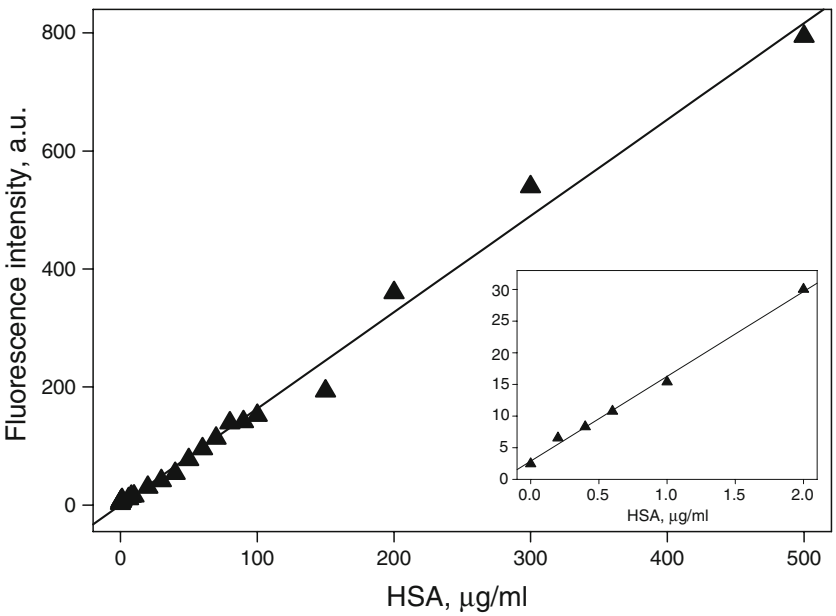

Fig. 3 Linear dynamic range of HSA detection for benzothiazole squaraine dye P-3. The fluorescence intensity of the dye was plotted against the increased amounts of protein. The result shows an approximately linear dynamic range from 0.2 to 500 μg/ml

cent enhancement, the emission intensity level of formed with IgG complexes was quite low—up to 124 a.u., while emission intensity of dyes in the HSA complexes was significantly higher—up to 1,800 a.u. It should be noticed, that for benzothiazole squaraines emission intensity increase in IgG presence was higher, than for their benzoselenazole analogues.

It was also admitted, that all squaraines having *N*-hexyl pendent groups (P-1, P-2, P-3 and P-5) demonstrated emission intensity increase in 5–15.2 times upon avidin addition. Among dyes with *N*-ethyl groups only benzothiazole dye with *N*,*N*-diethylamino substituents into squaric ring P-15 enhanced fluorescence in 6.4 times in the presence of AVI, for other dyes I^{AVI}/I_0 values were insignificant. All studied dyes slightly response on the presence of insulin and ANH, the I^{INS}/I_0 and I^{ANH}/I_0 values didn't exceed 2.7.

Conclusions

1. Fluorescence properties of a series of benzothiazole and benzoselenazole squaraines in unbound state and in the presence of various proteins were studied. For the reported benzothiazole dyes intrinsic fluorescence intensity values were higher than that for corresponding benzoselenazole analogues.
2. It was shown that the length of the *N*-alkyl pendent group could effect on the binding affinity of the dyes. Generally, squaraines having long N-hexyl pendent groups demonstrate higher value of emission increasing in proteins presence than their analogues with short *N*-ethyl tails.
3. Majority of the studied squaraines demonstrated bright fluorescence both in HSA and BSA presence. For squaraine dyes with *N*-hexyl groups (P-1, P-2, P-3, P-5) about 100−540-fold fluorescence intensity increase upon albumins addition was observed.
4. For the studied dyes in presence of other proteins (IgG up to 43 times and AVI up to 15 times) fluorescence enhancement values were considerably lower comparing with albumins. In the presence of insulin and carbonic anhydrase studied squaraines slightly increased (up to 2.7. times) or even decreased their fluorescent intensity.
5. It was shown, that using of benzothiazole squaraine dye P-3 allows quantification of HSA in the dynamic range from 0.2 to 500 μg/ml. Detection limit for this dye is determined as 0.2 μg/ml, which is comparable with that of commercially available dyes as CBB and pyrogallol red protein. Also due to the noticeable selectivity of P-3 dye to albumins it could be proposed as fluorescent probe for HSA detection.

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