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N-alkyl functionalized squaraine dyes as fluorescent probes for the detection of serum albumins

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Aim. Here we synthesize a series of benzoindolium squaraine dyes with N-substituents (SQ) and examine these dyes as fluorescent probes to detect serum albumins. **Methods.** Organic synthesis, fluorescent spectroscopy, absorption spectroscopy, fluorescent microscopy. **Results.** The spectral-luminescent properties of SQ dyes in the aqueous solution were investigated in the presence of bovine serum albumin (BSA), human serum albumin (HSA), equine serum albumin (ESA), ovalbumin (OVA), beta-lactoglobulin (BLG) and lysozyme (LYS), as well as in the presence of nucleic acid. The maxima of excitation spectra of the studied dyes in the buffer solution ranged within 623–673 nm, with the fluorescence emission maxima lying between 635 and 690 nm. All these dyes showed a similar increase in the fluorescence intensity with serum albumins and at the same time with the red shift up to 12 nm, which could point out the binding of the dyes to proteins. These dyes demonstrate a noticeably lower fluorescence intensity in the presence of OVA, BLG, and LYS, which structurally differ from serum albumins. The studied dyes gave no significant fluorescent response to the addition of nucleic acids. The equilibrium constants of dyes binding to BSA (K) were estimated as $3.0 \pm 0.3 \times 10^5$ M^{-1} for SL-2411 and $2.4 \pm 0.6 \times 10^5$ M^{-1} for SL-2412. Based on the relative values of K, we could suppose that the mechanism of dye-BSA binding is the interaction of the chromophore of the dye with the protein globule. It was shown that SL-2411 penetrates the cell membrane and distributes in the cytoplasm without co-localization with MitoTracker Green. **Conclusion.** These dyes could apply fluorescent spectroscopy for protein detection and potentially visualize cell components with minimum to no autofluorescence.

K e y w o r d s: squaraine dyes, serum albumins, fluorescent spectroscopy, absorptions spectroscopy, fluorescent detection, fluorescence microscopy

Introduction

There are many fluorescent dyes with different structures, which can be successfully used for the detection and visualization of various biomolecules [1–5]. Squaraine (SQ) dyes are a class of organic dyes derived from the aromatic squaric acid core and containing electron-donating aromatic rings at the diametrically opposite sides of the four-member ring. These dyes are widely used due to their high molar extinction coefficient, absorption, and fluorescent emission in the visible and far-red regions. Depending on the structure, SQ dyes potentially can be applied in photodynamic therapy [6, 7], solar cells [8], optical bioimaging [9], NIR-fluorescent probes, and labels [10]. Besides, the development of far-red fluorescent probes based on SQ dyes for protein detection in solution has been also described [11–14]. Detection of proteins is essential in chemical and biochemical analyses, biotechnology, and immunodiagnostics. Serum albumins are the major transport proteins in blood plasma for many compounds like hormones, fatty acids, *etc.*; to perform this function, serum albumins can bind various ligands [15]. The binding of dyes to biomolecules is realized in two ways: covalent labeling and non-covalent interaction. Non-covalent binding of dyes to proteins generally leads to the long-wavelength shift of the absorption and emission spectra of the dyes and to an increase in the fluorescence quantum yield [16]; it affects the functional activity of the biomolecule to a lesser extent [17]. Serum albumins possess several sites for the ligand binding. The primary binding sites (first described for HSA) are sites I and II [18]. The complex formation between SQ dyes and bovine serum albumin (BSA) was studied by ligand displacement experiments using dansylamide (DNSA) for the site I selectivity and dansylproline (DP) for the site II binding [19].

The site-selectivity experiments demonstrated binding to the sites I and II of BSA (with preference to site II). The binding of SQ dyes with serum albumins is used for biomedical imaging applications [20], for the determination of drug-to-albumin binding constant [21], and for the investigation of conformational changes in proteins [21]. The selective detection of BSA by SQ dye using the fluorescence lifetime imaging microscopy (FLIM) method was applied to monitor the endocytosis of BSA in live cultured cells in real-time to explain some biochemical processes [22].

Previously we have studied a series of different classes of dyes, including benzothiazole aza-substituted squaraine dyes; they demonstrated an enhancement of emission intensity up to 400 times and the shift of excitation and emission maxima up to 60 nm in the presence of albumins [12, 23]. In the present paper, a series of squaraine benzoindolium dyes are studied (Fig. 1). The spectral-luminescent properties of SQ dyes were characterized in the buffer solution in the presence of bovine serum albumin (BSA), human serum albumin (HSA), equine serum albumin (ESA), ovalbumin (OVA), beta-lactoglobulin (BLG), and lysozyme (LYS) of distinct structure. The binding constants of the dyes were calculated, additionally, their permeability to cells and

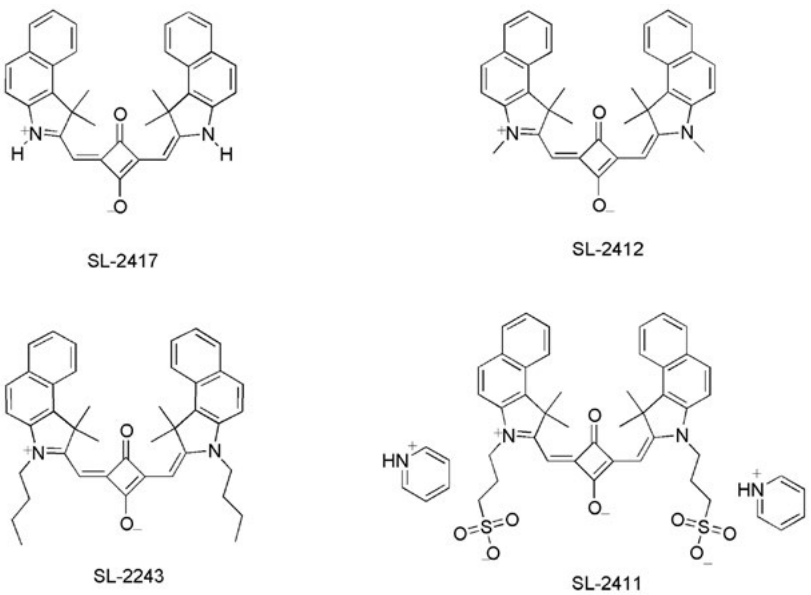


Fig. 1. Chemical structures of studied SQ dyes

distribution within them were determined by fluorescent microscopy.

Materials and Methods

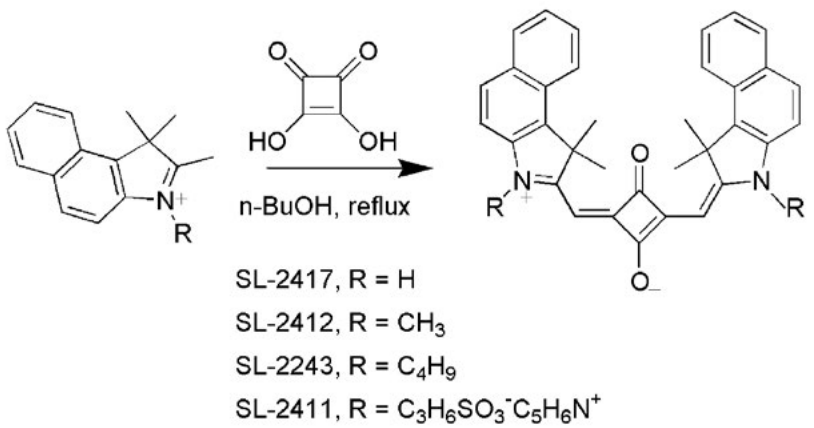
Synthesis of studied dyes

SQ dyes were synthesized by condensation reactions between the corresponding N-substituents benzoindolium salts and squaric acid (Scheme1). For obtaining the studied dyes, the mixture of the corresponding salt (5 mmol) and squaric acid (2 mmol) was boiled in an n-butanol/toluene (2:1 v/v) mixture using a Dean-Stark apparatus. The reaction solution was cooled and concentrated under vacuum. The resulting precipitate was washed with water and purified by preparative TLC with a mobile phase of CH_2Cl_2-CH_3OH (9:1). The structures of obtained dyes were confirmed by ^{1}H NMR.

(4*Z*)-4-[(1,1-dimethyl-1*H*-benzo[*e*]indolium-2-yl)methylene]-2-[(*E*)-(1,1-dimethyl-1,3-dihydro-2*H*-benzo[*e*] indol-2-ylidene)methyl]-3-oxocyclobut-1-en-1-olate (**SL-2417**), mp 271–275 °C (dec); ^{1}H NMR(400 MHz, DMSO-d_6): δ 7.98 (d, *J* = 8.6 Hz, 2H), 7.93 (dd, *J* = 8.7, 4.3 Hz, 4H), 7.63 (d, *J* = 8.8 Hz, 2H), 7.57 (t, *J* = 7.7 Hz, 2H), 7.36 (t, *J* = 7.5 Hz, 2H), 5.79 (s, 2H), 1.95 (s, 12H); LCMS: m/z 497.3 [M+H$^+$], R_f = 0.81 min.

(4*Z*)-3-oxo-4-[(1,1,3-trimethyl-1*H*-benzo[*e*] indolium-2-yl)methylene]-2-[(*E*)-(1,1,3-trimethyl-1,3-dihydro-2*H*-benzo[*e*]indol-2-ylidene)methyl] cyclobut-1-en-1-olate (**SL-2412**), mp 274-277 °C (dec); ^{1}H NMR(400 MHz, DMSO-d_6): δ 8.22 (d, *J* = 8.6 Hz, 2H), 8.00 (dd, *J* = 8.7, 4.3 Hz, 4H), 7.70 (d, *J* = 8.8 Hz, 2H), 7.60 (t, *J* = 7.7 Hz, 2H), 7.43 (t, *J* = 7.5 Hz, 2H), 5.81 (s, 2H), 3.68 (s, 4H), 3.30 (s, 4H), 2.52 (s, 2H), 1.95 (s, 10H); LCMS: m/z 525,3 [M+H$^+$], R_f = 1.268 min.

3-butyl-2-((*Z*)-{3-[(*E*)-(3-butyl-1,1-dimethyl-1,3-dihydro-2*H*-benzo[*e*]ind-2-ylidene) methyl]-2-hydroxy-4-oxocyclobut-2-en-1-yli-



Scheme 1. Synthesis of squaraine dyes

dene}methyl)-1,1-dimethyl-1*H*-benzo[*e*]indolium (**SL-2243**), mp 272-275 ^{0}C(dec); ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.21 (d, *J* = 8.5 Hz, 2H), 8.01 (d, *J* = 8.6 Hz, 4H), 7.70 (d, *J* = 8.9 Hz, 2H), 7.61 (t, *J* = 7.8 Hz, 2H), 7.44 (t, *J* = 7.6 Hz, 2H), 5.86 (s, 2H), 4.21 (s, 2H), 2.52 (s, 12H), 1.94 (s, 8H), 1.74 (s, 3H), 1.43 (q, *J* = 7.6 Hz, 3H), 0.95 (t, *J* = 7.3 Hz, 4H); LCMS: m/z 608,3 [M+H$^+$], R_f = 1.493 min.

dipyridinium 4-{(2*E*)-2-[(3*Z*)-3-{[1,1-dimethyl-3-(4-sulfonatobutyl)-1*H*-benzo[*e*] indolium-2-yl]methylene} -2-oxido-4-oxocyclobut-1-en-1-yl)methylene]-1,1-dimethyl-1,2-dihydro-3*H*-benzo[*e*]indol-3-yl}butane-1-sulfonate (**SL-2411**), mp 270-275 ^{0}C (dec); ^{1}H NMR(400 MHz, DMSO-d_6): δ 8.87 (d, *J* = 5.5 Hz, 4H), 8.49 (t, *J* = 8.0 Hz, 2H), 8.22 (d, *J* = 8.7 Hz, 2H), 7.99 (d, *J* = 8.8 Hz, 8H), 7.73 (d, *J* = 8.8 Hz, 2H), 7.60 (t, *J* = 7.7 Hz, 2H), 7.44 (q, *J* = 7.5, 6.8 Hz, 2H), 5.86 (s, 2H), 4.19 (d, *J* = 8.1 Hz, 4H), 1.94 (s, 10H), 1.83 (q, *J* = 7.5 Hz, 4H), 1.74 (p, *J* = 7.1, 5.8 Hz, 4H); LCMS: m/z 383,2 [M-2H$^+$]$^{2-}$/2, R_f = 0.78 min.

Materials used

Dimethyl sulfoxide (DMSO), methanol, and 0.05 M Tris-HCl buffer (pH 7.9) were used as solvents. Human serum albumin (HSA), bovine serum albumin (BSA), equine serum albumin (ESA) and ovalbumin (OVA), beta-lactoglobulin (BLG), lysozyme (LYS), as well as deoxyribonucleic acid (DNA) from salmon testes were purchased from Sigma-Aldrich (USA).

Preparation of the solutions

The 2 mM stock solutions were prepared by dissolving the dyes in DMSO. The stock solutions of proteins (HSA, BSA, ESA, BLG, LYS, and OVA) were prepared by dissolving in 0.05M TRIS-HCl buffer (pH 7.9) in a concentration 0.2 mg/ml. Protein concentrations in stock solutions were equal to 3 μM for BSA, HSA, and ESA and 4.5 μM for OVA, BLG, and LYS. DNA stock solution (6 mM b.p.) was prepared in 0.05M TRIS-HCl buffer (pH 7.9). The free dyes' working solution was prepared by diluting the dye stock solution in 0.05 M

TRIS-HCl buffers (pH 7.9). Working solutions of the dyes and proteins were prepared by adding dye stock solution to protein stock solution. The concentrations of dye in working solutions amounted from 5 to 10 μM. All working solutions were prepared immediately before the experiments.

General information

NMR spectra were acquired on a Bruker Avance III 600 spectrometer. ESI mass spectra were recorded on an ESI Q-tof Ultima API mass spectrometer (Waters). HPLC-MS analysis was performed using the Agilent 1100 LC/MSD SL separations module and Mass Quad G1956B mass detector with electrospray ionization (+ve or -ve ion mode as indicated). HPLC was performed using a Zorbax SB-C18, Rapid Resolution HT cartridge, 4.6x30 mm, 1.8 μm i.d. column at a temperature of 40 °C with gradient elution of 0–100 % CH_3CN in 0.1 % aq. HCOOH at a flow rate of 3 mL/min and a run time of 2.8 min. Absorption spectra of studied dyes were recorded on a SHIMADZU UV-VIS-NIR spectrophotometer UV-3600. Spectra were recorded at 350–850 nm regions. Fluorescence excitation and emission spectra were registered using the fluorescent spectrophotometer Cary Eclipse (Varian, Australia). All spectral measurements were performed at room temperature in quartz absorption cuvettes (1x1 cm).

Estimation of affinity of dyes Sl-2411 and Sl-2412 binding to BSA (binding constant K)

The equilibrium constant of the dyes Sl-2411 and Sl-2412 binding with BSA (binding constant, K) was estimated based on experimental measurements of the dependence of the fluorescence intensity of these dyes (5 μM) on BSA concentration in 0.05 M Tris-HCl buffer (pH 7.9) as described in [23]. Shortly, the calculation of K was performed, assuming that each protein globule has n sites for dye binding (and that the equilibrium constant of binding with different binding sites has equal values). The dye fluorescence intensity is proportional to the number of bound dye molecules. Under these conditions, the experimentally obtained curve of the dye fluorescence intensity was presented as the dependence of $(I - I_0)$ on C_P, and further fitted with the dependence:

$$Y = A \times \left[\frac{1}{2} + \frac{x \times n}{2 \times C_L} + \frac{1}{2 \times K \times C_L} - \sqrt{\left(\frac{1}{2} + \frac{x \times n}{2 \times C_L} + \frac{1}{2 \times K \times C_L}\right)^2 - \frac{x \times n}{C_L}}\right] \quad (1)$$

where $x = C_P$, $Y = (I - I_0)$, $A = (I_{max} - I_0)$; C_P and C_L are total concentrations of protein globules and dye molecules respectively; I is the observed fluorescence intensity of the dye at the protein concentration C_P, while I_0 and I_{max} are fluorescence intensities of the free dye in the absence of protein, and of all dye molecules bound to the protein, respectively. First, the n and A values were obtained as fitting parameters. Further, the values of n were fixed at the integer values the closest to those obtained by fitting, and so fitting was repeated to obtain the values of K and A.

Cell culture cultivation

The human ovarian cell line (A2780) was obtained from Sigma-Aldrich. A2780 cells were grown in RPMI-1640 medium supplemented with 10 % fetal bovine serum (FBS), 1 % l-glutamine, and 1 % penicillin/streptomycin. Cells were cultivated to 80–90 % confluence and detached from the flask by using

trypsin/EDTA solution (0.025 %/ 0.01 %, w/v, Biochrom GmbH, Germany) in PBS.

Live-cell fluorescent imaging

Cells were cultivated as described above. For the live-cell staining, the cells were resuspended in the RPMI-1640 medium containing 5 % FBS, 1 % L-glutamine, and 1 % penicillin/streptomycin and seeded at concentration 80 cells/μL per glass-bottom fluorescent dish (μ-Dish 35 mm, high, ibidi GmbH, Germany). Every dish with 2 ml total volume was left at 37 °C in the chamber filled with CO_2 (5 %) overnight. The cells were washed the next day (2 × 2 mL DPBS), and a fresh portion of HBSS (2 mL) was added. Solutions of Hoechst 33342, MitoTracker Green, and the dye SL-2411 in DMSO were added into separate and the same dishes for 30 min of incubation. Then the cells were washed twice with PBS, and the fresh HBSS (2 ml) was added. The fluorescence images were taken with a Zeiss Axio Vert.A1 and filter set: ex 625–655/em 665–715 nm (deep red) for the detection of SL-2411, ex 335–383/em 420–470 nm (blue) for the detection of Hoechst 33342, ex 450–490/em 500–550 nm (green) for the detection of MitoTracker Green. Objective: 40x/1.30. Oil (DIC). Obtained images were analyzed using free software Fiji/ImageJ v1.52b [24].

Result and Discussion

Spectral-luminescent properties of free dyes in methanol and aqueous buffer solution

Spectral properties of the studied dyes in methanol and Tris-HCl buffer (pH 7.9) are presented in Table 1. The absorption maxima of the SQ dyes in methanol are located in the range of 658–676 nm, and the molar extinction values were moderate in the range (0.41–0.70) $\times 10^5$ $M^{-1}cm^{-1}$. The exception is the dye 2411, for which the extinction coefficient is quite high and equals to 2.31×10^5 $M^{-1}cm^{-1}$. It should be mentioned that, though the absorption maxima of SL-2243 and SL-2411 have similar shape and maximum, their molar extinction coefficients differ about three times.

Table 1. **Molar extinction coefficient at λ_{max} of the dyes (5 μM) in methanol and buffer solutions**

	methanol		buffer	
	λ_{max}, nm	ε, 10^5 $M^{-1}cm^{-1}$	λ_{max}, nm	ε, 10^5 $M^{-1}cm^{-1}$
2417	676	0.41	644/690	0.2/0.17
2412	658	0.63	618/676	0.14/0.11
2243	662	0.70	628/674	0.17/0.15
2411	662	2.31	612/658	0.9/2.1

Fig. 2 represents the absorption spectra of the examined dyes in methanol (a) and Tris-HCl buffer (b). The optical density of the dyes in methanol (Fig. 2a) was normalized to unity at the corresponding dye's maximum wavelengths. The spectra of all studied compounds in methanol possess a close shape with a strong maximum and a weak short-wavelength shoulder, which is characteristic of the monomeric form of the dyes. The spectrum of SL-2417 is the most noticeably different (in the sense of both shape and maximum wavelength), apparently due to hydrogen atoms in N-positions instead of carbon ones. As for the solutions of the dyes in buffer (Fig.2b), the shape of absorption spectra of SL-2417, SL-2412 and SL-2243 undergoes a strong change. Namely, instead of a single sharp band, two broad bands appear, both shifted by 30-40 nm to the short-

and long-wavelength region compared to the corresponding spectrum in methanol (Table1). We believe that such changes probably occur due to the aggregation of the three mentioned dyes in an aqueous solution. At the same time, for the dye bearing sulfonate groups (SL-2411), the shape of the absorption spectrum in the buffer is similar to that in methanol (658 nm), except for the appearance of a stronger small short-wavelength minor maximum (612 nm). Hence, dye aggregation in the buffer is much less intensive than in three other dyes. This occurs most possibly due to the double negative charge of the SL-2411 dye molecule, which causes the coulomb repulsion of the molecules, this repulsion being destructive to dye aggregation.

Concentration-dependent aggregation in aqueous solution was studied for dyes with a more pronounced tendency to aggregation. All three dyes (SL-2417, SL-2412, and SL-2243) have similar aggregation behavior. Fig. 3c shows concentration-dependent aggregation on the example of a dye SL-2412. As shown in Fig. 3c, an increase of the concentration from 1μM to 25 μM at 20 °C results in a transition between the monomeric (λ max at 670 nm) and aggregated (λ max at 624 nm) states. At lower concentrations starting from 1 μM, monomeric species dominate in the dye spectrum. At a concentration of 7 μM, the optical density of the dye monomeric and aggregated maxima is in equilibrium. This may indicate a thermodynamic equilibrium between the monomeric and aggregated states. From 10 μM, the aggregated species slightly dominate in the spectrum.

All dyes in an aqueous buffer show weak fluorescence in a free state, except for the dye SL-2411 (Table 2). The excitation wavelengths of the studied dyes are located in the range of 662–673 nm, whereas the fluorescence emission maxima lie between 675 and 690 nm, with small Stocks shifts (12–17 nm). The fluorescence excitation maxima of all four dyes in the buffer are close to the corresponding maxima of absorption spectra in methanol. This means that the dyes fluorescent emission corresponds

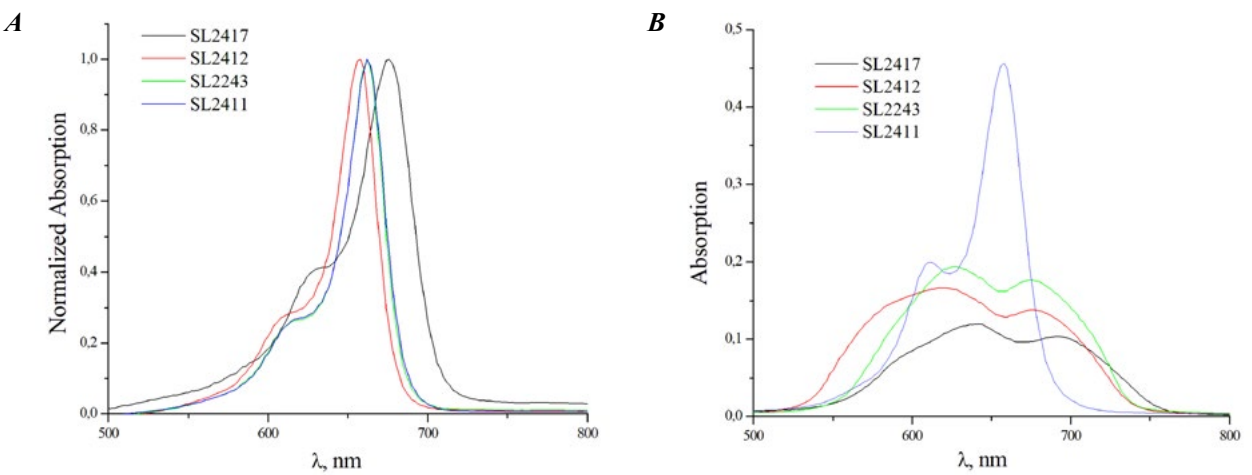


Fig. 2. The absorption spectra of the studied SQ dyes in methanol (normalized; *A*) and in 0.05 M Tris-HCl buffer pH 7.9 (*B*). Dye concentration 5 μM.

to the dye monomer form emission. Thus, the low intensity of fluorescent emission of the dyes SL-2417, SL-2412, and SL-2243 is due to the formation of non-fluorescent aggregates by most of these dye molecules in water solution. Such a concept explains that the fluorescence intensity of the dye SL-2411 (which stays mostly in monomer form) is much higher.

Spectral-luminescent properties of free dyes in the presence of globular proteins and DNA

Further, we studied spectral properties of the dyes in the presence of bovine, human and equine serum albumins as well as in the presence of other globular proteins (lysozyme, beta-lactoglobulin, ovalbumin) and DNA (Table 2). It is seen from Table 2 that the presence of proteins and DNA leads to the shift of maxima of excitation and emission spectra to either the long-wavelength (up to 28 nm) or the short-wavelength (up to — 19 nm) spectral range. All the studied dyes showed an increase of fluorescence intensity in the presence of serum albumins. The highest increase was observed for the dye SL-2412 (588, 169, and 460 times for BSA, HSA, and ESA, respectively). The dyes SL-2417 and SL-2243 demonstrate the specificity to ESA (73.5 and 19.5 times, respectively). Noteworthy, despite the similar structures of BSA, HSA, and ESA, the mentioned dyes SL-2412, SL-2417, and SL-2243 demonstrate different sensitivity to these serum albumins. The dye SL-2411 demonstrated approximately the same fluorescence response in the presence of BSA, HSA, and ESA; it is generally lower than those for other studied dyes. This can be explained by notably higher intrinsic fluorescence of the dye SL-2411.

All squaraine dyes demonstrated noticeably lower fluorescence intensity in the presence of OVA, which is structurally different from se-

Table 2. **Spectral properties of SQ dyes in buffer and [in] the presence of structurally different proteins or DNA.**

Dye	In buffer			In BSA presence				In HSA presence				In ESA presence			
	λ_{ex}, nm	λ_{em}, nm	I_0, *a.u.*	λ_{ex}, nm	λ_{em}, nm	I_{BSA}, a.u.	I_{BSA}/I_0	λ_{ex}, nm	λ_{em}, nm	I_{HSA}, a.u.	I_{HSA}/I_0	λ_{ex}, nm	λ_{em}, nm	I_{ESA}, a.u.	I_{ESA}/I_0
SL-2417	665	678	4.6	684	698	64	13.9	671	675	105	22.8	681	686	338	73.5
SL-2412	673	690	2.7	666	671	1600	588	668	674	461	169	667	673	1250	460
SL-2243	668	682	18.8	670	680	162	8.6	672	680	125	6.6	672	678	367	19.5
SL-2411	662	675	502	682	692	5250	10.5	678	688	4700	9.4	682	688	3850	7.7
Dye	**In LYS presence**				**In BLG presence**				**In OVA presence**				**In DNA presence**		
	λ_{ex}, nm	λ_{em}, nm	I_{LYS}, a.u.	I_{LYS}/I_0	λ_{ex}, nm	λ_{em}, nm	I_{BLG}, a.u.	I_{BLG}/I_0	λ_{ex}, nm	λ_{em}, nm	I_{OVA}, a.u.	I_{OVA}/I_0	λ_{ex}, nm	λ_{em}, nm	I_{DNA}/I_0
SL-2417	693	705	11.2	2.4	683	698	15.7	3.4	675	678	123	26.7	663	678	1.26
SL-2412	660	671	181	67	669	677	15	5.5	673	676	277	102	669	682	2.28
SL-2243	673	678	20.5	1.1	675	689	18.6	0.99	674	680	557	29.6	668	680	1.04
SL-2411	662	674	675	1.3	662	674	812	1.6	677	681	1700	3.4	664	676	1.16

λex (λem) — maximum wavelength of fluorescence excitation (emission) spectrum; I_0 — emission intensity of dye in a free state (in a.u.-arbitrary units); $I_{PROTEINS/DNA}/I_0$ — increment of the dyes fluorescent intensity in the presence of proteins/DNA (in numbers of times)

rum albumins. The exception is the dye SL-2412 which showed a 102-fold increase in emission in complexes with OVA. As for the other three dyes (SL-2417, SL-2243, and SL-2411), the enhancement of their fluorescence intensity in the presence of OVA does not exceed 29.6 times. The fluorescent response of the studied dyes to lysozyme is also strongly different, with intensity enhancement up to 67 for SL-2412 and below 2.4 for the other three dyes. For all four studied dyes, the presence of BLG led only to slight or no enhancement of fluorescence intensity (up to 5.5, respectively). Finally, all studied SQ are not sensitive to DNA since they do not increase fluorescence intensity. Since two squaraine dyes, SL-2411 and SL-2412, are specific to all serum albumins with BSA preference, we have estimated their equilibrium binding constants to this serum albumin.

For dye SL-2412, the most sensitive and specific to serum albumins, we measured its absorption and fluorescence spectra in the presence of BSA to study the effect of a free dye aggregation on its sensitivity to the protein. Absorption and fluorescence spectra of SL-2412 in the presence of BSA in concentrations from 0 to 30 μM are presented in Fig.3a

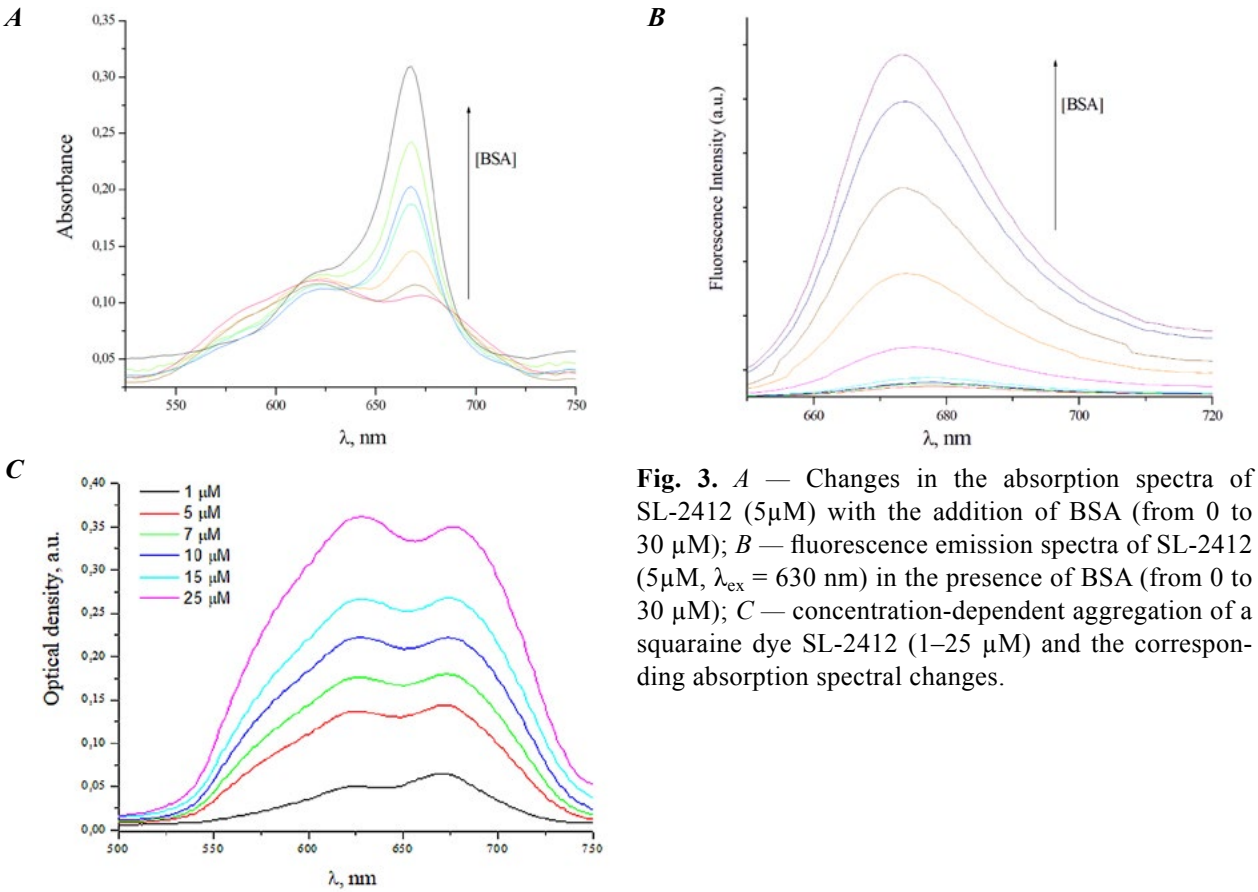


Fig. 3. *A* — Changes in the absorption spectra of SL-2412 (5μM) with the addition of BSA (from 0 to 30 μM); *B* — fluorescence emission spectra of SL-2412 (5μM, λ_{ex} = 630 nm) in the presence of BSA (from 0 to 30 μM); *C* — concentration-dependent aggregation of a squaraine dye SL-2412 (1–25 μM) and the corresponding absorption spectral changes.

and Fig.3b, respectively. With the increasing BSA concentration, redistribution between the absorption maxima of the aggregate form of the dye (618 nm) and the monomers (676 nm) is observed [25]. Thus, we found an increase in the peak of the monomeric form of the dye following an increase in the protein concentration, and the peak of the aggregated form (618 nm) acquires the shape of the shoulder of the main peak (676 nm). In parallel, there is an raise in the fluorescence of the dye SL-2412 with increasing concentration of BSA near 668 nm corresponding to the monomer form of the dye. This can be related to the destruction of dye aggregates due to the interactions between protein and dye. As a result of this interaction with BSA binding sites, the fluorescence intensity increased due to (1) destruction of non-fluorescent aggregates and (2) further increase in monomer dye intensity because of the limited rotation of the dye molecule fixed on the protein. This correlates with the concentration-dependent aggregation behavior of the free dye SL-2412 (Fig. 3c).

Estimation of equilibrium constants of dyes binding to BSA

To characterize the affinity of the interaction of the dye with BSA, we estimated equilibrium constants of the binding of dyes SL-2411 and SL-2412 with this serum albumin. These two dyes, having the same chromophore, possess quite different N,N'-substituents, resulting in the difference in size and charge (SL-2412 is electrically neutral, SL-2411 has a double negative charge). At the same time, though the responses of both dyes to the BSA presence are quite different (I_{BSA}/I_0 being 588 for SL-2412 and 10.5 for SL-2411, Table 2), this does not necessarily mean a weaker binding of SL-2412 as compared to SL-2411, since the lower value of I_{BSA}/I_0 of the former is to the high extent due to its high value of free dye fluorescence I_0. This is because SL-2411 is mostly monomeric in water solution, whereas SL-2412 strongly aggregates (as discussed above). Thus, the equilibrium constants of the SL-2411 and SL-2412 dyes binding to BSA were estimated by fitting the data of experimental measurements of the fluorescence intensity of the dyes with the increasing amounts of BSA (Fig. 4).

Fitting was performed using fixed integer values of the number of dye molecules per BSA globule *n*. The best fitting was obtained for $n = 6$ in the case of SL-2411 and $n = 3$ for SL-2412. For these values of *n*, the *K* obtained by fitting was equal to $(3.0 \pm 0.3) \times 10^5$ M^{-1} for SL-2411 and $(2.4 \pm 0.6) \times 10^5$ M^{-1} for SL-2412. Based on the relative values of K, we could suppose that the mechanism of dye-BSA binding is the interaction of the chromophore of the dye with the protein globule, without noticeable participation of N,N'-substituents of SL-2411 in the binding. At the same time, it should not be forgotten that for each dye, binding to different binding sites of the globule with the same binding constant was supposed. Meanwhile, it is quite possible that for each dye, binding to different sites in a globule is characterized by different values of binding constant.

The fluorescence microscopy study.

The human ovarian cancer A2780 cell line was used to study the ability of dye Sl-2411 to penetrate the cell membrane and their applicability as the probes for fluorescence imaging

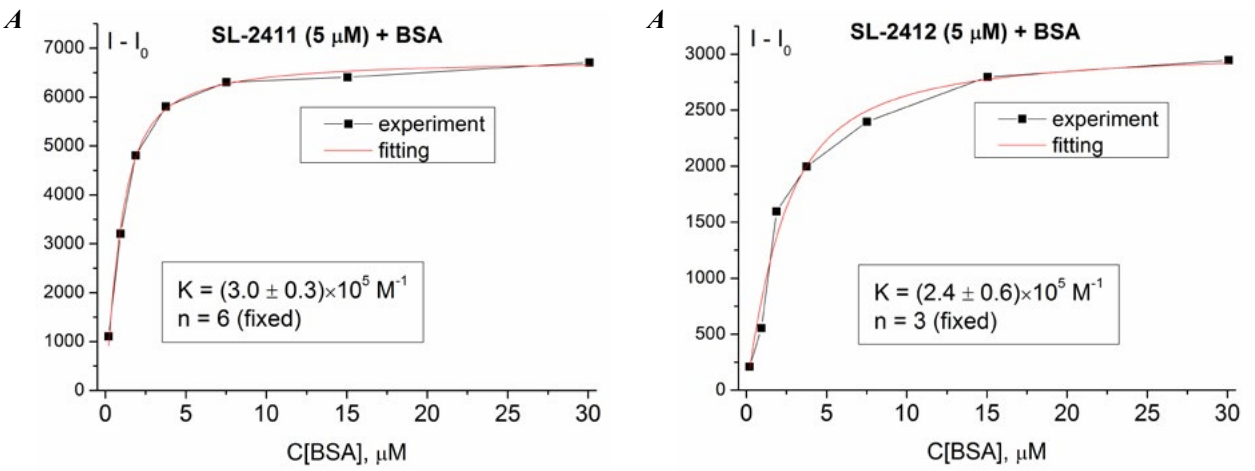


Fig. 4. Dependence of $I-I_0$ on BSA concentration and its approximation by the equation (1) for the dyes SL-2411 (*A*) and SL-2412 (*B*). I and I_0 are fluorescence intensities of the dye in the presence of specific BSA concentrations and free dye, respectively. Approximation was performed for the fixed values of n = 6 for SL-2411 and n = 3 for SL-2412 (see Materials and Methods for details).

of living cells. The two standard dyes — blue fluorescent dye Hoechst binding to nuclear DNA and green fluorescent mitochondria-specific dye MitoTracker Green — were used for the co-staining (Fig. 5). Irradiation with 335–383 nm wavelength was used to excite the Hoechst fluorescence, and a blue filter was used to detect the emission of the dye (420–470). We have found that dye Sl-2411 can penetrate through the cellular membrane and stain the cell components, but not the nucleus. The dye does not accumulate in the nucleus as shown by co-staining with Hoechst: no co-localization with the nuclear DNA dye is observed. Co-staining of the cells with the studied dyes and Mitotracker Green (a dye specific for mitochondria) was carried out to understand the organelle-specificity of the dyes. Irradiation with 625–655 nm wavelength was used to excite the dye fluorescence, and a deep red filter was used to detect the emission of the dye (665–715 nm); for MitoTracker Green,

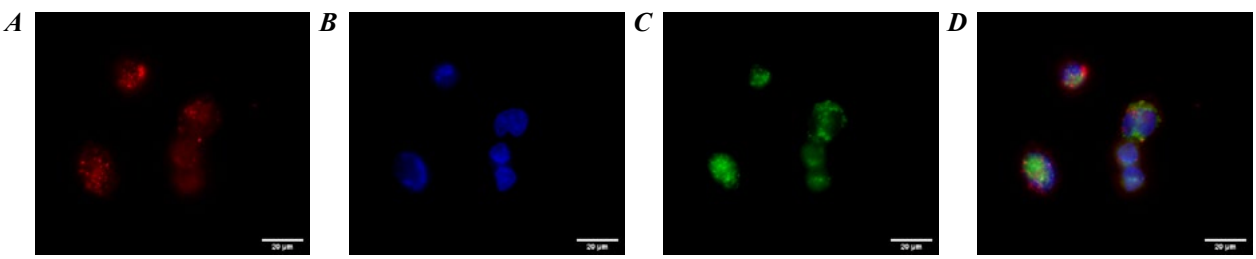


Fig. 5. Sl-2411 (*A*) staining A2780 cells at a concentration of 1 μM. Co-localization with Hoechst (*B*) and MitoTracker Green (*C*), their channels merged image (*D*). Scale bars — 20 μm.

the excitation 450–490 nm wavelength was used to excite the fluorescence and a green filter 500–550 nm to detect the emission. As seen from Fig.5, Sl-2411 showed no co-localization with mitochondria-specific dye MitoTracker Green. Although the specificity of the dye Sl-2411 to any organelle remains unclear, the staining pattern's shape allows assuming the dye to bind to lysosomes.

Conclusions

The spectral-luminescent properties of SQ dyes in the aqueous solution and in the presence of bovine serum albumin (BSA), human serum albumin (HSA), equine serum albumin (ESA), ovalbumin (OVA), beta-lactoglobulin (BLG) and lysozyme (LYS), and nucleic acid (DNA) were investigated. The maxima of excitation spectra of the studied dyes in the buffer are located at 623–673 nm, with the fluorescence emission maxima lying between 635–690 nm. The addition of serum albumins leads to the shift of excitation and emission maxima of the dyes to the long-wavelength spectral region for 8–12 nm, which could point out the binding of the dyes to proteins. The highest increase in fluorescent intensity was observed for the dye SL-2412 (588, 169, and 460 times for BSA, HSA, and ESA, respectively). The dyes SL-2417 and SL-2243 demonstrate the specificity to ESA (73.5 and 19.5 times, respectively). Studied dyes SL-2417, SL-2411, and SL-2412 demonstrate noticeably lower fluorescence intensity in the presence of OVA, which is structurally different from serum albumins. These dyes gave no significant fluorescent response upon the addition of DNA. Two squaraine dyes, SL-2411 and SL-2412, are specific to all serum albumins with a preference for BSA. Thus, we have estimated their equilibrium constants of binding to this serum albumin. The equilibrium constants of dyes binding to BSA (K) were estimated as $3.0 \pm 0.3 \times 10^5$ M^{-1} for SL-2411 and $2.4 \pm 0.6 \times 10^5$ M^{-1} for SL-2412. Based on the close values of K, we could suppose that the mechanism of dye-BSA binding is the interaction of the chromophore of the dye with the protein globule. For the most sensitive and most specific to serum albumins dye SL-2412, its absorption and fluorescence spectra were studied. With the increasing BSA concentration, the redistribution between the absorption maxima of the aggregate form of this dye (618 nm) and the monomers (676 nm) is observed towards an increase in the monomeric maximum. There is also an increase in the fluorescence of the dye SL-2412 with an increasing concentration of BSA near 668 nm, which is related to the destruction of dye aggregates due to the interaction with BSA binding sites. It was shown that the dye SL-2411 penetrates the cell membrane and distributes in the cytoplasm without co-localization with MitoTracker Green. Although the specificity of the dye SL-2411 to any organelle remains unclear, the shape of the staining pattern allows assuming the dye binding to lysosomes.

These dyes could be applicable for the protein detection by fluorescent spectroscopy and for the potential visualization of the cell components with minimum or no autofluorescence.

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N-алкільні функціоналізовані сквараїнові барвники як флуоресцентні зонди для детекції сировоткових альбумінів.

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Мета. Синтез та дослідження серії бензоіндолієвих скварейнових барвників із N-замісниками (SQ) як флуоресцентних зондів для виявлення сироваткових альбумінів. **Методи.** Органічний синтез, флуоресцентна спектроскопія, абсорбційна спектроскопія, флуоресцентна мікроскопія. **Результати.** Досліджені спектрально-люмінесцентні властивості барвників SQ у водному розчині та в присутності бичачого сироваткового альбуміну (BSA), сироваткового альбуміну людини (HSA), кінського сироваткового альбуміну (ESA), овальбуміну (OVA), бета-лактоглобуліну (BLG) та лізоциму (LYS), а також в присутності нуклеїнової кислоти. Максимуми спектрів збудження досліджуваних барвників у буферному розчині розташовані в діапазоні 623-673 нм, при цьому максимуми флуоресценції знаходяться в області 635-690 нм. Практично усі барвники демонструють збільшення інтенсивності флуоресценції із сироватковими альбумінами одночасно із червоним зміщенням до 12 нм, що може вказувати на зв'язування барвників із білками. Показано, що барвники проявляють помітно нижчу інтенсивність флуоресценції в присутності OVA, BLG та LYS, що структурно відрізняються від сироваткових альбумінів. Досліджені барвники не дають значної флуоресцентної реакції в присутності нуклеїнової кислоти. Константи зв'язування барвників з BSA (K) становлять $3{,}0 \pm 0{,}3 \times 10^5$ M^{-1} для SL-2411 і $2{,}4 \pm 0{,}6 \times 10^5$ M^{-1} для SL-2412. Виходячи з відносних значень K, можна припустити, що механізм зв'язування барвника з BSA полягає у взаємодії хромофора барвника з білковими групами. Було показано, що SL-2411 проникає через клітинну мембрану та розподіляється в цитоплазмі без спільної локалізації з MitoTracker Green. **Висновки.** Представлені барвники можуть бути застосовні для флуоресцентної спектроскопії при виявленні білків і для потенційної візуалізації компонентів клітини з мінімальною або зовсім відсутньою автофлуоресценцією.

Ключові слова: скваraїнові барвники, сироваткові альбуміни, флуоресцентна спектроскопія, абсорбційна спектроскопія, флуоресцентне детектування, флуоресцентна мікроскопія

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