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Variation of photostability of DNA-sensitive styrylcyanine dyes caused by N-alkyl functionalizationY. V. Snihirova¹, M. Yu. Losytskyy¹, D. V. Kryvorotenko¹, M. V. Kuperman¹,
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Aim. Synthesis and characterization of benzothiazole-based styrylcyanines functionalized by different N-alkyl tail groups as potential photostable probes for nucleic acids (NA) detection. **Methods.** Chemical synthesis, absorption and fluorescence spectroscopy, irradiation with a visible light source, confocal microscopy. **Results.** Styrylcyanine dyes are weakly fluorescent when free, but in the presence of NA they show emission intensity increase of up to 83 times; the most pronounced emission increase was observed for the dyes Sbt1 and Sbt3 bearing positively charged N-alkyl tail group. Photostability of the dyes Sbt1 and Sbt3 when non-bound to NA is low while binding to DNA results in a strong increase in their photostability. Dyes with uncharged and negatively charged N-alkyl groups are more photostable when free, and DNA slightly affects their photostability. This is also due to low binding of dyes with negatively charged tail groups to DNA. Sbt1 brightly stains cytoplasm and nuclear components (probably nucleoli) of mesenchymal stem cells; therefore this dye may be used to visualize cytoplasmic RNA and nuclear RNA clusters. **Conclusions.** Variation of chemical nature of N-alkyl tail group allows to design styrylcyanines of different photostability. Functionalized styrylcyanine dyes may be used for detection of nucleic acids in solution and visualization of RNA-containing components in microscopy.

Keywords: fluorescence, styrylcyanine dyes, NA detection, photostability, microscopy

Introduction

Fluorescent detection and visualization of biological molecules, organelles, cells and tissues is a powerful method of research that is widely used for different biomedical and medical purposes [1]. One of the necessary components this method does require is efficient fluorescent probes for the detection of various biological objects; particularly important are nucleic acids (NA) probes [2]. The dyes used as NA probes have to enhance strongly the fluorescence intensity upon binding to target NA molecule, possess high affinity to nucleic acid, low detection limit, high molar extinction coefficient and fluorescent quantum yield when bound, and sufficient photostability when exposed to the high intensity light necessary for microscopy [1].

A common problem with various cyanine fluorescent dyes is low photostability during continuous irradiation in the presence of oxygen that causes irreversible chemical destruction of the fluorophore molecule which thus loses its ability of fluorescence [3, 4]. On the other hand, biological object can be damaged by direct excitation of the luminescent probe in close contact with that object. These processes limit the applicability of fluorescent dyes for practical applications such as fluorescence microscopy and labeling of molecules.

Fluorescent cyanine dyes are molecules commonly used as probes or labels in different biological applications such as imaging, biomolecular labeling, and proteomics [5]. As a result of several advantages of styryl dyes as compared to classical cyanine dyes (better photostability [6], large Stokes shift, relative ease of synthesis), styrylcyanines are extensively

studied as possible fluorescent probes for NA detection and visualization. Thus, styrylcyanines were shown to be low-toxic NAs-sensitive dyes, they demonstrated fluorescence intensity enhancement upon binding to NAs as well as high fluorescence quantum yield in the NAs presence [7-10]. Applicability of benzothiazole styryl dyes for two-photon excited fluorescent visualization of living cells was also demonstrated [11]. Earlier, it was shown that the binding affinity of styrylcyanine to NAs could be modified by variation of the structure of N-alkyl tail group of the dye [12-14].

Here, a series of the benzothiazole-based styrylcyanine dyes (Fig. 1) functionalized by different N-alkyl tail groups was synthesized and characterized for their photostability and fluorescent sensitivity to nucleic acids. The long alkyl linkage (*n*-butyl) was chosen to eliminate the effects of terminal side groups on electronic transitions of chromophore. We studied UV-VIS absorption and fluorescent spectra of these dyes both in the absence and in the presence of NA (both dsDNA and RNA). We evaluated photostability of dyes as a change in the dye absorption after 150 min of irradiation by VIS source, and analyzed the influence of interaction between dyes and DNA on dyes photostability. By confocal laser scanning microscopy, we evaluated ability of styrylcyanine to penetrate into eukaryotic living cells and visualize cell components.

Materials and Methods

General. dsDNA (salmon testes) and yeast total RNA were purchased from Sigma-Aldrich Co. Solvents were of analytical grade. ¹H NMR

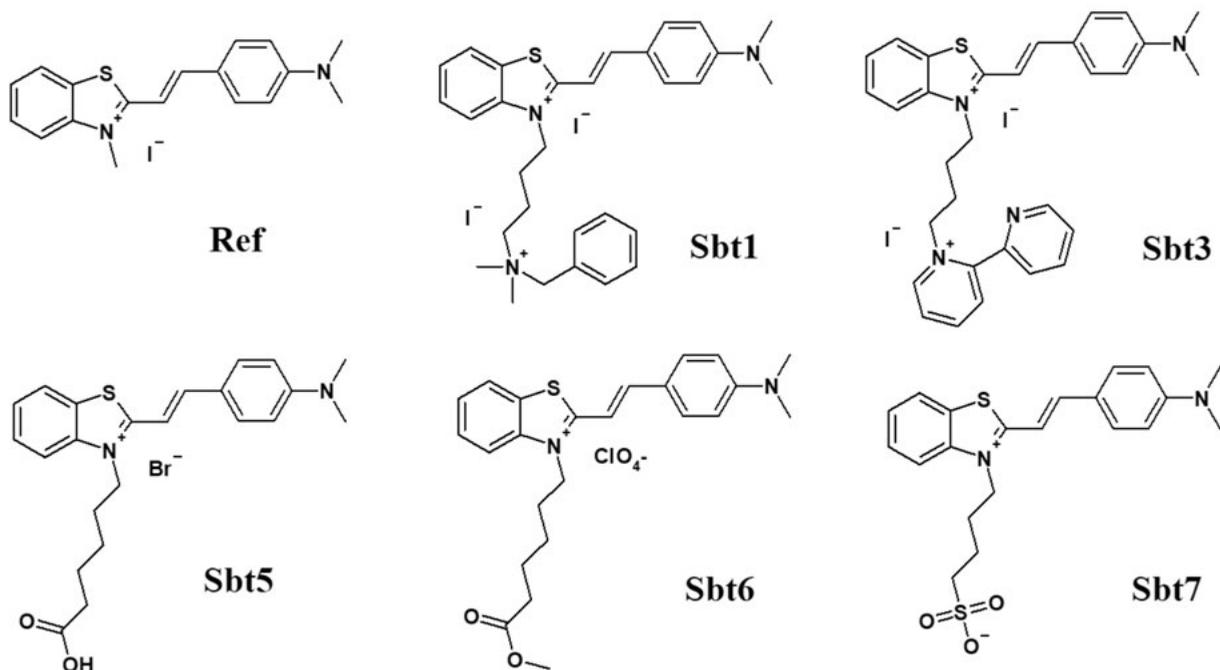


Fig. 1. Structures of the studied dyes.

spectra were recorded on Bruker ARX 400 spectrometers; chemical shifts (δ) were given in ppm relative to SiMe₄. 50mM Tris-HCl buffer (pH 7.9) was used in all assays described.

Synthesis of the dyes

Referent dye with N-methyl substituent (**Ref**) was synthesized as described previously [15].

Monomeric benzothiazole styryl dyes (**Sbt1**, **Sbt3**) with positively charged tail groups were synthesized as described in [12]. The structures of the dyes were confirmed by ¹H NMR spectra, LC-MS and element analysis.

The dyes **Sbt5**, **Sbt6** and **Sbt7** were synthesized as follows:

3-(5-carboxypentyl)-2-[2-(4-dimethylaminophenyl)-vinyl]-benzothiazol-3-ium bromide (**Sbt5**)

The mixture of 103 mg (0.3 mmol) of 3-(5-carboxypentyl)-2-methylbenzothiazol-3-ium bromide and 44.3 mg (0.3 mmol) of *p*-dimethylaminobenzaldehyde in 0.5 ml of pyridine and catalytic amount of morpholine was boiled during 2 h. The precipitate was filtered off and washed with ethyl alcohol. Yield 80 mg (56 %).

2-[2-(4-dimethylaminophenyl)-vinyl]-3-(5-methoxycarbonylpentyl)-benzothiazol-3-ium perchlorate (**Sbt6**)

40.5 mg (0.25 mmol) of carbonyldiimidazole were added to 100 mg (0.21 mmol) of **Sbt5** in 0.2 ml of DMF at 40°C. After 5 min 0.1 ml of methanol were added and stored during 24 h at room temperature. The water solution of sodium perchlorate was added. The precipitate was filtered off, washed with water and recrystallized from methanol. Yield 102 mg (95 %).

2-[2-(4-dimethylaminophenyl)-vinyl]-3-(4-sulfobutyl)-benzothiazol-3-ium inner salt (Sbt7)

Mixture of 143 mg (0.5 mmol) of 2-methyl-3-(4-sulfobutyl)-benzothiazol-3-ium and 74.5 mg (0.5 mmol) of *p*-dimethylaminobenzaldehyde in 1 ml of *n*-butanol and catalytic amount of morpholine was boiled during 30 min. The precipitate was filtered off, washed with ethyl alcohol and recrystallized from methanol. Yield 190 mg (91 %).

The results of characterization of the dyes **Sbt5**, **Sbt6** and **Sbt7** are provided below.

Sbt5

m. p. (dec.): > 200 °C; ¹H NMR (DMSO-d₆) δ(ppm): 1.45(2H, m), 1.56(2H, m), 1.82(2H, m), 2.20(2H, t, *J*=7.5 Hz), 3.11(6H, s), 4.79(2H, t, *J*=7.0 Hz), 6.83(2H, d, *J*=8.2 Hz), 7.58(1H, d, *J*=15.3 Hz), 7.67(1H, dd, *J*=8.0 Hz), 7.77(1H, dd, *J*=7.7 Hz), 7.93(2H, d, *J*=8.7 Hz), 8.10(2H, m), 8.28(1H, d, *J*=8.2 Hz), 12.10(1H, broad s).

Sbt6

m. p. (dec.): 181-184 °C; ¹H NMR (DMSO-d₆) δ(ppm): 1.44(2H, m), 1.58(2H, m), 1.81(2H, m), 2.29(2H, t, *J*=7.7 Hz), 3.11(6H, s), 3.54(3H, s), 4.78(2H, t, *J*=8.5 Hz), 6.83(2H, d, *J*=8.3 Hz), 7.57(1H, d, *J*=15.3 Hz), 7.68(1H, dd, *J*=7.8 Hz), 7.77(1H, dd, *J*=8.0 Hz), 7.90(2H, d, *J*=8.5 Hz), 8.11(2H, m), 8.28(1H, d, *J*=8.0 Hz).

Sbt7

m. p. (dec.): > 200 °C; ¹H NMR (DMSO-d₆) δ(ppm): undissolved, LC-MS : *m/z* (%) = 417 (100 %) [MH]⁺.

Anal. calcd. for C₂₁H₂₄N₂O₃S₂: C, 60.55; H, 5.81 ; N, 6.72. Found: C, 60.21; H, 5.61; N, 6.82.

Spectroscopic measurements. The absorption spectra were recorded on GENESYSTM

20 Visible Spectrophotometer (Thermo Fisher Scientific, USA). The fluorescence excitation and emission spectra were collected on a Cary Eclipse fluorescence spectrophotometer (Varian, Australia). The fluorescence spectra were measured at the excitation and emission slit widths equal to 5 nm. The spectra were acquired using standard quartz cuvettes (1 × 1 cm) at room temperature (20 °C). All measurements were made at the respective excitation maxima of each dye. Each experiment was performed three times.

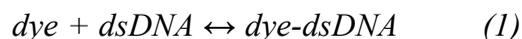
Preparation of the solutions. Dye stock solutions were prepared by dissolving the dyes at 2 mM concentration in DMSO. Stock solutions of dsDNA and RNA were prepared by dissolving the NA in Tris-HCl buffer (50 mM, pH 7.9) at the concentration of 6.15 mM b.p. for dsDNA and 12.3 mM b. for RNA. Working solutions of free dyes were prepared by dilution of the dye stock solutions with Tris-HCl buffer (pH 7.9) to the concentration of 2 μM for absorption and fluorescence spectrophotometry and 5 μM for the photostability experiments. The working solutions of dye/NA mixtures for absorption and fluorescence spectrophotometry were prepared by mixing a dye aliquot (1 μL) and an aliquot of DNA or RNA stock solutions (10 μL) in Tris-HCl buffer (final concentration of dsDNA was 61.5 μM b.p. and RNA – 123 μM b.). The working solutions of dye/NA mixtures for the photostability experiments were prepared by mixing a dye aliquot (7 μL) and an aliquot of DNA solution (30 μL) in 3 mL of Tris-HCl buffer (final concentration of dsDNA was 61.5 μM b.p.)

Photostability. Photostability of the dyes in solution and in the presence of DNA was studied using the laboratory-designed equip-

ment; its instrumental setup was as follows. The transparent glass vials with the samples (3 mL of each sample) were placed into the container with the reflecting inner surface, while on the top of the container the irradiating unit was installed. The irradiating unit consisted of 27 light-emitting diodes (blue light, 470 nm). To prevent the heating of the samples, cool water was placed inside the container that served as water bath for the glass vials with the samples. The distance between the samples and lamp was 2 cm. This way, photostability of the studied dyes was estimated both in a free state and in the presence of dsDNA (5 μ M dye, 61.5 μ M b.p. dsDNA) in 0.05 M Tris-HCl buffer (pH 7.9) and in 0.01 M phosphate buffer (pH 7.0); experiments were also performed for the dyes Sbt1 and Sbt3 in methanol. After irradiation of the mentioned samples during certain time (0.5, 1.5 and 2.5 hours), the irradiated solutions were placed into the quartz cell, absorption and fluorescence spectra of these solutions were measured, and the values of optical density (D) and fluorescence intensity (I) were obtained. Photostability of the dyes was then characterized by the ratios D/D_0 and I/I_0 , where D_0 and I_0 were optical density and fluorescence intensity of corresponding solutions prepared in the same way without irradiation. For each sample, the photostability measurements were repeated two to four times, and standard deviations were calculated.

Determination of the binding constant (K_b) for the association of dsDNA with dyes Sbt1 and Sbt6. To estimate the stability of the association of selected dyes **Sbt1** and **Sbt6** with dsDNA, we conducted fluorescent titration of **Sbt1** and **Sbt6** with increasing dsDNA

concentrations (0.2 - 171 μ M). Each experiment was performed three times. The titration curve is provided for the average values along with the standard deviations (SD). For the calculation of binding constant, we used the points corresponding to the excess of DNA. Thus we assumed that only negligible amount of the dye molecules will bind to dsDNA close to each other and affect each other's binding. Based on this assumption, the binding of dye to DNA could be described by the following equilibrium:



the constant of this equilibrium (binding constant, K_b) can be expressed with the equation (law of mass action [16]) below:

$$\frac{C_{bd}}{C_{fdNA} \times C_{fd}} = K_b \quad (2)$$

where C_{bd} , C_{fd} and C_{fdNA} are concentrations of a bound dye, a free dye and free dsDNA binding sites, respectively. For the DNA concentrations starting from 20 μ M, that is significantly higher than that of the cyanine dye (2 μ M), the concentration of free dsDNA base pairs is roughly equal to the total dsDNA concentration C_{DNA} ; $C_{fdNA} \approx C_{DNA}$. Concentration of the free dye in equilibrium is $C_{fd} = C_d - C_{bd}$ (where C_d is the total dye concentration). The measured fluorescence intensity (I) of the dye in the presence of dsDNA at the DNA concentration of C_{DNA} can be expressed with the following equation $I = I_{\max} \times C_{bd}/C_d + I_0 \times C_{fd}/C_d$, where I_0 is the fluorescence intensity of the dye (2 μ M) in the absence of dsDNA and I_{\max} is the fluorescence intensity of the dye (2 μ M) in the presence of the indefinitely large dsDNA concentration. Equation (2) can be transformed into (3):

$$I - I_0 = \frac{A \times K_b \times C_{DNA}}{1 + K_b \times C_{DNA}} \quad (3)$$

where $A = I_{\max} - I_0$.

K_b and A values can be calculated as approximation parameters by fitting the experimentally obtained data $I - I_0$ versus C_{DNA} by using equation (3). Fitting was performed and the values of K and A with their standard deviations were estimated by using Origin 8.0 program.

Eukaryotic cell staining. Mesenchymal stem cells were obtained like it was done before [17]. After transferring the slides to room temperature, the medium was removed. The cells were fixed on microscope slides at room temperature, and incubated with fluorescent dyes for 30 min. The washed cell samples were covered with a cover slip.

Confocal Laser Scanning Microscopy analysis (CLSM). CLSM analysis of the samples was done using Leica TCS SPE Confocal system with coded DMi8 inverted microscope (Leica, Germany). Images were acquired using

excitation at 488 nm and emission collected at 510-605 nm for SYBR Green I, and excitation at 532 nm and emission collected at 600-740 nm for Sbt1.

Results and discussion

Absorption characteristics of the dyes and dyes' fluorescent properties in free state and in complexes with NA

Earlier a series of styrylcyanine dyes were characterized as the fluorescent probes for DNA detection, and some of them showed good ability to enhance the fluorescent signal upon DNA binding [12]. Spectral properties of the dyes studied in this work (including **Sbt1**, **Sbt3** and **Ref** dyes reported also in [12]) are provided in the Table 1. The absorption maxima of the styrylcyanines in 50 mM Tris-HCl buffer (pH 7.9) are located in the range of 510-524 nm, with moderate values of the molar extinction coefficients at maximum wavelengths $(2.6-7.2) \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$. Maxima of fluorescence excitation spectra of free dyes

Table 1. Optical properties of styrylcyanine dyes (free and in the presence of dsDNA and RNA) in the same buffer at room temperature.

Dye*	$\lambda_{\max}^{\text{abs}}$, nm	ε , $10^4 \text{ M}^{-1}\text{cm}^{-1}$	$\lambda_{\max}^{\text{ex}}$, nm	$\lambda_{\max}^{\text{em}}$, nm	$\lambda_{\text{DNA}}^{\text{ex}}$, nm	$\lambda_{\text{DNA}}^{\text{em}}$, nm	I_0	I^{DNA}	I^{DNA}/I_0	I^{RNA}
Sbt1	524	3.8	535	596	557	605	20.0	1660	83.0	1400
Sbt3	515	6.0	532	596	562	605	15.0	750	50.0	680
Sbt5	514	5.6	529	592	549	596	31.3	100	3.2	196
Sbt6	515	7.2	529	595	558	601	26.8	721	26.9	639
Sbt7	510	4.3	530	593	547	596	14.0	68.6	4.9	62.1
Ref	512	2.6	539	593	556	604	20.6	845	41.0	861

* $C_{\text{dye}} = 2 \mu\text{M}$; $C_{\text{DNA}} = 61.5 \mu\text{M b.p.}$; $C_{\text{RNA}} = 123 \mu\text{M b.}$; 50 mM Tris-HCl buffer (pH 7.9); $\lambda_{\max}^{\text{abs}}$ — absorption spectra maxima, ε — molar extinction coefficients, $\lambda_{\max}^{\text{ex}}$, $\lambda_{\max}^{\text{em}}$ — fluorescence spectra excitation and emission maxima of free dyes; $\lambda_{\text{DNA}}^{\text{ex}}$, $\lambda_{\text{DNA}}^{\text{em}}$ — fluorescence spectra excitation and emission maxima of dyes in the presence of DNA; I_0 , I^{DNA} , I^{RNA} — fluorescence intensity (in arbitrary units) of free dyes and of the dyes in the presence of dsDNA and RNA; I^{DNA}/I_0 — fluorescence intensity enhancements.

are shifted for 11-27 nm to the long-wavelength region as compared to corresponding absorption maxima and are situated at 529-539 nm, whereas the maxima of fluorescence emission spectra are between 592 and 596 nm. The studied styrylcyanine dyes were found to be weakly fluorescent (14-31 a.u.) in the unbound state.

The presence of dsDNA leads to the long-wavelength shift of the maxima of fluorescence excitation and emission spectra for 17-30 nm and 3-11 nm as compared to corresponding maxima of the free dyes; the excitation and emission maxima for the dyes in the presence of dsDNA are thus at 547-562 nm and 596-605 nm respectively. Upon binding with DNA, the dyes increased their fluorescence intensity up to 83 times (the most significant enhancement was observed for **Sbt1** and **Sbt3** dyes, the least significant – for **Sbt7** and **Sbt5**). It could be concluded that the less significant values of fluorescence intensity enhancement were observed for zwitterionic dyes (**Sbt5** and **Sbt7**), whereas the other dyes, which are positively charged, demonstrate higher enhancement values, and these values are higher for **Sbt1** and **Sbt3** containing the charge +2 compared to the dyes **Sbt6** and **Ref** containing the charge +1. At the same time, the comparison of the dyes with the same charges points to a negative effect of $-(\text{CH}_2)_4\text{COOCH}_3$ (comparison of **Sbt6** and **Ref**), and to better effect of $-(\text{CH}_2)_4\text{N}^+(\text{CH}_2)_2\text{CH}_2\text{Ph}$ group (**Sbt1**) as compared to $-(\text{CH}_2)_4$ dipyridyl one (**Sbt3**). Noteworthy, the presence of RNA (with the same concentration of nucleotide bases) results for the studied dyes (except for **Sbt5**) in about the same increase in fluorescence intensity as in the presence of dsDNA.

Photostability of the dyes

To investigate the relationship between molecular structures of the studied styrylcyanine dyes and their photostabilities, the effect of irradiation of the dyes solutions by the blue light (working wavelength about 470 nm) on the absorption and fluorescence spectra of these solutions was studied. The change of the characteristics (maximum wavelength and optical density) of the absorption spectra of the dyes (free and in the dsDNA presence) after 30 minutes and 150 minutes of irradiation could be seen in the Table 2.

It could be seen from the Table 2 that 30-min irradiation leads to a small shift of the absorption maxima of some samples. At the same time, irradiation during 2.5 hours results in the short-wavelength shift for 1-17 nm (without any change in the peak shape) for all the studied samples. Noteworthy, for the dyes **Sbt1**, **Sbt3** and **Ref** the shift for the free dye (10, 15 and 17 nm respectively) significantly exceeds that for the dye in dsDNA presence (3, 4 and 9 nm respectively). As for the dyes **Sbt5**, **Sbt6** and **Sbt7**, the shift for the dyes in the dsDNA presence (3, 3 and 1 nm respectively) is about the same as for the free dyes (3 nm for all three dyes).

Besides the wavelength shift, irradiation of the dyes solutions also leads to the change in the optical density of the dyes. For all the dyes (except **Sbt7**), the optical density after 30-min irradiation became equal to 63-95 % of the corresponding value before irradiation for free dyes (the lowest values were observed for **Sbt1** and **Sbt3**) and 75-93 % for dyes in the presence of DNA (Table 2; Fig. 2). Noteworthy, for the dyes **Sbt1** and **Sbt3**, the addition of DNA caused noticeable increase in photosta-

Table 2. Optical density (D) of dyes in a free state and in the presence of dsDNA

Dye	λ_{\max}^0 (nm)	$\lambda_{\max}^{0.5}$ (nm)	$D/D_0 \times 100$ % [0,5 hour]	$\lambda_{\max}^{2.5}$ (nm)	$D/D_0 \times 100$ % [2,5 hours]
Sbt1	524	522	66 ± 7	514	11 ± 11
Sbt1+DNA	549	548	89 ± 4	546	42 ± 19
Sbt3	516	513	63 ± 4	501	8 ± 8
Sbt3+DNA	527	527	75.2 ± 1.4	523	35 ± 19
Sbt5	514	514	95 ± 5	511	81 ± 15
Sbt5+DNA	514	514	93 ± 3	511	74 ± 10
Sbt6	515	515	88 ± 11	512	61 ± 6
Sbt6+DNA	521	520	86 ± 11	518	59 ± 6
Sbt7	517	517	127 ± 22	514	116 ± 14
Sbt7+DNA	518	518	113 ± 23	517	129 ± 29
Ref	513	508	75 ± 15	496	38 ± 46
Ref+DNA	523	524	80 ± 5	514	44 ± 4

* in % from that of non-irradiated sample, D_0 after irradiation by LED during 30 and 150 min, λ_{\max}^0 , $\lambda_{\max}^{0.5}$, $\lambda_{\max}^{2.5}$ – maximum wavelengths of absorption spectra for non-irradiated samples and those irradiated during 30 and 150 min

bility, whereas for other dyes the mentioned effect exceeding the experimental error was not observed. As for the dye **Sbt7**, irradiation during 30 minutes caused an increase in the optical density of this dye both in the presence and in the absence of DNA (Fig. 3). This result could be explained by bad solubility of **Sbt7**

in the buffer, which could be increased because of local heating as the result of light absorption by the low-fluorescent dye.

After 150 min of irradiation, for all the dyes (except **Sbt7**) further photodestruction was observed, and two tendencies described above became still more noticeable (Table 2, Fig. 2).

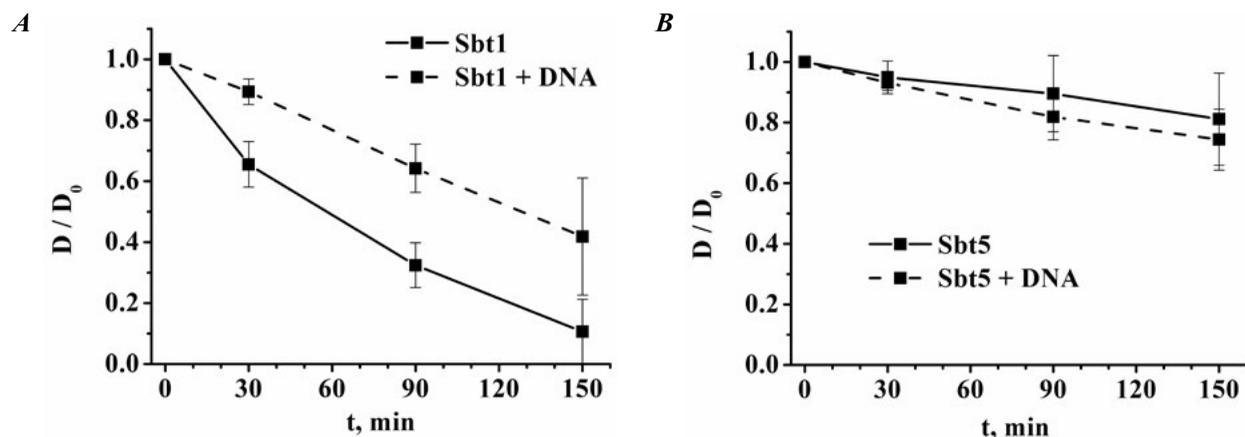


Fig. 2. Graphs of the ratio D/D_0 upon irradiation time, where D is the optical density (at maximum wavelength) of the sample after irradiation during 0, 30, 90, and 150 min, and D_0 is the optical density of the sample before irradiation, for dyes **Sbt1** (A) and **Sbt5** (B) with and without dsDNA in solution ($C_{\text{dye}} = 5 \mu\text{M}$, $C_{\text{dsDNA}} = 61.5 \mu\text{M b.p}$) in Tris-HCl buffer ($C = 0.05 \text{ M}$, pH 7.9).

First, strong difference in a decrease of the optical density for the free dyes took place. For **Sbt1** and **Sbt3** the optical density after 150 min of irradiation becomes respectively 11 % and 8 % of corresponding value before irradiation whereas for **Ref**, **Sbt6** and **Sbt5** this value is 38 %, 61 % and 81 % respectively. Second, an addition of DNA causes a strong increase in the photostability for the same dyes **Sbt1** and **Sbt3** (optical density is 42 % and 35 % respectively compared to the corresponding values before irradiation), whereas for other dyes this increase is either absent or below the experimental error. As for the dye **Sbt7**, irradiation during 150 min does not make the changes in its optical density exceeding the experimental error both for free dye and in DNA presence (Table 2, Fig. 3).

Thus, the obtained results regarding the irradiation effect on the dyes absorption spectra

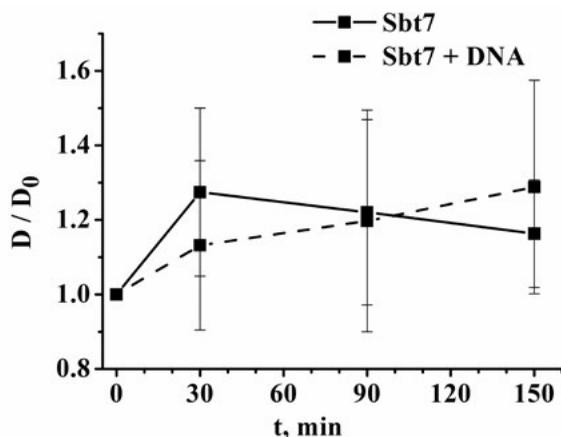


Fig. 3. Graph of the ratio D/D_0 upon irradiation time, where D is the optical density (at maximum wavelength) of the sample after irradiation during 0, 30, 90, and 150 min, and D_0 is the optical density of the sample before irradiation, for the dye **Sbt7** with and without dsDNA in solution ($C_{\text{dye}} = 5 \mu\text{M}$, $C_{\text{dsDNA}} = 61.5 \mu\text{M}$ b.p.) in Tris-HCl buffer ($C = 0.05 \text{ M}$, pH 7.9).

could be summarized as follows. First, it could be seen that the dyes with aromatic charged substituents **Sbt1** (with N-alkyldimethylbenzyl substituent) and **Sbt3** (with N-alkyldipyridyl substituent) bearing double positive charge are, on one hand, the least photostable in free state and, on the other hand, an addition of DNA to these dyes results in a strong increase of their photostability. On the other hand, the unsubstituted dye **Ref** as well as the dye **Sbt6** (with uncharged aliphatic substituent) both bearing single positive charge are more photostable in free state than **Sbt1** and **Sbt3**, whereas the dyes **Sbt5** and **Sbt7** (which are zwitterionic due to negatively charged N-substituents), are the most photostable. At the same time for these four dyes (**Ref**, **Sbt6**, **Sbt5** and **Sbt7**), the changes in their photostability induced by the DNA presence do not exceed the experimental error.

Though the detailed mechanisms of dyes photodestruction are not completely understood, this process is generally considered to be connected with the transition of the excited dye to the triplet state. The dye in the triplet state can then either induce reactive oxygen species which will further damage the dye, or directly react with redox-active molecules present in the solvent [4]. Thus, the obtained dependence of the free dye photostability on its electric charge and/or substituent could be explained by the effect of charge/substituent on chromophore interaction with the possible redox-active molecules or generated reactive oxygen species in the solution (due to the interaction of the substituent group either with the chromophore, or with reactive molecules). It should be added that photostability of the free dyes in phosphate buffer (data not pre-

sented) does not essentially differ from corresponding values in Tris-HCl buffer. At the same time, photostability of **Sbt1** and **Sbt3** in methanol is significantly higher (optical density after 150 min of irradiation is $89 \pm 7\%$ and $103 \pm 2\%$ respectively of corresponding values before irradiation) than in both Tris and phosphate buffers. These facts could point to the role of the redox-active molecules present in water in photodestruction of the dyes.

As for the different influence of the DNA presence on the photostability of different studied dyes, two factors should be considered. First, the dyes **Sbt1** and **Sbt3** which increase their photostability in the DNA presence have also the highest increase in their fluorescence intensity upon DNA addition (Table 1). Taking into account that these dyes possess double positive charge, we could suppose that they bind to DNA with higher affinity as compared to other dyes. If so, the reason of the increase in the dye photostability should be its binding with DNA (despite the anticipated increase in the excited state lifetime). The possible explanation could be the restriction (at least partial) by DNA of the access to the dye molecule of the generated reactive oxygen species or redox-active molecules. On the other hand, one cannot also exclude the fixation of the dye structure upon binding that does not permit the excited dye to acquire the reaction-suitable conformation. As for other studied dyes which either contain single positive charge or are zwitterionic, we could suppose that they bind with DNA with lower affinity. This means that lower percent of these dyes form complexes with DNA, and thus DNA influence on the dyes photostability is less significant. Another factor is that the dyes **Sbt5**, **Sbt6** and **Sbt7** are much

more photostable in a free state as compared to **Sbt1** and **Sbt3**, so that the influence of DNA on their photostability would not be as noticeable for the former dyes as for the latter ones.

For the dyes **Sbt1**, **Sbt3** and **Ref** in the presence of dsDNA, we also compared the influence of irradiation on dye optical density and on its fluorescence intensity (Table 3). It is seen from the Table 3, that while for the dyes **Sbt1** and **Sbt3** fluorescence intensity is decreased to much lower extent as compared to optical density, for the dye **Ref** emission is suppressed almost as strongly as absorption (the difference between D/D_0 and I/I_0 for **Ref** is due to the nonlinear dependence of fluorescence intensity on optical density). Such difference between **Ref** and two other dyes corresponds to the difference between them in DNA effect on their photostability. In the case when the DNA-bound dye molecules are more photostable than the free ones, irradiation of dye-DNA solution leads to the destruction of mostly non-bound dye molecules. Since the emission intensity of DNA-bound dyes significantly exceeds that of the free dyes, such photodestruction makes stronger effect on dye absorption than on its emission. This feature

Table 3. Optical density (D) and fluorescence intensity (I) of dyes in the presence of dsDNA

Dye	Absorption, $D/D_0 \times 100\%$		Fluorescence, $I/I_0 \times 100\%$	
	0.5 hour	2 hours	0.5 hour	2 hours
Sbt1+DNA	89 ± 4	42 ± 19	96 ± 4	77 ± 6
Sbt3+DNA	75.2 ± 1.4	35 ± 19	97.3 ± 1.4	83 ± 5
Ref+DNA	80 ± 5	44 ± 4	75.8 ± 0.2	55 ± 6

* 50 mM Tris-HCl buffer (pH 7.9) (in % from that of non-irradiated sample, D_0 and I_0 respectively) after irradiation by LED during 30 and 150 min. $C_{dye} = 5 \mu\text{M}$, $C_{dsDNA} = 61.5 \mu\text{M}$ b.p.

may be used for differentiation and contrast of structures stained by dyes that burn out more and less quickly.

Determination of binding constant

Based on the results of the dye fluorescence intensity increase in the dsDNA presence, we supposed above that increased photostability of the dyes **Sbt1** and **Sbt3** in the DNA presence is due to higher affinity of these dyes binding to DNA as compared to other studied dyes. To support this assumption, we have compared the affinity of dye binding to dsDNA for the dyes **Sbt1** and **Sbt6**. Both dyes increase their fluorescence intensity upon the DNA binding, but while the presence of DNA strongly enhances the photostability of **Sbt1**, that of **Sbt6** does not depend on the DNA presence. The equilibrium constant (K_b) of binding to dsDNA was determined for **Sbt1** in [12]; for **Sbt6**, K_b was found here using the approximation of the dye fluorescent titrations by dsDNA with the equation (3) (Fig. 4), in the same way as it was done for **Sbt1** in [12]. Thus, the values of K_b for

Stb1 and **Sbt6** were found to be $5.0 \times 10^4 \text{ M}^{-1}$ and $1.0 \times 10^4 \text{ M}^{-1}$ respectively. Such values of K_b are typical for intercalating dyes, the usual range being between 10^4 and 10^6 M^{-1} , while groove binders have higher binding constants ($10^5 - 10^9 \text{ M}^{-1}$) [18].

Since the K_b value for **Sbt6** is 5 times lower than the one for **Sbt1**, higher percent of dye molecules are bound to DNA in the case of the latter as compared to the former. For the obtained K_b values and used concentrations ($61.5 \text{ } \mu\text{M}$ DNA and $5 \text{ } \mu\text{M}$ of dye), the rough estimation based on the mass action law results in 75 % and 38 % of the bound dye in the dye-DNA solution for **Sbt1** and **Sbt6** respectively. That may explain (at least partially) the fact that the DNA effect on the dye photostability was significant for **Sbt1** and was not observed for **Sbt6**. Other alternative (or additional) explanations could be e.g. more rigid fixation in DNA or longer time of being bound (since dynamic equilibrium between free and DNA-bound dyes takes place in dye-DNA solution) for **Sbt1** as compared to **Sbt6**, or different

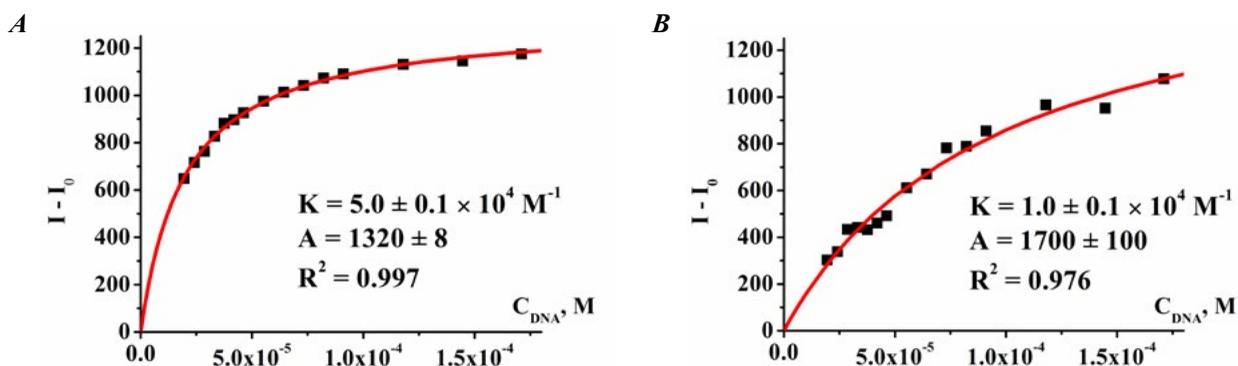


Fig. 4. Plots of $(I - I_0)$ dependence on C_{DNA} (black squares) and its approximation by the Eq. (3) (red line) for **Sbt1** (A) and **Sbt6** (B) ($C_{\text{dye}} = 2 \text{ } \mu\text{M}$, $C_{\text{DNA}} = 19.5\text{--}171 \text{ } \mu\text{M}$). $(I - I_0)$ is the difference between the measured fluorescence intensity (I) of the dye ($2 \text{ } \mu\text{M}$) in the presence of dsDNA at the certain DNA concentration (C_{DNA}) and fluorescence intensity of the free dye at $2 \text{ } \mu\text{M}$ concentration (I_0).

mechanisms of fixation making different groups of the chromophore accessible to reactive oxygen species or redox-active molecules.

Staining by the dyes

Since the dye **Sbt1** bearing N-alkylbenzylamine group has demonstrated rather high constant of binding to dsDNA, as well as strong increase in fluorescence intensity and photostability upon this binding, we have studied the possibility to use this dye as fluorescent stain for fluorescent microscopy. Thus, we have performed the staining of mesenchymal stem cells with **Sbt1** in co-staining with DNA-sensitive stain SYBR Green I (Fig. 5); the obtained images showed that **Sbt1** brightly stains cytoplasm, so it could be suggested to visualize cytoplasmic RNA. The dye seemed to penetrate less efficiently into the nucleus, but nucleus components (probably nucleoli) stained by this dye were highly discernable. In contrast to styrylcyanine dye **Sbt1**, SYBR Green I stained mainly nucleus, and discrimination of nucleolus components with this dye was less pronounced (Fig. 5).

Conclusions

A series of the benzothiazole-based styrylcyanines functionalized with N-alkyl tail groups of different nature was synthesized and characterized for their photostability and fluorescence response upon binding to nucleic acids. These styrylcyanine dyes are weakly fluorescent in the unbound state, whereas the presence of NA caused an increase of the dyes fluorescence intensity up to 83 times. The intensity of dye fluorescence response depends on N-alkyl tail substituent of the dye. The most significant enhancement was observed for the dyes with positively charged aromatic substituents **Sbt1** (with N-alkyldimethylbenzyl substituent) and **Sbt3** (with N-alkyldipyridyl substituent), the least significant – for the dyes with negatively charged groups **Sbt7** (sulfo-substituent) and **Sbt5** (carboxy-substituent).

The dyes **Sbt1** and **Sbt3** are, on one hand, the least photostable in a free state and, on the other hand, addition of DNA to these dyes results in strong increase of their photostability. The dyes, which either bear single positive

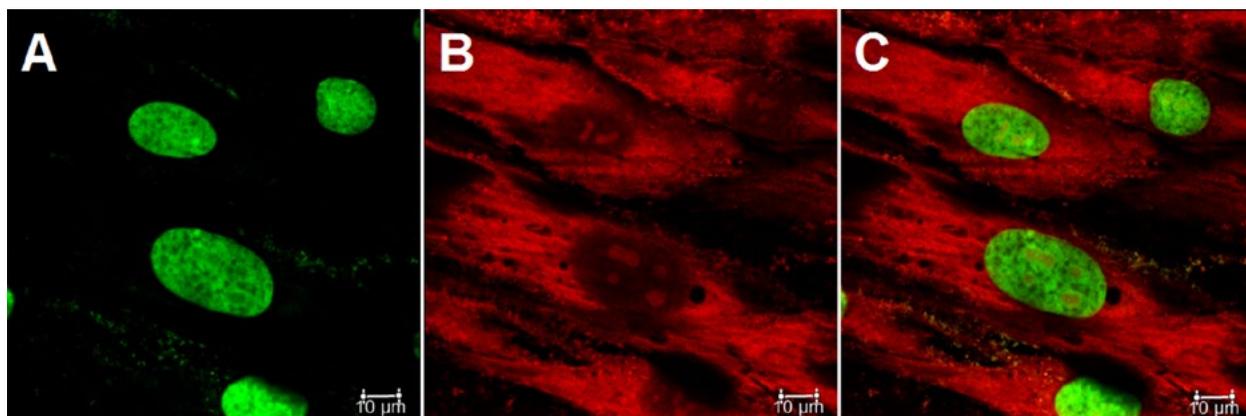


Fig. 5. Images of mesenchymal cells co-stained by **Sbt1** and SYBR Green I: green emission of SYBR Green I (A), red emission of dye **Sbt1** (B), and merging of both emissions (C).

charge on chromophore (uncharged N-alkyl tail group) or are zwitterionic (due to negatively charged N-alkyl tail group), are more photostable when free, and the presence of DNA weakly affects their photostability. Thus, the charge and/or the nature of N-alkyl tail group makes strong effect on the photostability of benzothiazole styrylcyanine dye in water solution. The dye bearing positively charged N-alkyl tail groups binds to DNA with higher affinity (binding constant for **Sbt1** is $5 \times 10^4 \text{ M}^{-1}$) comparing to the dye **Sbt6** with uncharged tail group (binding constant 10^4 M^{-1}).

Staining of mesenchymal stem cells with the dye **Sbt1** in co-staining with sensitive to DNA dye SYBR Green I has shown that **Sbt1** brightly stains cytoplasm and nucleus components (probably nucleoli), whereas SYBR Green I stains mainly the nucleus. Presumably, **Sbt1** is suggested to visualize cytoplasmic RNA and clusters of RNA in the nucleus.

Thus, variation of chemical nature of N-alkyl tail group is a way to design styrylcyanine dyes of different photostability. Functionalized styrylcyanines are suggested as handy and photostable fluorescent stains for microscopy techniques and nucleic acid detection in solution.



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Conflicts of interest

Authors declare no conflict of interests.

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Варіація фотостабільності ДНК-чутливих стирилціанінових барвників, зумовлена природою N-алкільних замісників

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Мета. Синтез та характеристика серії N-алкільних функціоналізованих бензтіазол стирилціанінових барв-

ників у якості потенційних фотостабільних зондів для детекції нуклеїнових кислот (НК). **Методи.** Синтез, спектроскопія поглинання і флуоресценції, опромінення барвників видимим світлом, конфокальна мікроскопія. **Результати.** Барвники, що мають слабку флуоресценцію у вільному стані, підвищують інтенсивність емісію при зв'язуванні з ДНК до 83 разів; найбільше підвищення спостерігалось для барвників з позитивно зарядженими N-алкільними замісниками — Sbt1 і Sbt3. Барвники Sbt1 і Sbt3 виявились найменш фотостабільними у вільному стані, але додавання ДНК значно підвищило їх фотостабільність. Барвники з незарядженими та негативно зарядженими N-алкільними замісниками є більш фотостабільними в незв'язаному стані, і присутність ДНК значно не впливає на їх фотостабільність (зокрема завдяки слабкій взаємодії з ДНК барвників з негативно зарядженим замісником). При фарбуванні мезенхімальних ставбурових клітин, Sbt1 яскраво забарлював цитоплазму та компоненти ядра (можливо, ядерця), так що імовірно він фарбує цитоплазматичну РНК та кластери РНК у ядрі. **Висновки.** Варіація хімічної природи N-алкільних замісників є способом створення стирилціанінових барвників з різною фотостабільністю. Функціоналізовані стирилціанінові барвники є перспективними як легкі в застосуванні флуоресцентні зонди для детекції нуклеїнових кислот у розчині та візуалізації РНК-містких об'єктів у мікроскопії.

Ключові слова: Флуоресценція, стирилціаніни, детекція НК, фотостабільність, мікроскопія.

Изменение фотостабильности ДНК-чувствительных стирилцианиновых красителей, обусловленное природой N-алкильных заместителей

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Цель. Синтез и характеристика серии N-алкил функционализированных бензтиазол стирилцианиновых красителей как потенциальных фотостабильных зондов для детекции нуклеиновых кислот (НК). **Методы.** Химический синтез, спектроскопия поглощения и флуоресценции, облучение красителей видимым светом,

конфокальная микроскопия. **Результаты.** Слабо флуоресцирующие в свободном состоянии, красители повышают интенсивность эмиссии при связывании с ДНК до 83 раз; наибольшее повышение наблюдалось для красителей с позитивно заряженным Т-алкильным заместителем — Sbt1 и Sbt3. Красители Sbt1 и Sbt3 оказались наименее фотостабильными в свободном состоянии, но добавление ДНК значительно увеличило их фотостабильность. Красители с незаряженными и негативно заряженными N-алкильными заместителями более фотостабильны в несвязанном состоянии, и присутствие ДНК слабо влияет на их фотостабильность (в частности благодаря слабому взаимодействию с ДНК красителей с негативно заряженным заместителем). При окрашивании мезенхимальных стволовых клеток,

Sbt1 ярко окрашивал цитоплазму и компоненты ядра (возможно, ядрышки), так что предположительно он окрашивает цитоплазматическую РНК и кластеры РНК в ядре. **Выводы.** Изменение химической природы N-алкильных заместителей является способом создания стирилцианиновых красителей различной фотостабильности. Функционализированные стирилцианиновые красители являются перспективными в качестве легких в использовании флуоресцентных зондов для детекции нуклеиновых кислот в растворе и визуализации РНК-содержащих объектов в микроскопии.

Ключевые слова: Флуоресценция, стирилцианины, детекция НК, фотостабильность, микроскопия.

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