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ORIGINAL PAPER

Fluorescence of Styryl Dyes-DNA Complexes Induced by Single- and Two-Photon Excitation

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Abstract The series of novel monomer and homodimer styryl dyes based on (*p*-dimethylaminostyryl) benzothiazolium residues were synthesized and studied as possible fluorescent probes for nucleic acids detection. Spectral-luminescent and spectral-photometric properties of obtained dyes in the unbound state and in DNA presence were studied. Fluorescence emission induced by two-photon excitation of dye-DNA complexes in aqueous buffer solution was registered. Two-photon absorption cross section values of the studied dyes in DNA presence were evaluated.

Keywords Styryl dyes · Nucleic acids detection · Fluorescent probes · Two-photon excitation

Introduction

Nowadays the detection of nucleic acids (NA) using fluorescent probes is widely applied. The most sensitive NA probes belong to the cyanine dyes class. Earlier the styryl benzothiazolium dyes were shown to interact with DNA with strong fluorescence intensity enhancement [1]. Due to the capacity of styryl benzothiazolium dyes to penetrate through cell membranes, these dyes were proposed for the DNA visualization in fluorescent microscopy [1]. In previous paper [2] we described series of novel homodimer styryl dyes containing non-charged aliphatic linkage group that give noticeable fluorescent response in DNA presence. Fluorescent emission of these probes is traditionally excited by UV or visible light.

Recently two-photon excitation (TPE) of the probes fluorescence by near IR radiation was proposed. This method has several important advantages over the commonly used single-photon excitation (SPE) [3]. Hence, the efforts were aimed on the development of DNA-sensitive fluorescent dyes with high two-photon absorption cross section (δ) values. Despite we have shown the applicability of DNA binding monomethine cyanine dye Cyan 40 for the nucleic acids visualization in live cells [4], the DNA-sensitive cyanine fluorescent probes are considered not to be efficiently excited by TPE [4]. At the same time styryls are among the dyes having high two-photon absorption cross-section values [3, 5]; for benzothiazolium styryl dye strong fluorescence upon TPE by 1064 nm radiation was observed [6].

Hence we propose to design the DNA-sensitive fluorescent dyes with high δ values on the basis of benzothiazole styryl residue. As is well known, the presence of spermine-like groups (containing charged quaternary nitrogen) in dye molecule could significantly increase its affinity to DNA [7]. Thus we used positively charged linkage/tail group to design series of DNA sensitive homodimer/monomer dyes.

Here we present the synthesis of novel monomer and homodimer styryl dyes based on the (*p*-dimethylaminostyryl) benzothiazolium template and containing spermine-like linkage or tail group of various length. These dyes were studied as possible two-photon excited fluorescent probes for DNA detection. First, spectral-luminescent and spectral-photometric properties of obtained dyes in the unbound state

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and in presence of DNA upon SPE were measured. Next the TPE-induced fluorescence emission of dye-DNA complexes in buffer solutions was registered. Two-photon absorption cross section values of the styryl dyes in DNA presence were estimated.

Experimental and calculations

Synthesis of dyes

Procedure 1

Mixture of 1.27 ml (0.01 mol) of 2-methylbenzothiazole and 3.3 ml (0.025 mol) of 1,4-diiodobutane in 5 ml dioxane was boiled during 8 hours. Obtained salt was precipitated with diethyl ether, filtered off and washed with isopropyl alcohol and ether.

Procedure 2

Mixture of 0.46 g (0.001 mol) of 3-(4-iodobutyl)-2-methyl-1,3-benzothiazol-3-ium iodide and 1.38 g (0.001 mol) of *p*-dimethylaminobenzaldehyde in 3 ml of acetic anhydride was boiled during 10 min. The dye was precipitated with diethyl ether, filtered off, than washed and recrystallized from methanol.

Procedure 3

To the solution of 0.295 g (0.0005 mol) of SI-1 (Scheme 1) in 4 ml of dimethylformamide 0.00025 mol of corresponding N,N,N′,N′-tetramethyldiaminoalkane was added; the obtained mixture was heated during 10 hours on boiling water bath. Reaction mixture was cooled; precipitate was filtered off, washed with methanol and ether.

Procedure 4

Mixture of 1.27 ml (0.01 mol) of 2-methylbenzothiazole and 3.0 g (0.01 mol) of 3-bromopropyl(triethyl)ammonium bromide was heated during 5 hours at 130°C. Then, 5 ml of dioxane was added to the obtained alloy and than boiled during 3 hours. Obtained salt was precipitated with diethyl ether, filtered off and washed with isopropyl alcohol and diethyl ether.

Procedure 5

Mixture of 0.45 g (0.001 mol) of 2-methyl-3-(3-triethylammoniopropyl)-1,3-benzothiazol-3-ium dibromide and 1.38 g (0.001 mol) of *p*-dimethylaminobenzaldehyde in 3 ml of acetic anhydride was boiled during 10 min. The precipitate was filtered off, washed with isopropyl alcohol and recrystallized from methanol.

Preparation of working solutions

The dye stock solutions with concentration 2×10^{-3} M were prepared in dimethylformamide (DMF). Concentration of dye in buffer working solutions was 5×10^{-6} M for the absorption and SPE fluorescence study, and 1.5×10^{-5} M for the TPE fluorescence study (homodimers concentration was calculated in chromophores). 0.05 M Tris-HCl buffer (pH = 8.0) was used for the measurements. Total DNA from chicken erythrocytes was purchased from Sigma. The DNA concentrations in the working solutions were 6×10^{-5} M base pairs (b.p.) for absorption and SPE fluorescence measurements, and 1.8×10^{-4} M b.p. for TPE experiments. Thus, dye to DNA concentrations ratio was 1 dye molecule per 12 DNA b.p. for all the experiments performed. The solution of Rhodamine 6G in ethanol at the concentration of 5×10^{-6} M was used as the reference in TPE experiment.

Spectral-luminescent studies

Absorption spectra were registered with Specord M40 spectrophotometer (Carl Zeiss, Germany). Fluorescence excitation and emission spectra in the SPE fluorescence study were obtained with a Cary Eclipse fluorescence spectrophotometer (Varian, Australia). These spectra were not corrected for the sensitivity of registration system. But the fluorescence spectra of all the studied dyes have the similar shape, and the difference between the maxima of the fluorescence spectra of the different dyes both in buffer and in DNA presence does not exceed 14 nm. Thus, the obtained SPE fluorescence spectra of all the studied dyes both in buffer and in DNA presence could be compared.

The fluorescence intensity values obtained during the SPE fluorescence study (Table 1) are presented in arbitrary units (a.u.). These values reflect the electrical current generated by the Cary Eclipse photomultiplier tube and thus are proportional (for the given wavelength) to the quantity of light photons emitted by the sample. Though the values measured in arbitrary units are generally not proportional to the fluorescence quantum yield of the sample (because of the possible change in absorption between the samples), they reflect the intensity of the fluorescence emission. The comparison of the fluorescence emission intensities is the most direct way to study the dyes applicability as fluorescent probes.

Table 1 Spectral-luminescent and spectral-photometric properties of dyes in the unbound state and in DNA presence

DYE	Free state				In DNA presence				
	λ_{abs}	λ_{ex}	λ_{em}	I_0	λ_{abs}	λ_{ex}	λ_{em}	I^{DNA}	I^{DNA}/I_0
DBos-21	487	533	595	3.5	514 547*	555	607	1730	494
DBos-24	432	533	596	5.4	437* 545	557	607	1090	202
DBos-25	493	536	596	4.3	520 545*	557	607	1500	349
DBos-28	494	540	593	1.0	515* 553	557	607	955	955
DBos-30	493	538	596	1.6	515 555*	558	606	1360	850
Bos-1	533	545	599	20.9	560	567	606	1360	65

Note. λ_{abs}, λ_{ex}, $\lambda_{em,}$ absorption, fluorescence excitation and emission spectra maxima wavelengths (nm); $I_{0,}$ intrinsic fluorescence intensity of dyes at maximum wavelength (a.u.); I^{DNA}, fluorescence intensity of dye-DNA complexes at maximum wavelength (a.u.).

*More intensive maximum.

TPE fluorescence studies

Two-photon excited fluorescence measurements were carried out with the help of YAG:Nd^{3+} laser generating 15 ns pulses with the wavelength 1064 nm at a repetition rate about 6 Hz. Fluorescence emission was detected at right angle as it passed the telecentric system of lenses and the entrance slit (2mm) of the monochromator (Cherney-Turner scheme with the grid 600 lines/mm). Behind the exit slit (2 mm) of monochromator the fluorescence emission was directed to the photomultiplier tube, then the signal was amplified and measured. The reference SPE fluorescence spectra to be used for the calculation of the δ values were measured on the same equipment using the second harmonic generator (efficiency of conversion 10%). The obtained TPE and reference SPE spectra were corrected for the sensitivity of registration system.

For the measurements of fluorescence induced by two-photon excitation as well as the reference SPE measurements, the solutions with the same chromophore number densities were used. Fluorescence spectra of Rhodamine 6G and other dyes were registered at the same conditions (the values of photon fluxes of the exciting radiation stayed permanent).

All measurements (except the investigation of the temperature destruction of dyes aggregates) were carried out at room temperature.

Calculation of two-photon absorption cross-section value

The two-photon absorption cross section (δ, cm^4s) can be determined by comparison the relative fluorescence intensities Fl_1 and Fl_2 produced by single- and two-photon absorption respectively [8, 9]. In order to make general evaluation the contribution of self-absorption was neglected.

If one accepts that the two-photon absorption cross section value for Rhodamine 6G in ethanol solution δ_{Rh6G} is known and amounts to 5.5×10^{-50} cm^4s [10, 11], then the two-photon absorption cross section for the new dye δ_x can be given as

$$\delta_x = 5.5 \times 10^{-50} \frac{Fl_{1Rh6G} Fl_{2x} \sigma_x}{Fl_{2Rh6G} Fl_{1x} \sigma_{Rh6G}} \quad (1)$$

where σ is the single-photon absorption cross section (cm^2).

Results and discussion

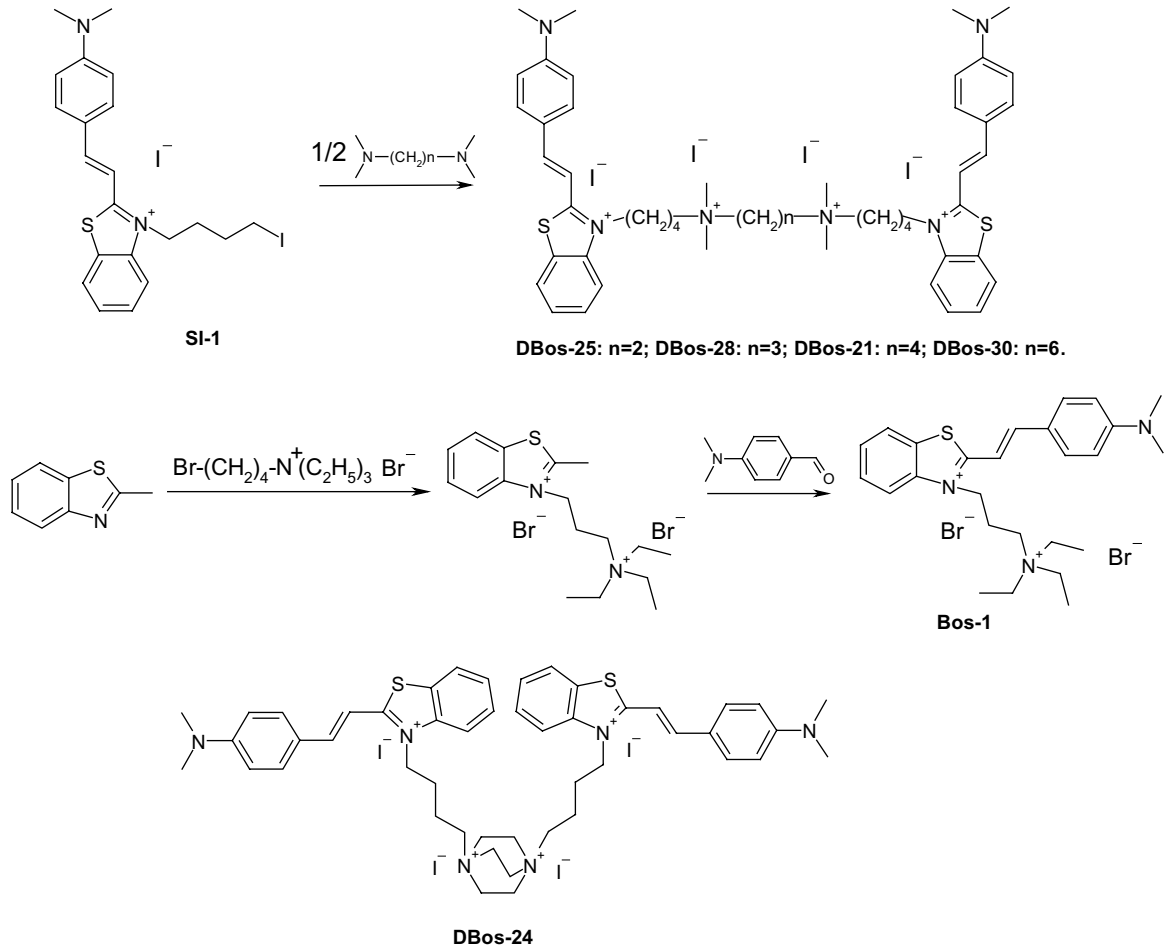
Synthesis of dyes

General scheme of the dyes synthesis and their structures are presented at Scheme 1. As the first stage of the homodimers synthesis the quaternary salt with iodo-alkyl substituent was obtained by heating of 2-methylbenzothiazole with exceed of 1,4-diiodobutane in dioxane (procedure 1). By condensation of this salt with *p*-dimethylaminobenzaldehyde in acetic anhydride we obtained the dye SI-1 (procedure 2). Homodimer styryl dyes were obtained by alkylation of corresponding N,N,N′,N′-tetramethyldiaminoalkane with SI-1 (procedure 3). Reaction was carried out in conditions used by Glazer et al. [12]. For the obtaining of the dye DBos-24, 1,4-diazabicyclo[2.2.2]octane was used instead of tetramethyldiaminoalkane.

Monomer benzothiazole styryl dye with positively charged tail group (Bos-1) was synthesized by the fusion of methylbenzothiazole and 3-bromopropyl (triethyl)ammonium bromide with next condensation of obtained quaternary salt with *p*-dimethylaminobenzaldehyde (procedure 4, 5). The structure of the dyes was confirmed with ^1H NMR spectra and element analysis.

General spectral-luminescent study of dyes

Spectral-photometric and spectral-luminescent properties of the dyes are presented in Table 1. As was expected all the studied dyes (homodimers and monomer) demonstrate



Scheme 1 Synthesis and chemical structures of homodimer and monomer styryl dyes

close fluorescence emission characteristics, namely maximum wavelengths are situated between 593 and 599 nm for free dyes and at 606–607 nm for dyes in the presence of DNA. Fluorescence excitation maximum wavelengths of homodimer styryls are close as well (533–540 nm for the free dyes and 555–558 nm for dyes in DNA presence), while for monomer this maximum is slightly red-shifted (545 nm for free dye and 567 nm for dye-DNA complex). The shapes of fluorescence excitation as well as fluorescence emission spectra (Fig. 1) are similar for monomer and all the homodimer styryls and should correspond to the excitation and emission of the single dye chromophore.

The binding of the studied dyes with DNA resulted in the increase in dyes fluorescence intensity of 2-3 orders of magnitude. All the studied dyes demonstrate high fluorescence intensity in presence of DNA (I^{DNA}), but the intensity of the free dye emission (I_0) is higher for an order of magnitude for monomer Bos-1 comparing with homodimers. Thus the value of emission enhancement (I^{DNA}/I_0) for monomer styryl is significantly lower than that for homodimer dyes.

In previous paper we firstly studied benzothiazolium homodimers with uncharged aliphatic linkage group as fluorescent dyes for DNA detection [2]. Discussing the influence of charged linkage group on the sensitivity of homodimer dyes to DNA, it should be noted that emission intensity of DNA complexes of the dyes with charged spermine-like linkage group exceeds that of homodimers with uncharged linkage group for more than an order of magnitude. Besides, for the dyes with charged linkage group the emission increase for several hundred times was recorded in DNA presence, while for the dyes with uncharged linkage group this enhancement does not exceed dozens times [2]. Comparing the properties of benzothiazole monomers with and without charged tail group, the same tendency is observed. The dye containing spermine-like group is characterized by considerably higher values of I^{DNA} and I^{DNA}/I_0 than the non-modified monomer. Using of various chemical substituents that do not influence on spectral properties of the dye but could increase its binding affinity to biomolecules was earlier proposed as "affinity-modifying group" approach [13]. Previously this

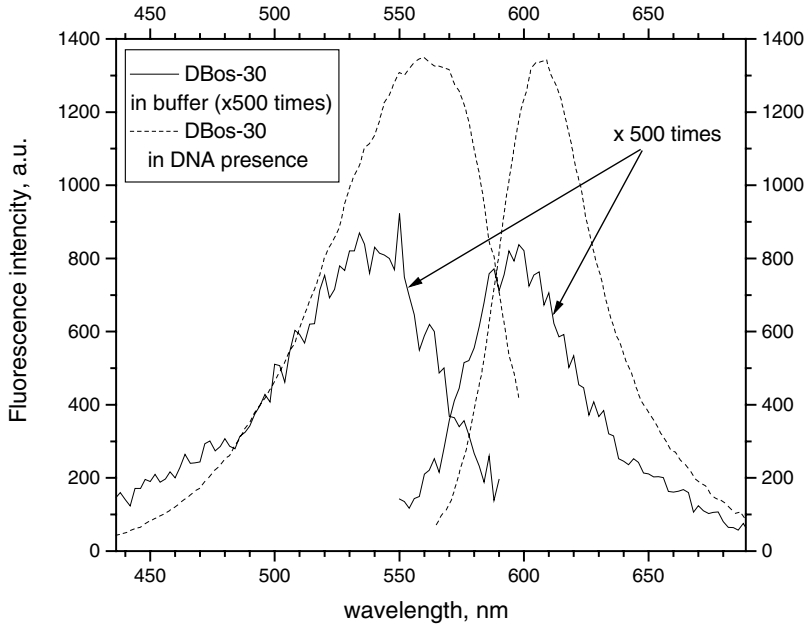


Fig. 1 Fluorescence excitation (left) and emission (right) spectra of DBos-30 in buffer solutions in the unbound state and in DNA presence (single-photon excitation). The fluorescence intensity of free dye solution is multiplied in 500 times

approach was successfully applied for designing the DNA sensitive probes on the base of cyanine dyes [14].

The shapes of absorption spectra of monomer Bos-1 in the unbound state and in the DNA presence are structureless and close to each other as well as to the corresponding fluorescence excitation spectra, though in DNA presence the absorption spectrum maximum is red-shifted on 27 nm as compared to the free dye. At the same time, in absorption spectra of homodimer styryls several peculiarities could be noticed. First of all, in the case of free dyes the band dominating in the absorption spectrum is blue-shifted as compared to that of monomer; it is situated at 432 nm for DBos-24 and at 487–494 nm for other homodimer styryls (Figs. 2 and 3). Besides, the low intensive wide shoulder red-shifted to the dominating band was observed in the free dyes absorption spectrum (near 490 nm for DBos-24 and near 550 nm for the rest of homodimer styryls). At the same time, the DNA presence causes strong changes in the absorption spectrum of homodimer styryl dyes, namely the maximum near 545–555 nm appears in the spectrum. This maximum could be attributed to the absorption of the styryl dye chromophores fixed on the DNA molecule. For the dyes DBos-21, DBos-25, DBos-28 and DBos-30 in presence of DNA the short-wavelength band becomes less intensive and shifts to about

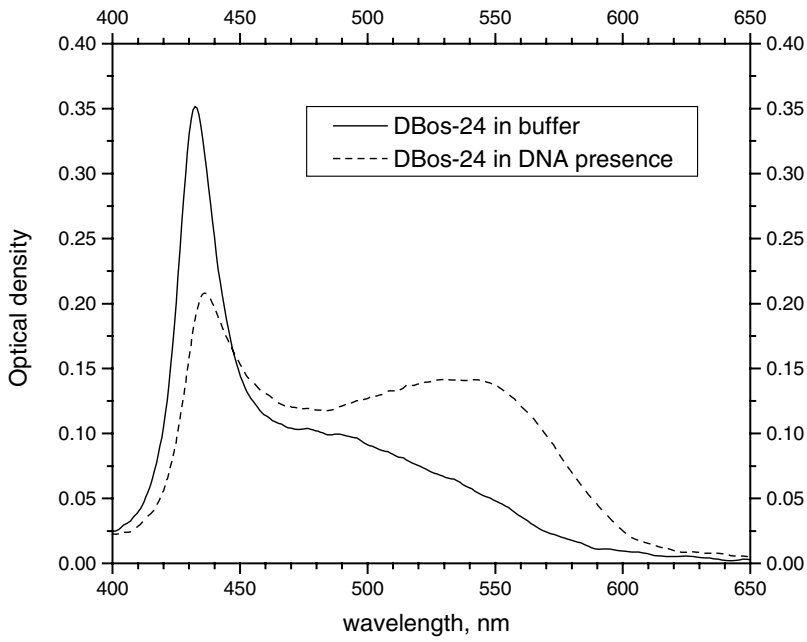


Fig. 2 Absorption spectra of DBos-24 in buffer solutions in the unbound state and in DNA presence

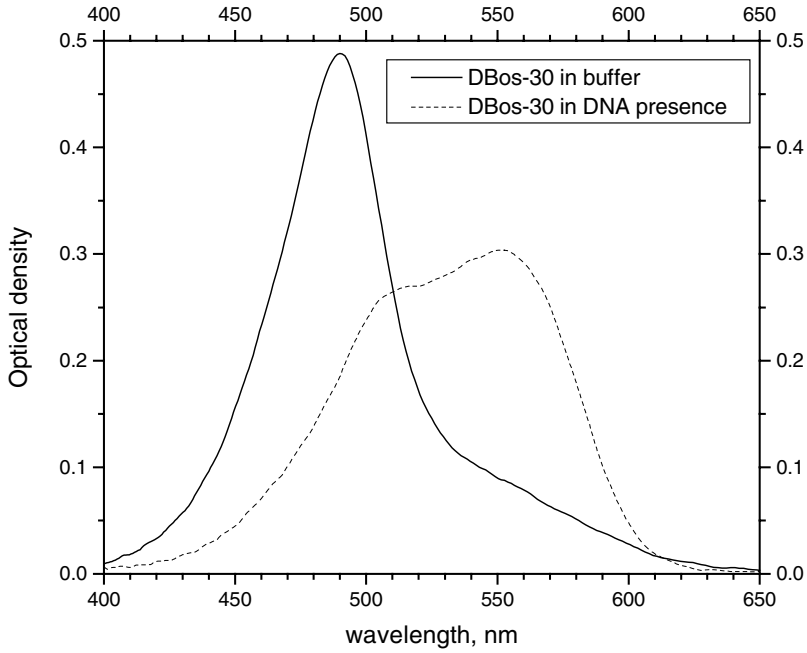


Fig. 3 Absorption spectra of DBos-30 in buffer solutions in the unbound state and in DNA presence

520 nm, while in the spectrum of DBos-24-DNA complex this band is situated at 437 nm.

As can be seen from Fig. 4, absorption bands of the monomer dye Bos-1 in both aqueous solution and organic solvent DMF have similar shape, close maxima (near 530 nm) and correspond to the absorption of the separate styryl dye chromophores. However, the shape of the absorption band of the homodimer styryls strongly changes going from water buffer to DMF that often takes place for the organic dyes forming aggregates in aqueous media [15]. Shortwave band with the maximum near 430 or 490 nm that is prevalent in spectra of unbound homodimers in buffer should belong to the H-aggregates of the styryl chromophores. At the same time, both the change from buffer to DMF and the addition of DNA to the buffer solution influence the homodimer absorption spectrum in the same way, leading to the decrease in the aggregate band intensity and increase in the separate chromophore one (Fig. 4). This should be explained by the partial destruction of the H-aggregates, that takes place both in organic solvent and at the fixation of separate chromophores on DNA [15].

For the dye DBos-21 the more detailed study was performed, that showed that the shape of its absorption spectrum stayed permanent while the solution was diluted down

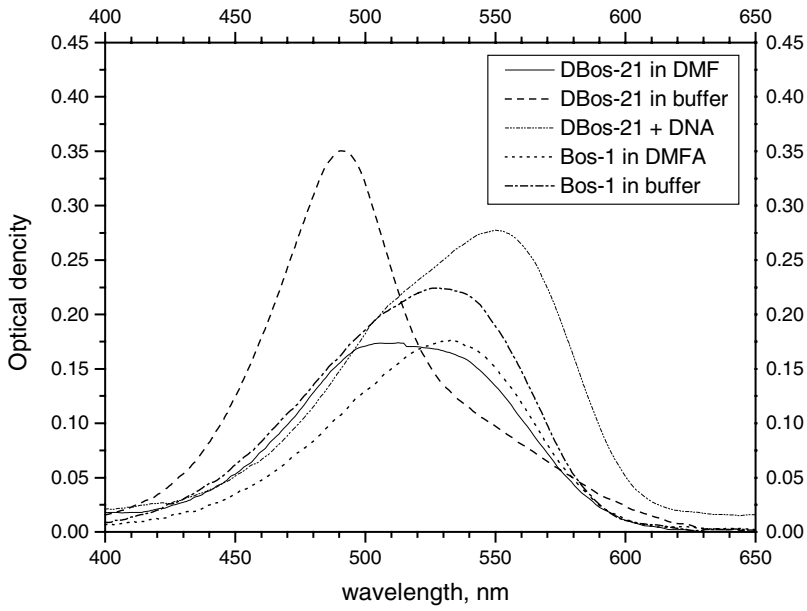


Fig. 4 Absorption spectra of homodimer styryl DBos-21 in DMF, buffer and in DNA presence; and of monomer styryl Bos-1 in DMF and buffer

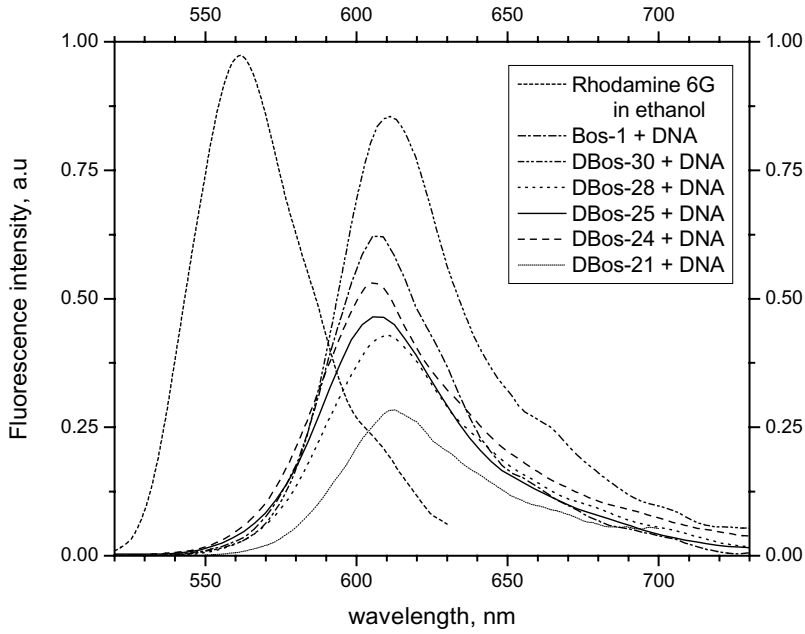


Fig. 5 Two-photon excited fluorescence spectra of styryl dyes in buffer in DNA presence and Rhodamine 6G in ethanol

to 2.5×10^{-7} M (until the optical density of the solution stayed high enough to be registered). Hence, the facts written above could evidence that the observed H-aggregates are the intramolecular dimers formed by the two chromophores of the same homodimer styryl dye molecule in the water buffer solution, while homodimer styryls binding with DNA destructs these aggregates. The temperature increase for the DBos-21 solution in buffer resulted in partial destruction of the aggregates beginning only from 80°C. Thus we could estimate that the binding energy of the intramolecular dimer overcomes 2.9 kJ/mol.

Two-photon excited fluorescence study

Since the studied styryl dyes demonstrate both strong fluorescent signal and sharp fluorescence intensity enhancement in DNA presence upon SPE, the fluorescence properties of the dyes solutions in DNA presence upon TPE were studied. As the result of the dyes-DNA complexes excitation at 1064 nm, efficient fluorescence was observed. Since the dyes-DNA buffer solutions have no single-photon absorption around 1064 nm, it can be concluded that dyes-DNA complexes efficiently fluoresce upon TPE.

The fluorescence spectra of the studied dyes in DNA presence induced by TPE are presented at Fig. 5. TPE-fluorescence maxima are situated between 607 and 609 nm and the shapes of SPE and TPE fluorescence spectra are close. Thus the emission centers are the same in case of both SPE and TPE fluorescence. The intensities of the SPE fluorescence spectra are higher for up to 35 times as compared to the TPE ones. The values of two-photon absorption cross section (δ) were calculated for the studied dyes complexes with DNA. The δ values are close for all the styryl dyes studied and are between 4.7×10^{-50} cm^{4}s and 7.4×10^{-50} cm^{4}s, being thus of the same order of magnitude as the respective value of Rhodamine 6G ethanol solution (see Table 2). These results demonstrate the possibility of using the studied dyes for the two-photon excited fluorescent detection and imaging of nucleic acids.

Conclusions

1. For the designing of fluorescent probes for DNA detection upon TPE, series of novel monomer and homodimer styryls based on (*p*-dimethylaminostyryl) benzothiazolium moiety and containing charged spermine-like linkage/tail groups were synthesized.
2. Spectral-luminescent and spectral-photometric properties of these dyes in the unbound state and in DNA complexes were studied. Homodimer as well as monomer styryls are low fluorescent in unbound state but give strong fluorescent response on DNA presence upon SPE experiment (emission intensity enhances up to three orders of magnitude for homodimer DBos-28). Using of spermine-like

Table 2 Two-photon absorption cross section (δ) of styryl dyes buffer solutions in DNA presence

Dye	δ, 10^{-50}cm^{4}s
Bos-1	7,4
DBos-21	5,5
DBos-24	4,7
DBos-25	5,8
DBos-28	7,0
DBos-30	6,0

linkage/tail groups for the designing of the dyes permitted to increase significantly emission intensity of the dye in DNA presence as well as the value of fluorescence intensity enhancement comparing with homodimers and monomers non-modified with charged group.

3. In aqueous buffer homodimer dyes form stable intramolecular aggregates that are present even at low dye concentrations and manifested as dominating short wavelength band in absorption spectra (near 490 nm or 430 nm). This aggregation process results in the decrease of intrinsic fluorescence of homodimers. In opposite to them, in the spectrum of monomer dye the single chromophore band prevails. Thus intrinsic emission intensity of monomer is considerably higher than that of homodimers.
4. The complexes of the studied dyes with DNA demonstrate intensive emission upon the TPE. The values of two-photon absorption cross sections of the studied dyes in DNA presence are in the range 4.7–7.4 $\times 10^{-50}$ cm^{4}s. Thus synthesized styryl dyes could be proposed as efficient fluorescent probes for DNA detection and imaging under both SPE and TPE.

Appendix

Data of H[1]NMR and CHN analysis for the synthesized dyes is presented below:

2-[2-(4-dimethylaminophenyl)-1-ethenyl]-3-{4-[6-[4-{2-[2-(4-dimethylaminophenyl)-1-ethenyl]-1,3-benzothiazol-3-ium-3-yl}butyl(dimethyl)ammonio]hexyl (dimethyl)ammonio]butyl}-1,3-benzothiazol-3-ium tetraiodide (DBos-30)

Yield: 79%; m. p.: 198–200°C; ^{1}H NMR (DMSO-d$_6$) δ(ppm): 1.39(4H, m), 1.82(8H, m), 2.08(4H, m), 3.03(12H, s), 3.09(12H, s), 3.42(8H, m), 4.87(4H, t, $J=7.5$), 6.76(4H, d, $J=9.3$), 7.61(2H, d, $J=14.9$), 7.70(2H, t, $J=7.7$), 7.79(2H, t, $J=7.5$), 8.02(6H, m), 8.25(2H, d, $J=7.8$), 8.33(2H, d, $J=8.1$). Anal. calcd. for. $C_{52}H_{72}I_4N_6S_2$: C, 46.16; H, 5.36; N, 6.21. Found: C, 46.25; H, 5.39; N, 6.15.

2-[2-(4-dimethylaminophenyl)-1-ethenyl]-3-{4-[3-[4-{2-[2-(4-dimethylaminophenyl)-1-ethenyl]-1,3-benzothiazol-3-ium-3-yl}butyl(dimethyl)ammonio]propyl (dimethyl)ammonio]butyl}-1,3-benzothiazol-3-ium tetraiodide (DBos -28)

Yield: 62%; m. p.: 232–234°C; ^{1}H NMR (DMSO-d$_6$) δ(ppm): 1.86(4H, m), 2.17(4H, m), 2.31(2H, m), 2.97(12H, s), 3.19(12H, s), 3.43(4H, m), 3.56(4H, m), 4.79(4H, t, $J=8.4$), 6.64(4H, d, $J=8.4$), 7.50(2H, d, $J=15.3$), 7.68(2H, t, $J=7.5$), 7.75(2H, t, $J=7.5$), 7.95(6H, m), 8.19(2H, d, $J=8.0$), 8.28(2H, d, $J=7.8$). Anal. calcd. for. $C_{50}H_{64}I_2N_4$: C, 44.90; H, 5.07; N, 6.41. Found: C, 44.85; H, 5.01; N, 6.49.

2-[2-(4-dimethylaminophenyl)-1-ethenyl]-3-(3-triethylammoniopropyl)-1,3-benzothiazol-3-ium dibromide (Bos-1)

Yield: 64%; m. p. (dec.): 261–263°C; ^{1}H NMR (DMSO-d$_6$) δ(ppm): 1.25(9H, t br), 2.17(2H, m), 3.13(6H, s), 3.29(6H, q br), 3.64(2H, m), 6.85(2H, d, $J=8.0$), 7.70-7.87(3H, m), 8.05(2H, d, $J=8.4$), 8.16(1H, d, $J=15.6$), 8.33(2H, m). Anal. calcd. for. $C_{26}H_{37}Br_2N_3S$: C, 53.52; H, 6.39; N, 7.20. Found: C, 53.50; H, 6.37; N, 7.31.

2-[2-(4-dimethylaminophenyl)-1-ethenyl]-3-{4-[2-[4-{2-[2-(4-dimethylaminophenyl)-1-ethenyl]-1,3-benzothiazol-3-ium-3-yl}butyl(dimethyl)ammonio]ethyl (dimethyl)ammonio]butyl}-1,3-benzothiazol-3-ium tetraiodide (DBos-25)

Yield: 69%; m. p.: 125–127°C; ^{1}H NMR (DMSO-d$_6$) δ(ppm): 1.88(4H, m), 2.18(4H, m), 3.07(12H, s), 3.25(12H, s), 3.57(4H, m), 4.04(4H, s), 4.87(4H, t, $J=7.0$), 6.77(4H, d, $J=9.3$), 7.61(2H, d, $J=14.6$), 7.70(2H, t, $J=7.8$), 7.79(2H, t, $J=8.0$), 8.05(6H, m), 8.28(2H, d, $J=8.7$), 8.33(2H, d, $J=8.0$). Anal. calcd. for. $C_{48}H_{64}I_4N_6S_2$: C, 44.46; H, 4.97; N, 6.48. Found: C, 44.53; H, 5.01; N, 6.39.

2-[2-(4-dimethylaminophenyl)-1-ethenyl]-3-{4-[4-(4-{2-[2-(4-dimethylaminophenyl)-1-ethenyl]-1,3-benzothiazol-3-ium-3-yl}butyl)-1,4-diazoniabicyclo[2.2.2]oct-1-yl]butyl}-1, 3-benzothiazol-3-ium tetraiodide (DBos-24)

Yield: 67%; m. p.: 234–236°C; ^{1}H NMR (DMSO-d6) δ(ppm): 1.89(4H, m), 2.01(4H, m), 3.15(12H, s), 3.63(4H, m), 3.91(12H, s), 4.87(4H, t, $J=6.5$), 6.87(4H, d, $J=9.3$), 7.63(2H, d, $J=15.3$), 7.72(2H, t, $J=7.5$), 7.82(2H, t, $J=7.8$), 8.00(4H, d, $J=9.3$), 8.16(2H, d, $J=15.3$), 8.23(2H, d, $J=7.5$), 8.36(2H, d, $J=8.0$). Anal. calcd. for. $C_{48}H_{60}I_4N_6S_2$: C, 44.60; H, 4.68; N, 6.50. Found: C, 44.52; H, 4.65; N, 6.57.

2-[2-(4-dimethylaminophenyl)-1-ethenyl]-3-{4-[4-[4-{2-[2-(4-dimethylaminophenyl)-1-ethenyl]-1,3-benzothiazol-3-ium-3-yl}butyl(dimethyl)ammonio] butyl(dimethyl) ammonio]butyl}-1,3-benzothiazol-3-ium tetraiodide (DBos-21)

Yield: 76%; m. p. (dec.): 242–244°C; ^{1}H NMR (DMSO-d$_6$) δ(ppm): 1.83(8H, m), 2.12(4H, m), 3.06(12H, s), 3.09(12H, s), 3.42(8H, m), 4.87(4H, t, $J=7.1$), 6.78(4H, d, $J=9.3$), 7.62(2H, d, $J=15.0$), 7.69(2H, t, $J=7.5$), 7.78(2H, t, $J=7.6$), 7.98-8.11(6H, m), 8.27(2H, d, $J=7.8$), 8.32(2H, d, $J=7.7$). Anal. calcd. for. $C_{50}H_{68}I_4N_6S_2$: C, 45.33; H, 5.17; N, 6.34. Found: C, 45.25; H, 5.19; N, 6.29.

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