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ORIGINAL PAPER

Styryl Dyes as Two-Photon Excited Fluorescent Probes for DNA Detection and Two-Photon Laser Scanning Fluorescence Microscopy of Living Cells

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Abstract Spectral-fluorescent properties of benzothiazole styryl monomer (Bos-3) and homodimer (DBos-21) dyes in presence of DNA were studied. The dyes enhance their fluorescence intensity in 2–3 orders of magnitude upon interaction with DNA. Studied styrylcyanines in DNA presence demonstrate rather high values of two-photon absorption (TPA) cross-section, which are comparable with the values of TPA cross section of the rhodamine dyes. An applicability of the styrylcyanines as probes for the fluorescence microscopy of living cells was studied. It was shown that both dyes are cell-permeable but homodimer dye DBos-21 produces noticeably brighter staining of HeLa cells comparing with monomer dye Bos-3. Molecules of DBos-21 initially bind to the nucleic acids-containing cell organelles (presumable mitochondria) and are able to penetrate into the cell nucleus. Thus, homodimer styryl DBos-21 dye is viewed as efficient stain for single-photon and two-photon excitation fluorescence imaging of living cells.

Keywords DNA detection · Fluorescent probes · Two-photon excitation · Fluorescence microscopy

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Introduction

Fluorescent imaging of living cells is a powerful tool in modern biology studies. Use of cell-permeable probes strongly enhancing fluorescence intensity while binding with target biomolecules permits to obtain fluorescent image of certain organelles (e.g. nucleus) [1]. Two-photon laser scanning microscopy, where fluorescence is excited by the two-photon excitation (TPE), is known as one of the most powerful tools for cell and tissue imaging. This method allows deeper penetration of exciting light beam into the tissue; excitation of visual fluorescence in near infrared spectral region, where the biological objects are transparent; decreased photodamage of the studied object; and obtaining of three-dimensional image of biological object [2]. Besides an ability to selectively bind with target biomolecules with increase in the inherent fluorescence, fluorescent probes in TPE fluorescence microscopy should also demonstrate high enough values of two-photon absorption cross-section (δ_{TPA}). Despite the fact that determination of the δ_{TPA} values of different type molecules for various applications has been extensively reported [3–14], only few works describe the TPE efficient probes for fluorescence detection and imaging of biological molecules [15–17].

Earlier we have proposed styrylcyanine dyes as efficient probes for TPE fluorescence detection of DNA in solution

[2, 18, 19]. Series of monomer and homodimer styryl dyes demonstrated considerable fluorescence intensity enhancement upon binding to DNA as well as high fluorescence quantum yield in DNA presence [20–23]. In addition to that monomer and homodimer benzothiazole styryls in presence of DNA demonstrate intense TPE fluorescence, their two-photon absorption cross-section values were estimated to be rather high [21]. The data on δ_{TPA} at 1064 nm for series of benzothiazole, naphtathiazole, benzoimidazole, pyridinium, quinoline and 4-oxo-thieno[2,3-d]pyrimidinium styryl dyes have been reported in [24].

In the work presented here, previously described homodimer benzothiazole styryl DBos-21 [21] as well as the newly synthesized monomer benzothiazole styryl dye containing spermine-like tail group Bos-3 (Fig. 1) were investigated. Absorption and both single-photon excitation (SPE) and TPE fluorescence spectra in DNA presence were obtained, and δ_{TPA} values at 880 nm and 1064 nm were determined. A possible application of DBos-21 homodimer dye in the TPE fluorescence microscopy of living cells was shown previously [24]. Here, the properties of homodimer DBos-21 as TPE fluorescence probe for the cell staining were studied and compared with monomer analogue Bos-3.

Experimental

Materials and synthesis of dyes

The total DNA from chicken erythrocytes was purchased from Sigma. A mitochondria specific fluorescent dye, MitoTracker Green, was obtained from Invitrogen. Dye DBos-21 (Fig. 1) was synthesized as described in [21]. The synthesis of 2-[2-[4-(dimethylamino)phenyl]ethenyl]-3-[4-[dimethyl[6-(trimethylammonio)hexyl]ammonio]butyl]-benzothiazol-3-ium triiodide (Bos-3) was performed as follows (Scheme 1). To the solution of 0.295 g (0.0005mol) of SI-1 [21] in 4 ml of dimethylformamide 0.0005 mol of N,N,N-trimethyl- 6-dimethylamino-1-hexanaminium iodide was added; the obtained mixture was heated during 10 h on boiling water bath. Reaction mixture was evaporated under low pressure and crystallized from methanol. Yeld 72% m.p. 259–265 °C. ^{1}H NMR (DMSO-d_6) δ(ppm): 1.36 (2H,m), 1.72(4H,m), 1.86(2H,m), 2.01(2H,m), 3.06(6H,s), 3.09(9H,s), 3.13(6H,s), 3.35(10H,m), 4.86(2H,t,J=7.0), 6.85 (2H,d,J=8.8), 7.62(1H,d,J=14.2), 7.7(1H,t,J=7.5), 7,8(1H,t, J=7.6), 8.01(2H,d,J=8.8), 8.12(1H,d,J=14.3), 8.22(1H,d, J=7.7), 8.35(1H,d,J=7.6). Anal. calcd. for. $C_{32}H_{51}N_4SI_3$: C, 42.49; H, 5.68; N, 6.19. Found: C, 42.39; H, 5.71; N, 6.24.

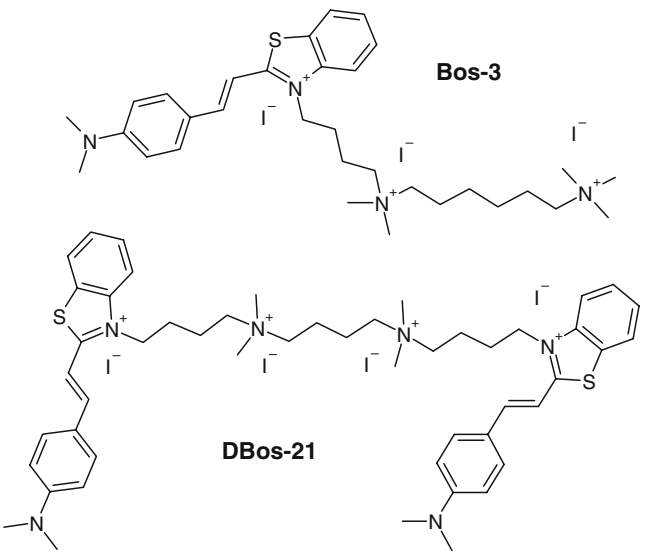


Fig. 1 Chemical structures of the studied dyes

Samples preparation

The dye stock solutions with concentration of 2×10^{-3} M were prepared in dimethylformamide (DMF). DNA stock solution (6×10^{-3} M base pairs, b.p.) was prepared in 0.05 M TRIS-HCl buffer, pH 8.0. Samples for spectroscopic measurements were prepared by dissolving corresponding stock solutions in 0.05 M TRIS-HCl buffer, pH 8.0. Concentrations of dyes and DNA were 5×10^{-6} M and 6×10^{-5} M b.p., correspondingly, in the SPE experiments and $1{,}5\times10^{-5}$ M and $1{,}8\times10^{-4}$ M b.p. in the TPE experiments. Concentrations given for dimer dye is per monomer chromophore.

Spectral measurements and calculation of δ_{TPA} values

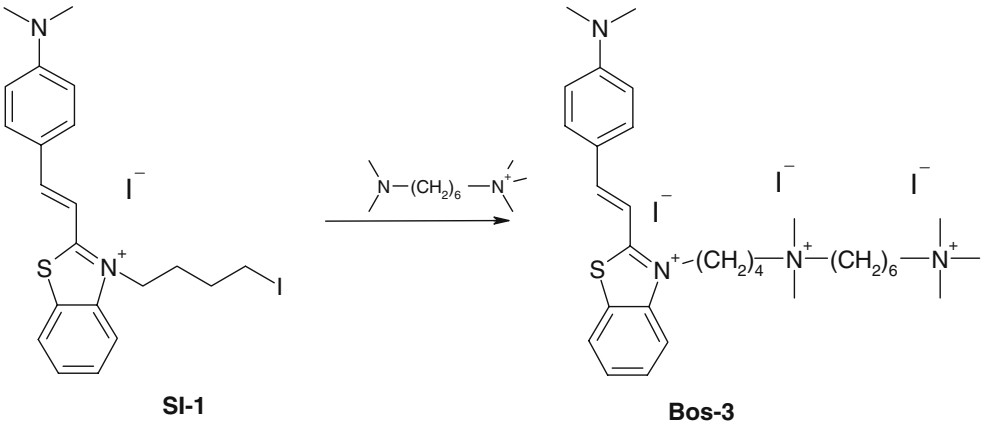
All the SPE and TPE measurements were carried out at room temperature.

Absorption spectra were recorded using a Specord M 40 spectrophotometer (Carl Zeiss, Germany). Fluorescence excitation and emission spectra upon SPE were obtained on a Cary Eclipse fluorescence spectrophotometer (Varian, Australia)

TPE fluorescence measurements of the studied dyes in presence of DNA and without it were performed using the 1064 nm irradiation of the 20 ns pulsed YAG: Nd^{3+} laser (laboratory designed using the Sagnac ring interferometer scheme) as the excitation source. Experimental setup and method of determination of the two-photon absorption cross-sections were the same as described in [21]. The values of δ_{TPA} of the dyes at 1064 nm were calculated using the known value for the Rhodamine 6G solution in ethanol to be equal to 5.5×10^{-50} cm^4s [4].

TPE fluorescence spectra of the dyes were also acquired upon the excitation at 880 nm. These measurements were performed using a Ti:Sapphire laser (Mira Optima 900-F, Coherent) pumped up with the Nd:YVO_4 10-W 532-nm CW laser (Verdi V10, Coherent) and generating 90 fs $cosec^2$-

Scheme 1 Synthesis of monomer styryl dye Bos-3



shaped pulses with repetition rate 76 MHz. Parameters of laser were monitored by autocorrelator (APE Autocorrelator mini) and power meter (Field Master GS, LM-10, Coherent). Fluorescence emission was detected at right angle as it passed through the telecentric lens system and the entrance slit (100 μ) of the spectrograph (500 mm Imaging Spectrograph SP-2558, Acton). A CCD camera (CCD-Spec-10:256E/TEPLUS 1024×256 Open-electrode, Marconi CCD 30-11) was used as a detector of TPE-fluorescence.

The values of δ_{TPA} at 880 nm were evaluated as it is described in [3]. The time-averaged fluorescence photon fluxes <F(t)> from the studied dyes and from the Rhodamine B solution in ethanol were measured, dye concentrations were equal. For the Rhodamine B in ethanol, δ_{TPA} is equal to 40×10^{-50} cm^4s [3]. TPA cross-section values of the dyes were determined using the following expression:

$$\delta_{TPA(x)} = \delta_{TPA(RhB)} \times \frac{\langle F(t)\rangle_x}{\langle F(t)\rangle_{RhB}} \times \frac{\varphi_{RhB}}{\varphi_x},$$

φ_x and φ_{RhB} are the values of the fluorescence quantum yield of the studied dye in DNA presence and Rhodamine B, respectively.

Uptake of the dyes by living cells: imaging with single- and two-photon laser scanning fluorescence microscopy

HeLa (human cervix epitheloid carcinoma) cells (American Type Culture Collection, Manassas, VA) were cultured in a Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS), following the established protocol. For studying dye uptake and imaging, the cells were trypsinized and re-suspended in a MEM alpha medium with 10% fetal bovine serum (FBS) at a concentration of 7.5×10^5 cells/ml and plated in 35 mm culture plates, using 2.5 ml of the medium containing 0.10 ml of the cell suspension. The plates were incubated overnight at 37 °C with 5% CO_2. Next day, the cells (50% confluency) were carefully rinsed with phosphate buffered saline (PBS),

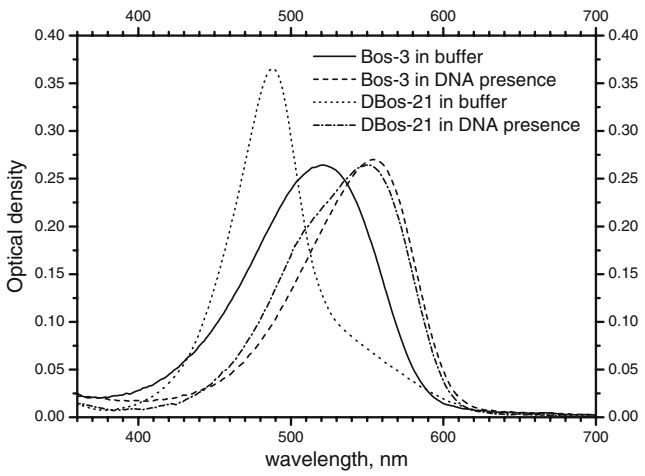


Fig. 2 Absorption spectra of free dyes and dyes in DNA presence in 0.05 M TRIS-HCl buffer, pH 8.0. Dye and DNA concentrations were 5×10^{-6} M and 6×10^{-5} M b.p. respectively

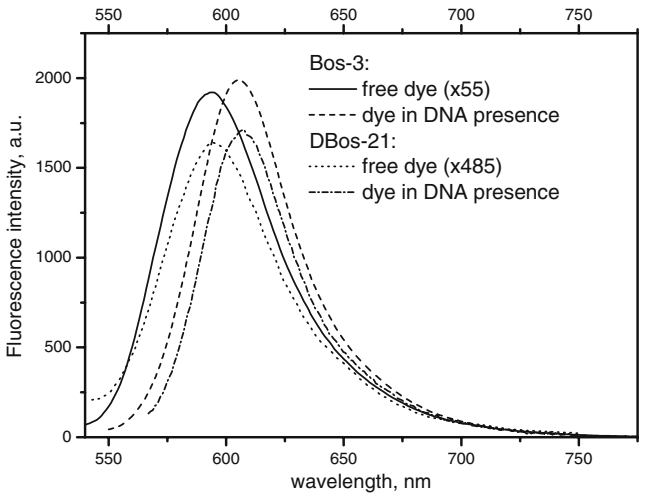


Fig. 3 SPE fluorescence spectra of free dyes and dyes in presence of DNA in 0.05 M TRIS-HCl buffer, pH 8.0. Dye and DNA concentrations were 5×10^{-6} M and 6×10^{-5} M b.p. respectively

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

Dye	Free dye				Dye in DNA presence				I/I_0
	λ_{abs}, nm	λ_{ex}, nm	λ_{em}, nm	I_0, a.u.	λ_{abs}, nm	λ_{ex}, nm	λ_{em}, nm	I, a.u.	
Bos-3	520	538	593	35	554	562	605	1989	56.8
DBos-21	487	533	595	3.5	547[a] 514	555	607	1727	488

λ_{abs}, λ_{ex}, λ_{em}—absorption, fluorescence excitation and emission maxima wavelengths (nm); I_0—fluorescence intensity of free dyes at maximum wavelength (a.u.); I—fluorescence intensity of dye-DNA complexes at maximum wavelength (a.u.)

[a] Higher intensity band

and 2.5 ml of the medium containing 1 or 5 μM of the dyes was added to plate. The treated cells were returned to the incubator and incubated for 1 h. Next, the plates were rinsed with sterile PBS, and fresh media added. The cells were then directly imaged using two-photon laser scanning microscopy (TPLSM) or confocal laser scanning microscopy (CLSM). The experimental setup and protocols used for TPLSM and CLSM are described elsewhere [25–27]. Two-photon excitation in TPLSM was performed at 880 nm using a Ti-sapphire laser (Tsunami, Spectra-Physics) pumped by a frequency-doubled diode-pumped solid-state laser (Millennia, Spectra-Physics). It provided 90 fs pulses at 82 MHz repetition rate. A spectrum analyzer (IST-REES, Germany) was used to monitor the wavelength and the bandwidth of excitation light. Confocal laser scanning microscope (Bio-Rad, model MRC-1024) with an upright microscope (Nikon, model Eclipse E800) was employed to obtain images; an 880 nm laser beam was introduced to the upper port of the MRC-1024. In case of single-photon excitation of the fluorescence in CLSM, excitation light (488 or 514 nm lines) from the Argon ion laser (Beamlock, Spectra Physics) was coupled to the upper port of the confocal microscope with single mode optical fiber. A water immersion objective (Nikon, Fluor-60X, NA 1.0) was utilized for cell imaging.

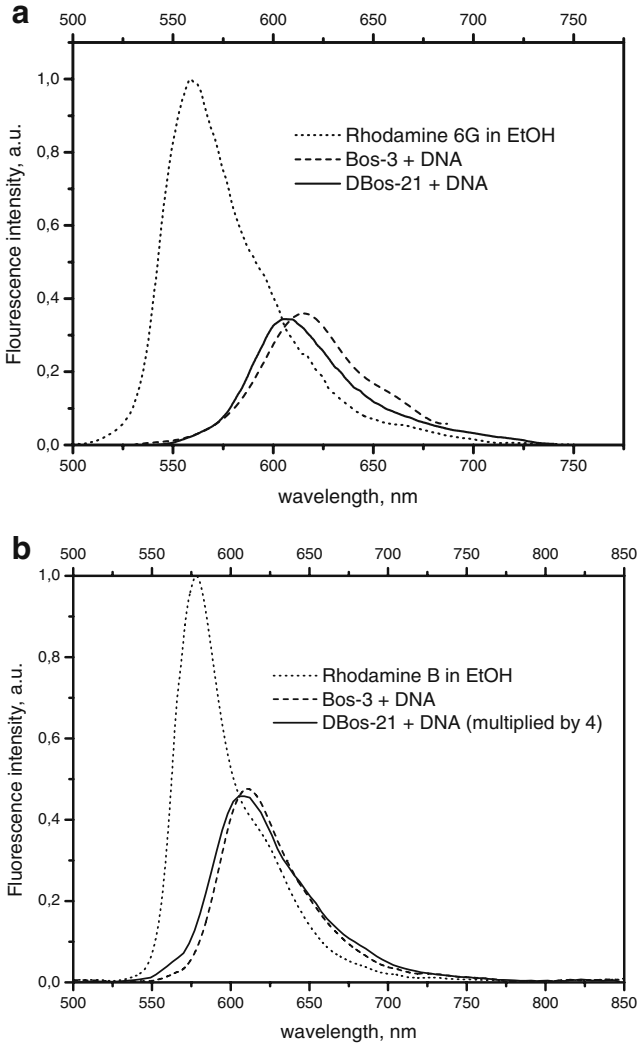


Fig. 4 TPE fluorescence spectra of dyes in DNA presence in 0.05 M TRIS-HCl buffer, pH 8.0. Dye and DNA concentrations were 1.5×10^{-6} M and 1.8×10^{-4} M b.p. respectively. Excitation with 1064 nm from YAG: Nd^{3+} 20 ns pulsed laser (**a**) and 880 nm from Ti:sapphire 90 fs pulsed laser (**b**). Samples of rhodamine dyes in ethanol were used as a reference

Results and discussion

Absorption and SPE fluorescence of free dyes and dye-DNA complexes

Absorption and SPE fluorescence spectra of free DBos-21 and Bos-3 as well as dyes bound with DNA are shown in Figs. 2, 3 and spectral characteristics are recapitulated in Table 1. The shape and maxima positions of Bos-3 spectra show that this dye in aqueous solution exists mainly in the

Table 2 TPA cross sections of Bos-3 and DBos-21 in DNA presence

Dye	TPA cross section δ_{TPA}, $10^{-50}cm^4s$	
	λ_{ex}=1064nm	λ_{ex}=880nm
Bos-3	2.1	68
DBos-21	1.7	24

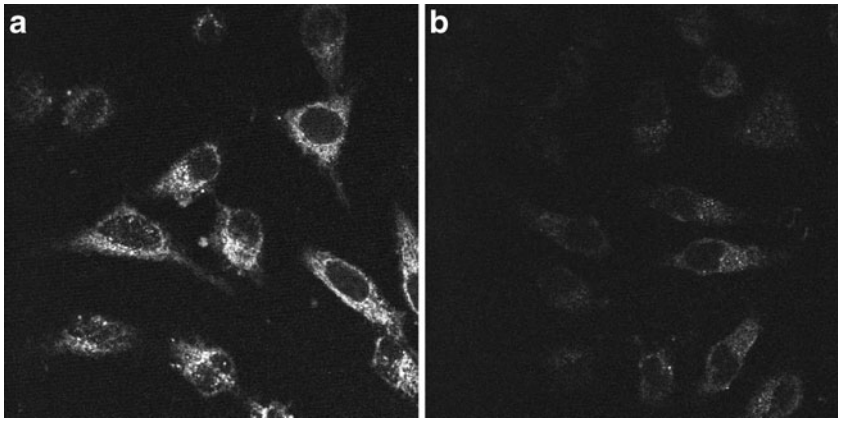


Fig. 5 TPE fluorescence laser scanning microscopy images of HeLa cells stained with 5 μM of DBos-21 (**a**) and Bos-3 (**b**) and incubated for 1 h. Concentration given for dimer is per monomer chromophore. Fluorescence was excited by 880 nm light from the femtosecond pulsed Ti-Sapphire laser

monomeric form, both in absence and presence of DNA [21]. At the same time, main peak of the absorption spectrum for the homodimer dye DBos-21 in the unbound state is strongly blue-shifted as compared to the monomeric Bos-3. Besides that, the fluorescence intensity for DBos-21 in unbound state is for an order of magnitude less as compared to Bos-3. These spectral peculiarities of the homodimer dye DBos-21 are explained by formation of the non-fluorescent aggregates in aqueous medium while dye interaction with DNA leads to the destruction of the aggregates [21]. Thus, the dominating band in the absorption spectrum of free DBos-21 belongs to aggregates, while fluorescence corresponds to the small portion of the non-aggregated DBos-21. At the same time, absorption and fluorescence spectra of dye in the presence of DNA are associated with non-aggregated dye molecules fixed on DNA. It is worth noting a 12–24 nm red spectral shift for fluorescence excitation and emission of dyes when bound with DNA as compared to the free dyes; similar shift is usually associated with dye-DNA complexation [20, 22].

TPE fluorescence of dye-DNA complexes

TPE fluorescence spectra of the dyes in DNA presence were acquired under excitation at 1064 nm and 880 nm (Fig. 4a and b respectively), and corresponding values of TPA cross-section were calculated (Table 2). TPE fluorescence spectra shape and maxima positions are close to those obtained under SPE (Fig. 3), hence both SPE and TPE fluorescence spectra correspond to the emission of the dye monomers complexed with DNA.

It is important to note that TPE fluorescence of DBos-21 is about of same intensity as that of Bos-3 when excited at 1064 nm, but four times less intense when excited with 880 nm (Fig. 4). Since SPE fluorescence is about the same for both dyes, the reason for difference in TPE fluorescence intensity is in difference in TPA cross-sections for both dyes. As one can see in the Table 2, TPE cross section at 880 nm is almost three times higher for Bos-3 than for DBos-21. In addition, the δ_{TPA} values at 880 nm are order of magnitude higher for both dyes than the corresponding values at 1064 nm. At the same time as it is seen in Fig. 2, absorption of both dyes in the DNA presence is much higher at 532 nm than at 440 nm. A possible explanation of such a discrepancy could be the fact that due to the selection rules two-photon transitions generally occur to the higher levels as compared to the single-photon ones [6]. Higher vibrational levels of the first excited singlet electronic level as well as higher excited singlet electronic levels could be allowed for the two-photon absorption

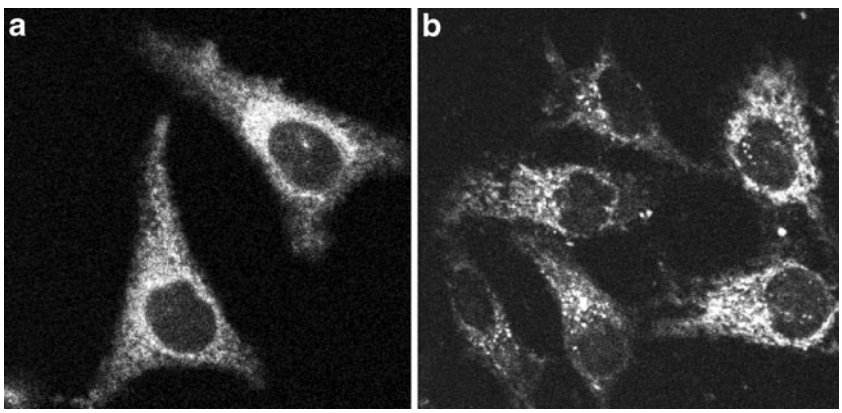


Fig. 6 SPE fluorescence confocal laser scanning microscopy images of HeLa cells stained with 1 μL of MTG (**a**) and TPE fluorescence laser scanning microscopy images of HeLa cells stained with 5 μL DBos-21 (**b**). Incubation with dyes for 1 h, fluorescence was excited with 488 nm to image MTG (**a**) and with 880 nm laser line for DBos-21 imaging (**b**)

transition [9, 10]. Thus, a spectral difference between maxima in single and two-photon absorption spectra could be rather significant, as it was shown for polymethine dyes [9, 10]. It should be also mentioned that δ_{TPA} values at 880 nm and 1064 nm were obtained using respectively femto- and nanosecond laser excitation, and were calculated basing on the reference values obtained by different research groups for different dyes.

Two-photon excited fluorescence imaging of the cells with Bos-3 and DBos-21

In order to check a possibility of using the developed dyes as probes for the TPE fluorescence imaging of living cells in vitro, HeLa cells were stained with Bos-3 and DBos-21 dyes. TPE fluorescence laser scanning microscopy images of the stained cells are presented in Fig. 5.

As can be seen in Fig. 5 both Bos-3 and DBos-21 are able to penetrate through cell membrane and stain the cells. Nevertheless, both dyes do not practically stain the cell nuclei and apparently produce staining of cell organelles in cytoplasm. TPE fluorescence images of homodimer dye DBos-21 are seen to be significantly brighter as compared to those of Bos-3. Keeping in mind four time less intense TPE fluorescence of DBos-21 as compared to Bos-3 under excitation at 880 nm (Fig. 4b), difference between two dyes in cellular uptake or efficacy of intracellular binding (which determines fluorescence enhancement) is really high. We believe that both possibilities can be considered: 1) much higher percentage of the homodimers bind with extranuclear nucleic acids, as compared with monomers; 2) much better penetration of the dimer molecules through cellular membrane; it can be also associated with aggregation of dimer molecules in solution, i.e. aggregation of DBos-21 may promote penetration of dye through cellular membrane. Additional studies are necessary to clarify this issue.

Figure 6 shows TPLSM image of DBos-21 in HeLa cells (B) in a comparison with CLSM image of HeLa cells stained with MTG, which is known to selectively stain mitochondria. As one can see, staining patterns are quite similar, supposing predominantly mitochondrial localization of DBos-21.

To further clarify behavior of DBos-21 within cells, we have performed co-staining of HeLa cells with DBos-21 and MTG and imaged cells using CLSM. As can be seen in Fig.7, a supplementing of the MTG probe into cell plate 20 min after starting treatment with DBos-21 results in a change in the intracellular localization of DBos-21; particularly, it is clearly showing nucleoli staining. In our opinion, MTG taken up by cell competes with DBos-21 when binding with mitochondria and, as a result, DBos-21 is being pushed towards cytoplasm and nucleoli, where it can bind with RNA. This effect can be considered as an evidence of the specificity of DBos-21 towards mitochondria.

Conclusions

1. Studied monomer and homodimer styrylcyanine dyes interact with DNA with significant increase in emission intensity. While the intrinsic emission of monomer Bos-3 is 10 times higher than that of homodimer dye DBos-21, both dyes demonstrate about the same emission intensities in DNA presence.
2. While the values of TPA cross sections at 1064 nm are close for both dyes in complexes with dsDNA ($2{,}1\times 10^{-50}\ cm^4 s$ for Bos-3 and $1{,}7\times 10^{-50}\ cm^4 s$ for Dbos-21), TPA cross sections at 880 nm are considerably different ($68\times 10^{-50}\ cm^4 s$ for Bos-3 and $24\times 10^{-50}\ cm^4 s$

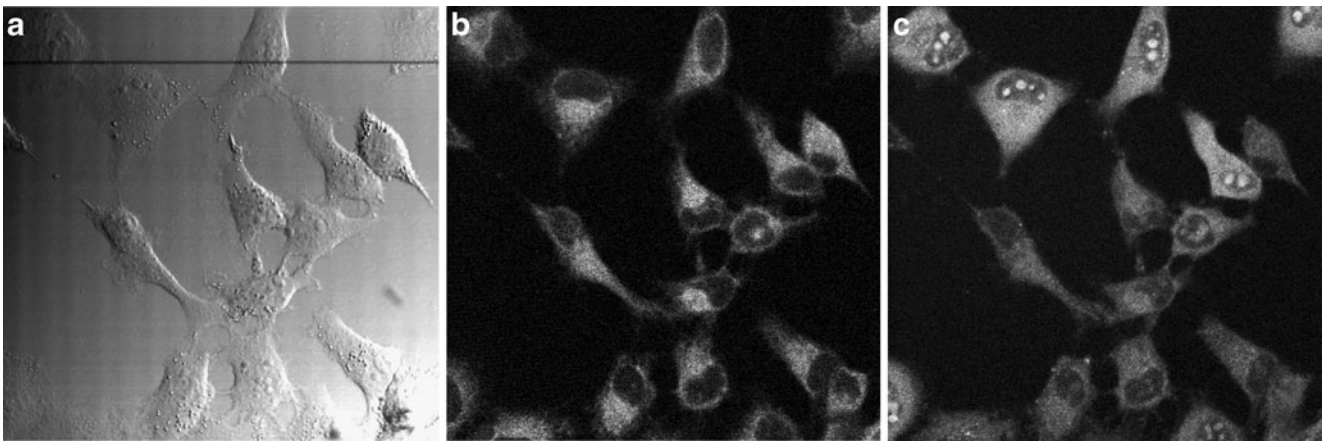


Fig. 7 HeLa cells stained with DBos-21 and with MTG added in 20 min after DBos-21: (**a**) transmission; (**b**) MTG fluorescence image; (C) DBos-21 fluorescence image. The fluorescence of MTG and DBos-21 was excited at 488 and 514 nm respectively and observed through the filters transparent at the range 507–537 nm and at more than 585 nm respectively

for DBos-21). Obtained values of TPA cross sections for styrylcyanines in DNA presence both at 1064 and 880 nm are of the same order as those for Rhodamine dyes.

3. Styrylcyanine dyes were shown to penetrate into the living cells, apparently displaying cytoplasmic staining of nucleic acid containing organelles. TPE fluorescence images of living cells stained with homodimer dye DBos-21 are significantly brighter as compared to images of cells treated with monomer dye Bos-3. Co-staining cells with DBos-21 and the mitichondria specific fluorescent probe allows us to suggest that DBos-21 initially binds to cell mitochondria. We propose homodimer styryclcyanine DBos-21 as efficient probe for TPE fluorescence cell-imaging applications.

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