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The photostability and phototoxic influence on DNA of several styryl dyes are investigated by studying the absorption, fluorescence and phosphorescence spectra of these dyes and DNA + dye systems. The dyes Bos-1, DBos-24, and DBos-30 exhibit

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a rather intensive fluorescence under the two-photon excitation. Changes of the optical density in the wavelength regions 250 ÷ 300 nm (DNA absorption) and 370 ÷ 650 nm (dye absorption) of DNA + dye solutions caused by the visible light irradiation are fixed. Bos-1 and DBos-30 dyes bound to DNA are more photostable than those in free state, being photochemically safe for DNA. DBos-24 and Dst-MdO dyes show a slight phototoxic effect on DNA.

Keywords: DNA; luminescent dyes; photostability; phototoxicity; two-photon excitation

INTRODUCTION

The detection of deoxyribonucleic acid (DNA) is an important problem in biophysics and medicine. This problem is often solved with the help of small luminescent molecular probes that can interact with DNA [1]. Such molecules bind to DNA by noncovalent interactions, being fixed either between the DNA nucleotides (intercalation) or between the sugar-phosphate chains (groove-binding) [2]. The molecule fixation on DNA for the majority of small molecules used as the luminescent probes for the DNA detection (often such probes are organic dyes) results in a strong increase of the dye luminescence intensity [3]. Recently, two-photon excitation (TPE) of the probes fluorescence by near IR radiation was proposed. The use of the two-photon excitation of fluorescent probes in the detection procedures of biological objects allows a deeper penetration of the exciting beam into a tissue and the excitation of visual fluorescence in the near infrared spectral region, where the biological objects are transparent; decreases a photodamage of the studied object; and allows one to obtain the three-dimensional image of a biological object. Hence, the development of fluorescent probes with a high cross-section of two-photon excitation, as well as with a low efficiency of the probe-induced damage of biomolecules, is an important task. At the same time, it is important that the luminescent probes applied to the study of living cells (e.g., with the fluorescent microscopy) have not to be toxic or phototoxic. In addition, the probe should be photostable in order that a biomolecule could be studied for enough a long period of time without damage of the probe.

It is known that the damage of a biological object can be induced by the direct excitation of a luminescent probe that contacts with this object. The phototoxic influence of the dye molecule on DNA can take place either directly via the excitation energy transfer from the dye to the DNA nucleotide bases or indirectly via the third molecule (e.g., by the triplet excitation energy transfer to an oxygen molecule resulting in the generation of toxic singlet oxygen [4,5]). The absorption bands

(connected with the first electronic transition) of DNA, RNA, and nucleotide bases are located in the near UV spectral region with maxima near 260 nm. At the same time, the corresponding absorption bands of the majority of dyes used as luminescent probes are located in the visual spectral region (more than 400 nm) [1]. The fact that the first excited singlet and triplet energy levels of dyes are situated essentially lower than the corresponding levels of any nucleotides (the DNA links) does not allow the excitation energy transfer from the dye to DNA, thus making impossible the direct phototoxic influence of the dye on DNA. Nevertheless, the small molecules (mostly porphyrins) that destroy DNA indirectly by generating the singlet oxygen are well known and used in the photodynamic therapy.

Recently, it was shown by us [6] that the fluorescence intensity of the benzothiazole styryl dyes increases by 2–3 orders of magnitude, when they are bound to DNA. In addition, these dyes demonstrate the sufficient TPE efficiency [7]. In this article, the results of investigations of the phototoxicity and photostability of several styryl dyes that could be used as the DNA probes will be studied. The influence of the irradiation of the dyes bound to DNA on the absorption spectra of dyes and DNA could characterize, respectively, the photostability and phototoxicity of these dyes.

EXPERIMENTAL

The total DNA from chicken erythrocytes was purchased from Sigma. Dyes Bos-1 and Dst-MdO (Fig. 1) were obtained by the boiling of

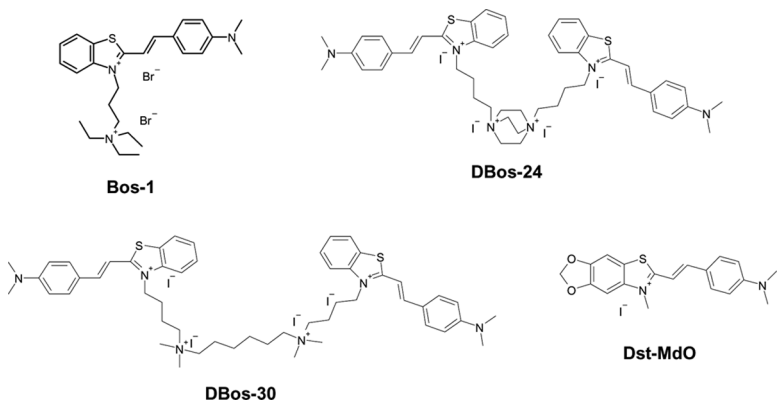

FIGURE 1 Chemical structures of monomer dyes Bos-1 and Dst-MdO, and dimer dyes Dbos-24 and Dbos-30.

quaternary salt and *p*-dimethylaminobenzaldehyde in acetic anhydride as described in [8]. Dyes Dbos-24 and Dbos-30 (Fig. 1) were obtained similarly to the TOTO dye [9] by the reaction of iodalkyl derivative of dimethylaminostyrylbenzothiazolium with the corresponding diamines by the long-term heating in DMF at 90°C. The structure of the dyes was confirmed with H^1 NMR and element analysis.

The samples were prepared in distilled water and 0.05 M TRIS-HCl buffer, pH 7.5. The concentrations of the dye and DNA were, respectively, 10^{-5} M and $6 \cdot 10^{-5}$ M b.p. (base pairs) for the absorption measurements and the photodamage experiment and 10^{-4} M and $6 \cdot 10^{-4}$ M b.p. for the luminescence measurements. For the low-temperature measurements, the prepared solutions were poured out into a special cell, so that the upper surface is open, and then frozen. The excitation beam was directed to the open surface, and the luminescence was registered from the same surface.

The steady-state fluorescence and phosphorescence measurements were performed using a laboratory-designed equipment; and the absorption spectra were recorded with the help of a Specord UV-VIS spectrophotometer. The photodamage of dyes and the DNA + dye system was performed by exposition of the corresponding solution (in a quartz cell) to the visible spectrum irradiation of a 1-kW Hg-lamp.

The measurements were carried out at 77 K and ambient temperatures.

RESULTS AND DISCUSSION

1. First Excited Singlet and Triplet Levels of DNA-Sensitive Luminescent Dyes

Recently, we have synthesized and studied novel monomer and homodimer styryls based on the (p-dimethylaminostyryl)benzothiazolium moiety and containing charged spermine-like linkage/tail groups [7]. For the monomer dye Bos-1, the 65-fold increase in the fluorescence intensity was revealed in the presence of DNA, while, for homodimers DBos-24 and DBos-30, the presence of DNA resulted in a dye fluorescence intensity increase by 200 and 850 times, respectively (Fig. 2).

In addition, for Bos-1, DBos-24, and DBos-30 in the presence of DNA, the fluorescence emission induced by TPE was registered (Fig. 3) [7]. TPE fluorescence measurements were carried out with the help of a YAG:Nd^{3+} laser generating 15-ns pulses. The two-photon absorption cross-sections (δ) of the dyes were estimated by using the known δ value of Rhodamine 6 G as the reference one. For Bos-1,

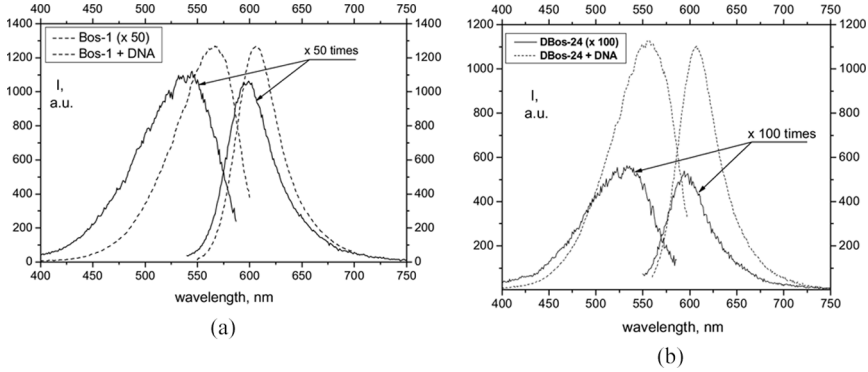

FIGURE 2 Fluorescence excitation (left) and emission (right) spectra of Bos-1 (a) and DBos-24 (b) in buffer solutions in the unbound state and in the presence of DNA. The concentrations of a dye and DNA are 5×10^{-6} M and 6×10^{-5} M b.p., respectively. The fluorescence intensity of a free dye solution is multiplied by 50 and 100 times for Bos-1 and DBos-24, respectively.

DBos-24 and DBos-30 in the presence of DNA, δ was found to be equal to 7.4, 4.7, and 6.0 GM respectively ($1\,\text{GM} = 10^{-50}\,\text{cm}^4\,\text{s}$) [7]. Hence, dyes Bos-1, DBos-24 and DBos-30 could be proposed for the application

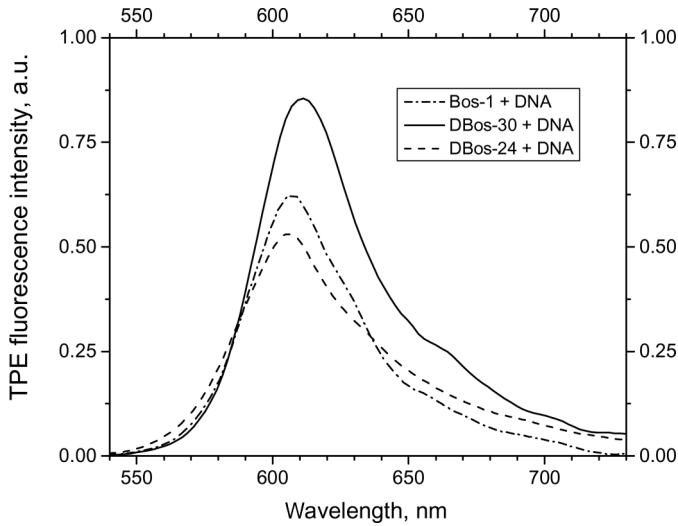

FIGURE 3 Two-photon excited fluorescence spectra of Bos-1, DBos-24, and DBos-30 in a buffer in the presence of DNA; the dye concentration is 1.5×10^{-5} M; the DNA concentration is 1.8×10^{-4} M b.p. [7].

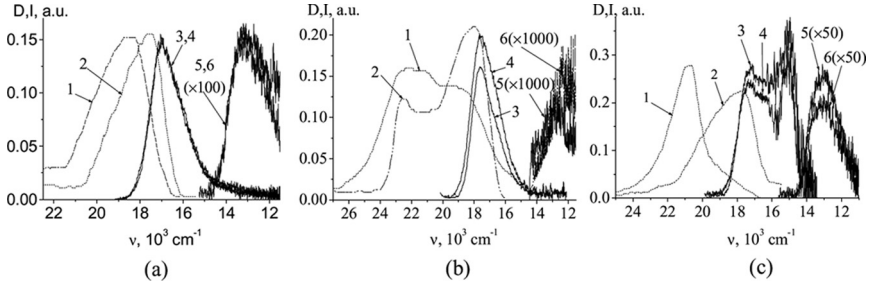

FIGURE 4 Absorption (1, 2), fluorescence (3, 4), and phosphorescence (5, 6) spectra (3–6 at T = 77 K) of free dyes (1, 3, 5) and the corresponding systems DNA + dye (2, 4 ,6) of: (a) Bos-1, (b) DBos-24, and (c) DBos-30.

as fluorescent probes in the TPE or SPE detection and imaging of DNA.

Nevertheless, in order to use the dyes as luminescent probes for the DNA detection and imaging in the study of living objects, the phototoxic influence of these dyes realized directly via the excitation energy transfer from the dye to DNA has to be absent. In order to avoid such a transfer, the first excited singlet and triplet levels of dyes must be situated lower than the corresponding levels of any nucleotide. The optical absorption, fluorescence, and phosphorescence spectra of the investigated dyes Bos-1, Dbos-24 and Dbos-30, as well as those of the corresponding DNA + dye systems, are given in Figure 4.

The absorption spectrum of dye Bos-1 both in the presence and in the absence of DNA in the visible spectral region consists of the single band with the maximum near 17540 and 18470 cm^{-1}, respectively (Fig. 4a, c.1,2). Since this band is the most long-wave band in the visible spectral region and the corresponding fluorescence spectrum (Fig. 4a, c.3,4) obeys the mirror symmetry rule respective to this absorption band, it should be attributed to the absorption transition to the first excited singlet level of the dye molecule. The shift between the maxima of the spectra of the free dye and the DNA + dye system points to the dye interaction with DNA. In the absorption spectra of dimer dyes Dbos-24 and Dbos-30 in the presence of DNA, the same band with the maximum near 18000 cm^{-1} (Fig. 4b, c.2) and 17750 cm^{-1} (Fig. 4c, c.2), respectively, can be observed. At the same time, the spectrum of Dbos-24 in the presence of DNA contains one more band with the maximum at 22500 cm^{-1} shifted to the short-wavelength region relative to the main band. This short-wavelength band with the maxima at 22200 and 20800 cm^{-1}, respectively, dominates in the absorption spectra of both Dbos-24 and Dbos-30 in the of DNA (Fig. 4b, c.1 and

Fig. 4c, c.1). Taking into account all the above-mentioned results, the short-wavelength band can be attributed to the aggregates of the styryl chromophore. The similar spectral manifestation of the aggregation was observed for cyanine dyes [10,11]. Since the tendency to form aggregates for dimer dyes is much higher than that for the corresponding monomers [12], the short-wavelength band is present in the spectra of Dbos-24 and Dbos-30 in free state, but is absent in the spectrum of Bos-1 containing the same chromophore. At the same time, the interaction of dimer dyes with DNA at a low ratio of the concentrations of dye and DNA leads generally to the fixation of separate chromophores on the DNA molecule, thus resulting in a decrease in the aggregates concentration in a solution [13]. This explains a decrease in the short-wavelength band contribution to the absorption spectrum of Dbos-24 in the presence of DNA, as well as the disappearance of this band in the same spectrum of Dbos-30.

Since, in the absorption spectra of Bos-1, no aggregate bands were observed, we can suppose that both the fluorescence and phosphorescence spectra of this dye also belong to the non-aggregated dye. As the spectra of dyes Bos-1, Dbos-24, and Dbos-30 contain the fluorescence and phosphorescence bands with the maxima near 17000 cm^{-1} and 13000 cm^{-1}, respectively, we can suppose that these bands correspond to the non-aggregated chromophore for all these dyes. For the fluorescence spectra, this is supported by the fluorescence excitation measurements at room temperature (Fig. 2) [7], while ones the additional studies should be performed for the phosphorescence.

The positions of the first excited singlet levels of the investigated dyes in the presence of DNA were obtained by the intersection of the absorption and fluorescence spectra, and those of triplet levels were calculated by the phosphorescence spectrum blue edge. The positions of both the singlet (19100 cm^{-1} for Bos-1, 18550 cm^{-1} for DBos-24, and 19100 cm^{-1} for DBos-30) and triplet (14800 cm^{-1} for Bos-1, 14450 cm^{-1} for DBos-24, and 14900 cm^{-1} for DBos-30) levels of the dyes in the presence of DNA were compared with the corresponding levels of the nucleotides obtained by us earlier (singlet levels: 33090 cm^{-1} for dCMP, 33030 cm^{-1} for dGMP, 33530 cm^{-1} for dTMP, and 34490 cm^{-1} for dAMP; triplet levels: 26630 cm^{-1} for dCMP, 26320 cm^{-1} for dGMP, 26160 cm^{-1} for dTMP, and 25950 cm^{-1} for dAMP [14]).

The comparison of the energy levels positions of the nucleotides with these of the dyes removes all doubts that even the triplet level of any nucleotide is situated much upper than the singlet level of any investigated dye. That is why the irradiation of a dye in the visual spectral region exciting the first singlet electronic level of the dye can not be resulted in the excitation energy transfer from the dye to DNA.

2. Irradiation of Bos-1 and Dbos-30 Dyes and the DNA + Dye Corresponding Systems. Photostability of Dyes

One of the spectral manifestations of the luminescent dye phototoxicity for DNA (i.e., the damage of DNA) is the optical density change in the DNA absorption band (maximum at 260 nm) corresponding to the $S_0 \rightarrow S_1$ electronic transition under the direct excitation of the dye bound to DNA. Moreover, the measure of the dye photostability is the change of the optical density of the dye absorption band corresponding to its $S_0 \rightarrow S_1$ electronic transition under the dye excitation at the wavelength of the same band. With the aim to investigate both the dye photostability and its phototoxicity for DNA, the dependence of the absorption spectra of the solutions of Bos-1, Dbos-24, and Dbos-30 dyes and the corresponding DNA + dye systems on the time of their irradiation by the visible light of a 1-kW Hg-lamp was studied. It is worth to note that the DNA absorption band related to the first electronic transition (at 260 nm) is located far from the same band of the majority of dyes used in this study (400–500 nm). This fact and the fact that the optical density of the absorption band of dyes connected with the second electronic transition (located at ~260 nm) is much lower than dyes corresponding value for the DNA band give the possibility to study the dynamics of the optical density of the dye and DNA absorption bands almost independently.

The dependences of the optical density D of the investigated compounds on the irradiation time are given in Figure 5. The gradual

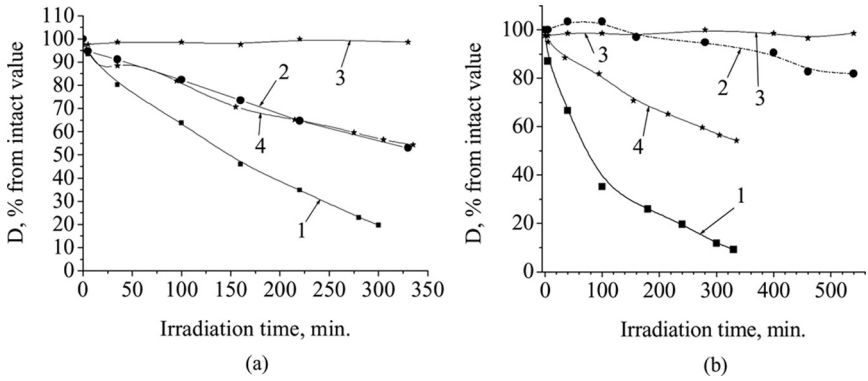

FIGURE 5 Dependence of the optical density of (a) styryl monomer Bos-1 and (b) dimer Dbos-30 (1-free dye, 2-dye with DNA, 3-the DNA maximum at 260 nm, 4-TO with DNA for the comparison) on the irradiation time.

decrease of D of the bands of the absorption spectra of free dyes is observed under increasing the dye irradiation time (Fig. 5; c.1). This is the evidence for that the damage of the π-electron systems of dyes takes place. Since, after 300 min of the irradiation, D for free dyes decreased by 80–90%, the mentioned dyes are not photostable in free form. For dyes in the presence of DNA, the gradual decrease of D is essentially slower than that for free dyes. In addition, for Dbos-30 in the presence of DNA (Fig. 5b; c.2), D decreases slower as compared with the same value for Bos-1 in the presence of DNA (Fig. 5a; c.2). Hence, the dimer Dbos-30 bound to DNA is more photostable than monomer Bos-1 bound to DNA. The comparing of the dependence of optical density curves on the irradiation time for the investigated styryl dyes and the well-known thiazole orange (TO) dye (Fig. 5, c.4) demonstrates that both Bos-1 and Dbos-30 dyes, when they are bound to DNA, are more photostable even than TO in the presence of DNA. The fact that a dye bound to DNA is more photostable than a free dye can be explained by the binding of a dye molecule with the DNA macromolecule. This causes the screening of the dye molecule against external influences and the decrease of contacts between the dye molecule and the surrounding molecules of oxygen. The last becomes an active oxidant after being transferred to the singlet excited state by the excitation transfer from a dye [15]. Moreover, the dye molecule fixation on DNA causes an increase in the fluorescence emission quantum yield. This entails a decrease of the probabilities of other excitation deactivation pathways (including pathways to the dye photoreaction). Our results are in agreement with the results of investigations of the cyanine dyes in polymeric matrices obtained in [12]. The difference in the Bos-1 and Dbos-30 photostabilities in the presence of DNA can be explained in the following way. It is known that the binding constant to DNA is often much higher for a dimer than for a monomer with the same chromophore group [9,16]. Thus, the number of due molecules bound with DNA is higher for Dbos-30 than that for Bos-1. Thus, the dimer molecules can be screened by DNA more efficiently than the monomer ones.

To study the phototoxic influence of the investigated dyes on DNA, the dependence of D of the DNA band maximum on the dye irradiation time (Fig. 5; c.3) was investigated. Figure 4 shows that D changes within 3% out of its intact value. Our experiments show that D of the absorption band of dyes related to the second electronic transition stays almost unchanged during the irradiation time. The changes of D within 3% at 260 nm may be connected with these negligible changes of the absorption band of dyes and experimental errors. It is known the DNA destruction is either the double strand untwisting or the strand

cutting that is reverse to the hypochromic effect, the complete untwisting resulting in the 37%-increase in D at 260 nm [17]. Thus, even if the 3%-changes mentioned above are connected with the DNA destruction, this destruction cannot be significant and can be included in experimental errors.

So, Bos-1 and Dbos-30 dyes can be considered as non-phototoxic for the DNA. On the other hand, DNA protects the investigated dyes against the photodamage.

3. Irradiation of Dbos-24 and Dst-MdO Dyes and the DNA + Dye Corresponding Systems. Phototoxicity for DNA

The dependences of the optical density D of Dbos-24 dye and the DNA + Dbos-24 system on the irradiation time (Fig. 6) are obtained from the optical absorption spectra (Fig. 6). During the irradiation of a free dye, D of the short-wavelength aggregate band decreased gradually, while the long-wavelength band of non-aggregated dyes showed some increase at first. In our opinion, this means that aggregates are damaged and turn into non-aggregated state. Dye Dbos-24 is more photostable than Bos-1 and DBos-30, D of DBos-24 decreasing by 40–50% after 600 min of the irradiation.

Figure 6b indicates that, in the presence of DNA, the optical density of the band of aggregates decreases gradually with increase in the irradiation time, while D of non-aggregated dyes remains constant. It can be explained by the assumption that non-aggregated dyes

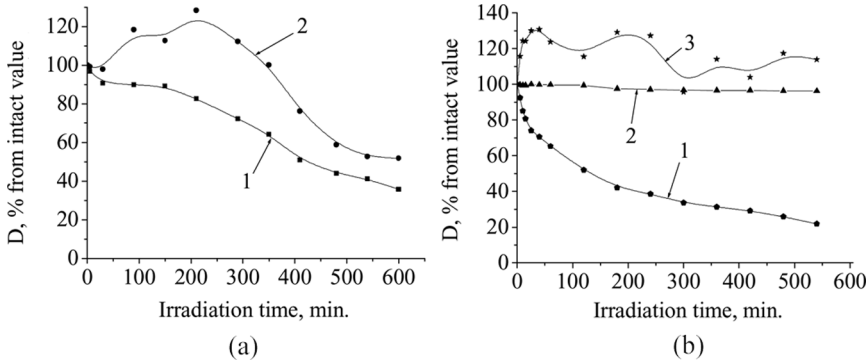

FIGURE 6 Dependence of the optical density of a dimer Dbos-24 on the irradiation time: (a) free dye, (b) bound to DNA (1-at 23100 cm^{-1}, 2-at 18500 cm^{-1}, 3-the DNA maximum at 38460 cm^{-1} (260 nm)).

intercalate into DNA and the aggregates bind outside the DNA chain. Therefore, the generated singlet oxygen damages the aggregated dyes. The non-aggregated dyes are protected from the influence of singlet oxygen and, therefore, are not damaged.

The dependence of the optical density D of the DNA band on the irradiation time (Fig. 6b) was obtained from the absorption spectra of the DNA + DBos-24 system (Fig. 7). This figure shows that the optical density of the DNA absorption band increases by 30% during the irradiation time. In our opinion, this fact is connected with cutting or untwisting the strands of a DNA macromolecule that interacts with DBos-24 dye molecules (this effect is reverse to the hypochromic effect). Our results are comparable with the 37%-increase for the complete DNA untwisting [17]. Thus, we can suppose that DBos-24 causes a significant DNA destruction. So, DBos-24 showed the phototoxic influence on DNA. On the other hand, the phototoxicity of dyes is manifested not only in cutting or untwisting the strands. Figure 8 shows that the optical density of the DNA absorption band in the DNA + Dst-MdO system decreases by 10%. In our opinion, this is connected with the fact that Dst-MdO molecules favor the destruction of DNA π-electron systems.

So, DBos-24 and Dst-MdO dyes can be used for the destruction of DNA in the photodynamic therapy.

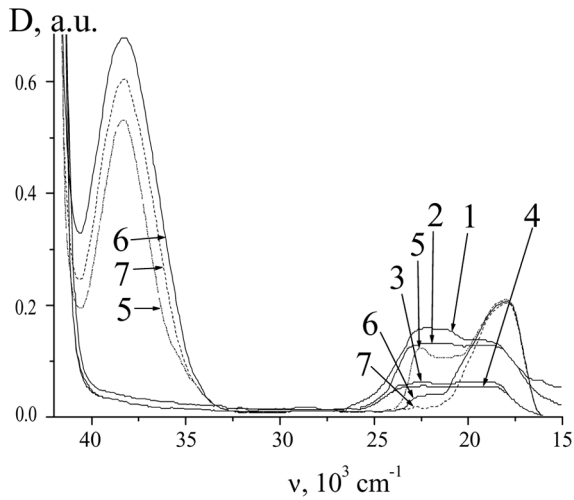

FIGURE 7 Absorption spectra of Dbos-24 during the irradiation. Free dye: (1) intact, (2) 210 min, (3) 540 min, (4) 600 min; dye with DNA, (5) intact, (6) 240 min, (7) 540 min.

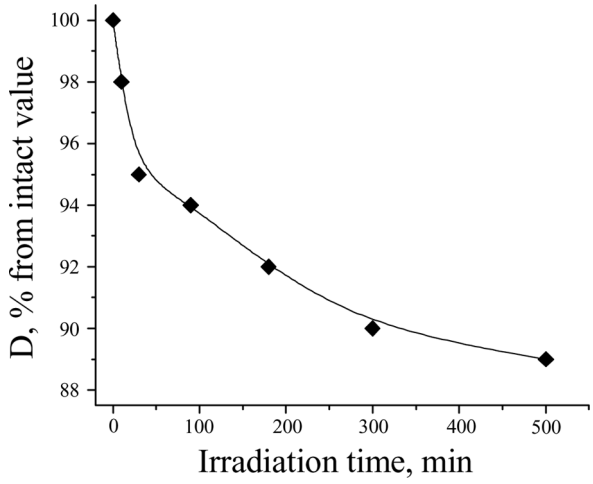

FIGURE 8 Dependence of the optical density of the DNA maximum in the DNA + Dst-MdO system.

CONCLUSIONS

A number of the newest luminescent monomer and homodimer styryl dyes was synthesized and investigated. The spectral investigations of the photostability and phototoxic influence of these dyes on DNA have been carried out. The studied benzothiazole styryl dyes Bos-1, DBos-24, and DBos-30 exhibit a rather intense fluorescent emission under the two-photon excitation at 1064 nm. The values of the two-photon absorption cross-section for these dyes are equal to 7.4, 4.7, and 6.0 GM, respectively (1 GM = 10^{-50} cm^4 s).

All the investigated dyes are rather photostable when they are bound to DNA, whereas the photostability of free dyes is much lower. This phenomenon is related, in our opinion, to the fact that the DNA macromolecule screens a dye molecule against external influences and decreases the contacts between the dye molecule and the surrounding molecules of oxygen that becomes an active oxidant, by being transferred to the singlet excited state due to the excitation transfer from a dye. DBos-24 dye is the most photostable among the investigated dyes. Dyes Bos-1 and DBos-30 are less photostable, since D of free dyes has decreased by 80–90% already after 300 min of the irradiation and by 20–50% for the DNA + dye systems. All the investigated styryl dyes are more photostable, when they are bound to DNA than the well-known thiazole orange (TO) dye.

Bos-1 and DBos-30 dyes are photochemically safe for DNA. This conclusion is confirmed by the fact D of the DNA absorption band for the DNA + dye systems stays almost invariable during the irradiation time. In contrast to them, DBos-24 dye showed the phototoxic influence on DNA. In this case, the optical density of the DNA band increases by 30% during the irradiation time. In our opinion, this fact is connected with cutting or untwisting the DNA macromolecule that interacts with DBos-24 dye molecules. But the phototoxicity of dyes is manifested not only in such an effect. It was shown that the optical density of the DNA band in the DNA + Dst-MdO system is decreased by 10%. In our opinion, this phenomenon is related to the fact that Dst-MdO molecules favor the destruction of DNA π-electron systems.

Dyes Bos-1 and DBos-30 can be used in biology and medicine as luminescent probes, while DBos-24 and Dst-MdO can be applied in the photodynamic therapy.

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