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Fluorescence Study of DNA–Dye Complexes Using One-Photon and Two-Photon Picosecond Excitation

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Preliminary results of investigation of one-photon- and two-photon-induced fluorescence of acridine orange (AO), epirubicin (ER), hypericin (HYP), and ethidium bromide (EB) in complexes with DNA are presented. A spectrofluorometer based on a picosecond Nd:YAG laser was used for investigations of two-photon (1064-nm, 1-mJ, 40-ps) and one-photon (532- and 355-nm) dye excitation. The spectra of two-photon-induced fluorescence of dyes and their complexes with DNA as well as the kinetics of dyes' fluorescence intensification during their interactions with DNA in dependence on the biomacromolecule concentration were obtained. The intensities of AO, HYP, and EB fluorescence were increased 2.4, 3.2, and 8 times, respectively, after binding with DNA at two-photon excitation, while at one-photon excitation the corresponding values were 2.5, 3.7, and 10 times. The difference in fluorescence enhancement during DNA–dye complex formation at linear and nonlinear excitation may possibly be associated with the fact that the cross sections of one-photon and two-photon absorption, in general, change unequally during the binding of dyes to organic molecules and bathocromic shift of the electronic transitions. It was shown that the peak of AO fluorescence shifted to a longer wavelength on 10 nm after two-photon excitation at 1064 nm in comparison with one-photon excitation at 532 nm.

KEY WORDS: DNA–dye complex; two-photon excitation; picosecond laser.

INTRODUCTION

Fluorescence spectroscopy of DNA–dye complexes is successfully used for structural and photochemical studies of deoxyribonucleic acid [1,2]. Here we present a study of one-photon and two-photon laser-induced fluorescence of several known dyes—acridine orange (AO), ethidium bromide (EB), hypericin (HYP), and epirubicin (ER)—during their interaction with DNA. These dyes are bound to DNA mainly by intercalation between two adjacent base pairs of the biomacromolecule and cause frame-shift mutation, DNA strand breaks, inhibition of nucleic acids synthesis, and other biological effects [1–4].

A modified Lerman's model of AO–DNA complexes as one of the possible modes of interactions has been discussed earlier [5]. It was shown that AO was absorbed on double-stranded DNA as a monomer, with the absorption maximum at 504 nm and the fluorescence maximum at 530 nm, while on single-stranded RNA and denatured DNA the dye was bound as a dimer, with the absorption maximum at 465 nm and the fluorescence maximum at 640 nm. The excitation of DNA–AO complexes with light which was absorbed only by DNA bases caused fluorescence of the dye, which indicated effective energy transfer from nucleotide bases to the AO molecules [2,3]. The lifetime of free monomer AO is $2.0 \cdot 10^{-9}$ s, and after binding with DNA it becomes $5.0 \cdot 10^{-9}$ s [4]. Intercalation of EB into DNA base pairs

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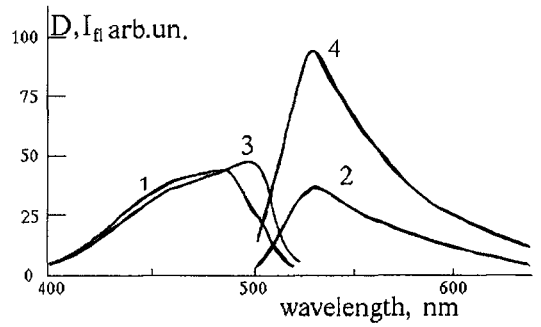

Fig. 1. Absorption [1,3] and fluorescence [2,4] spectra of AO [1,2] and AO–DNA complexes [3,4]. C_{DNA} = 20 mg/L; λ_{exc} = 355 nm.

brought a multiple increase in the quantum yield of the dye fluorescence [6]. It was also shown that HYP selectively interacted with the N7 sites of purins [7].

Rapid development of laser techniques provided the opportunity to elaborate principally new methods of study of biomolecules: pico and femtosecond time-resolved spectroscopy, nonlinear photophysics and photochemistry, etc.[8–16]. Classic spectroscopy is a study of transitions by which the electric dipole operator connects the initial and final states of atoms and molecules. For "allowed" transitions these states have to be of opposite parity, so transitions between like-parity states remain inaccessible for these methods. Two-photon absorption, depending quadratically on the momentum operator, removes this limitation. Linear (one-photon) and two-photon spectroscopies are complementary and these two approaches, with the application of sufficient powerful lasers, allow examination of all states of molecules [8,9]. A second useful difference between linear and nonlinear spectroscopies is the characteristic dependence of the strength of nonlinear absorption on the intensity of the applied light wave.

The inducible nature of two-photon absorption allows us to use more infrared light so that the excitation wavelength can lie in the transparency "window" of biological tissues (680–1100 nm). Two-photon spectroscopy extends the possibilities of optical methods and allows us to obtain supplementary information about the structure and photophysics of biomolecules, including DNA [11–16]. The only limitation of nonlinear spectroscopy is the optical damage of samples [9].

Advantages of nonlinear absorption and fluorescence spectroscopy lead to their fast inoculation not only in fundamental investigations, but also in three-dimensional confocal microscopy [14], diagnosis and therapy of tumors [17,18], etc.

MATERIALS AND METHODS

Computerized spectrofluorometer based on a passive mode-locked picosecond Nd:YAG laser was used in the experiments. After single-pulse selection the radiation of the generator (1064 nm; pulse duration, 40 ps) was increased up to E = 1 mJ and used for two-photon excitation (TPE). For one-photon fluorescence investigation the second (532-nm) and third (355-nm) harmonics of the laser were used. Laser radiation was focused by a lens with F = 150 mm into the quartz cuvette (10 mm) with the studied solution. The intensity of the fundamental harmonic of the laser in the focal region was estimated as 10–50 GW/cm^2. The scattered light from the object was collected by the lens and registered by a monochromator (MDR-23)–PMT (FEU-79)–computer system. A part of the laser beam was directed through a beam splitter to the second photodetector for normalization of the excitation radiation intensity. A personal computer and a two-channel analog-to-digital converter were used for control of the system and automatic data collection and processing. The spectra were corrected for the wavelength response of the detecting system. Spectral resolution of the system was 1 nm. Optical absorption measurements were carried out using a Shimadzu recording spectrophotometer, Model UV-2100.

In the experiments we used bull spleen highly purified DNA obtained from Belorus Bioorganic Chemistry Institute. Amounts of RNA and proteins in DNA preparation were no more than 0.1%. The DNA preparation in buffer (0.1 M NaCl + 0.01 M KH_2PO_4, pH = 7.2) was stored at +4°C before use. The concentration of DNA before dye addition was 70 mg/L. The dyes were also dissolved in the same phosphate buffer, pH 7.2. The concentrations of DNA and the dyes during the measurements were as follows: C_{DNA} = 2–25 mg/L; C_{HYP} = 20 μM; C_{AO} = 15 μM; C_{EB} = 12 μM; and C_{ER} = 20 μM.

RESULTS AND DISCUSSION

The fluorescence spectra of EB and AO as well as the quantum yield and decay time of fluorescence are known to be affected upon binding to DNA. Figure 1 presents the absorption and fluorescence spectra of AO and the AO–DNA complex. It is shown in the figure that the intensity of bound AO fluorescence increased up to 2.5-fold at 530 nm, which is in accordance with the literary data [1]. The shape of the fluorescence spectrum did not change, which indicated the absence of RNA molecules and single-stranded DNA in the solutions.

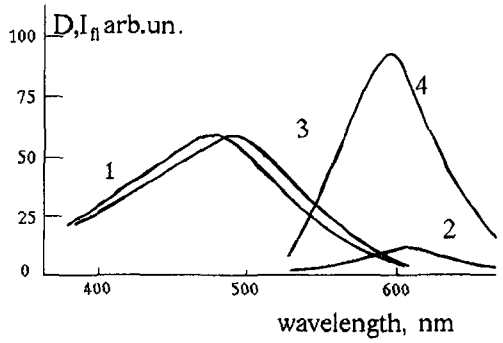

Fig. 2. Absorption [1,3] and fluorescence [2,4] spectra of EB [1,2] and EB–DNA complexes [3,4]. C_{DNA} = 20 mg/L; λ_{exc} = 532 nm.

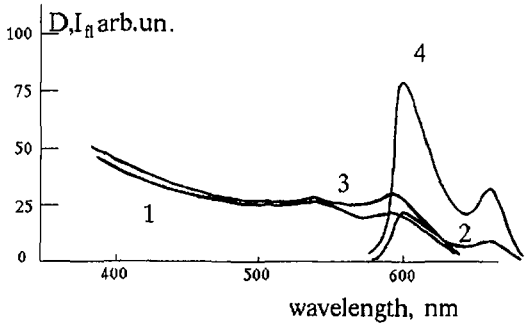

Fig. 3. Absorption [1,3] and fluorescence [2,4] spectra of HYP [1,2] and HYP–DNA complexes [3,4]. C_{DNA} = 10 mg/L; λ_{exc} = 532 nm.

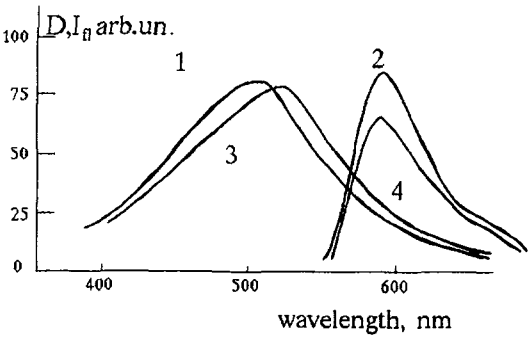

Fig. 4. Absorption [1,3] and fluorescence [2,4] spectra of ER [1,2] and ER–DNA complexes [3,4]. C_{DNA} = 10 mg/L; λ_{exc} = 532 nm.

The absorption peak of AO shifted from 492 to 503 nm. Significant changes in EB spectral properties were observed after binding of the dye with DNA (Fig. 2). The absorption peak of EB molecules shifted from 480 to 495 nm and the fluorescence intensity increased up to 10-fold. For HYP an increase in absorption in the 590- to 600-nm region after the interaction with DNA was observed. At the same time the fluorescence of HYP

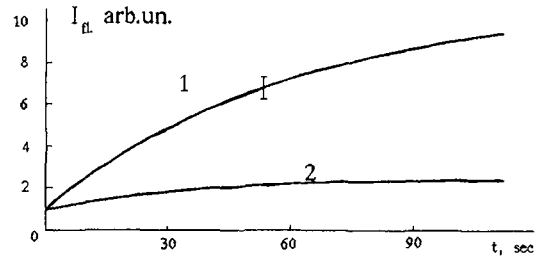

Fig. 5. Time-dependent change of EB [1] and AO [2] fluorescence intensity after DNA addition to the solutions of the dyes. C_{DNA} = 20 mg/L; λ_{exc} = 532 nm [1] and 355 nm [2].

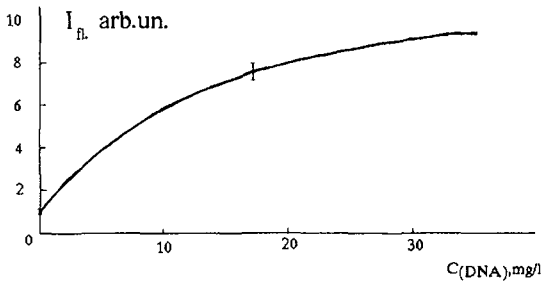

Fig. 6. Intensity of EB fluorescence in dependence of DNA concentration. λ_{exc} = 532 nm.

increased up to 3.7-fold (Fig. 3). A red shift of the absorption on 10 nm was observed after the addition of DNA to ER solution (Fig. 4).

Laser spectrofluorometer allows us to register the kinetics of dye interaction with DNA. Figure 5 presents the time-dependent change of EB and AO fluorescence intensity after DNA addition. It is obvious that these curves express the rate of formation of DNA–dye complexes at given concentrations of the reagents and therefore they provide information about diffusion coefficients and association constants of the compounds.

The intensity of EB fluorescence depending on DNA concentration is presented in Fig. 6. The measurement was carried out 2 min after preparation of the EB–DNA solution. Already most of the EB molecules were associated with DNA at a DNA concentration of 35 mg/L. According to Ref. 6, the fluorescence decay time of EB increases from 1.75 to 20.5 ns and the quantum yield of fluorescence increases 12-fold after binding with DNA. The value obtained in our experiments (10) is close to this datum. The small discrepancy can be explained by different experimental conditions.

Registration of two-photon-induced fluorescence of dense solutions of dyes and biomolecules or their crystals is easily carried out even with the use of nanosecond

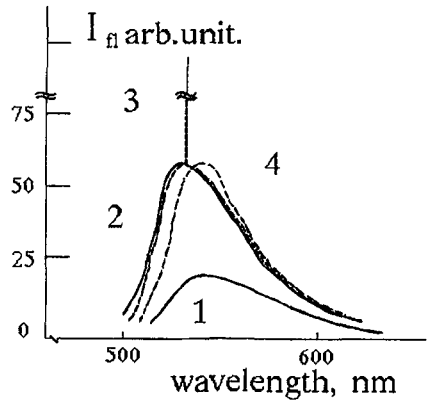

Fig. 7. Two-photon [1,4]- and one-photon [2,3]-induced fluorescence spectra of AO [1] and AO–DNA complexes [2–4]. $\lambda_{exc.}$ = 1064 nm [1,4], 532 nm [3], and 355 nm [2]; C_{DNA} = 20 mg/L.

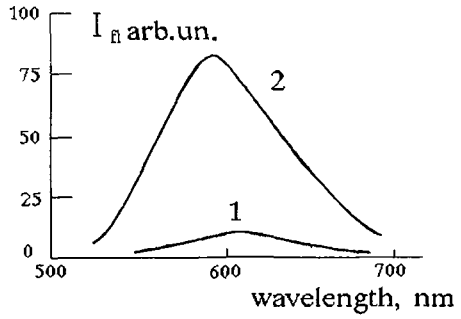

Fig. 8. Two-photon-induced fluorescence spectra of EB [1] and an EB–DNA complex [2]. C_{DNA} = 20 mg/L; λ_{exc} = 1064 nm.

lasers [9]. However, in biological investigations when the concentrations of studied compounds can not be increased because of possible aggregate formation, the problem becomes more complicated. The computerized picosecond laser spectrofluorometer allowed us to register two-photon fluorescence of HYP, AO, and EB at concentrations of $2 \cdot 10^{-5}$, $1.5 \cdot 10^{-5}$, and $1.2 \cdot 10^{-5}$ *M*, respectively, in the complexes with DNA at concentrations of 10–20 mg/L (see Figs. 7 and 8). So the concentrations of the compounds studied in our experiments were the same as in the investigations with application of linear optical methods [1–4]. The fluorescence intensity increased in proportion to the square of the laser intensity, which indicated the two-photon nature of the excitation.

We observed an interesting peculiarity of the fluorescence of some dyes at TPE. In the case of AO the "anti-Stokes" component of fluorescence was easily registered in the region of 500–530 nm at 1064-nm two-photon absorption. Figure 7 presents the AO fluorescence spectra after one-photon excitation and TPE. The sharp peak corresponds to the scattered laser light at 532-nm one-photon excitation. The fluorescence spectra of free and bound AO at the TPE shifted to a longer wavelength on 10 and 12 nm in comparison with that of 532- and 355-nm excitations, respectively. This result is unexpected, as the AO molecule obtains the same excitation energy (2.34 eV) in both cases: after two-photon absorption at 1064 nm and one-photon absorption at 532 nm. A similar phenomenon was observed at TPE of disodium salt of fluorescein (FL) and riboflavin. Deformation of the two-photon-induced fluorescence spectrum was not observed for dyes, the fluorescence of which lies in a longer-wavelength region than 532 nm [EB (see Fig. 8), HYP, chlorin e_6, HpD]. One could explain the shift of the fluorescence spectra at TPE by the phenomenon of light quenching, i.e., stimulated transitions from the excited electronic level under the influence of the laser radiation [10,19]. However, our theoretical estimations based on the approach suggested in Ref. 20 exclude this mechanism in the cases of AO, FL, and riboflavin at 1064-nm excitation.

The long-wavelength spectral shift of AO fluorescence on 10 nm at TPE could be qualitatively explained by the following. As is known [6], the excitation on the red edge of the molecule's absorption spectrum can lead to a shift of the fluorescence spectrum to a longer wavelength. This shift conditions as follows: excitation on the red edge of the spectrum is selectively possible for those molecules, which interact more intensively with the solvent and have shifted spectra of fluorescence and absorption. The portion of such molecules might be larger at two-photon excitation in comparison with one-photon excitation, which will bring to a shift of the fluorescence spectrum. In the case of AO, the shift of the fluorescence spectrum at-TPE could be explained by the influence of AO aggregates (dimers), which have a longer-wavelength fluorescence spectrum than monomers. This will occur if the ratio of two-photon absorption by dimers is larger than that of monomers. However, in the cases of riboflavine and FL, for which there were no aggregates observed with long-wavelength fluorescence, this mechanism cannot explain the observed shift in fluorescence spectra of the dyes at TPE.

The possibility of different mechanisms of absorbed energy transformation in molecules at one-photon and two-photon excitations has been indicated recently [13]. The authors showed that nonresonant two-photon excitation of 2,2-diphenylhexamethyltrisilane with a 532-nm laser light induced two major reactions with a selectivity quite different from that of one-photon (266-nm) excitation.

The spectra of EB and EB–DNA fluorescence at TPE are shown in Fig. 8. The intensity of bound EB fluorescence increased eight fold. This value for 20% is lower than in the case of one-photon excitation. These data reflect different changes of one-photon and two-photon absorption spectra of EB after its interaction with DNA. As regards AO and HYP, the coefficients of fluorescence enhancement at TPE during binding with DNA are approximately the same as for one-photon excitation.

Thus the results obtained from experiments with a picosecond Nd:YAG laser source show that the technique of two-photon fluorescence spectroscopy can be used in the study of interaction of DNA with fluorescent ligands. It should be noted that the shift of the excitation spectrum to the near-infrared region allows us to investigate molecules in biological media (blood, tissues, cells, etc.) which could not be studied before by one-photon spectroscopy because of high absorption in the visible region. Nonlinear laser spectroscopy provides the opportunity of studying such electron levels and structural transformations, which cannot be revealed and studied by common linear optical methods.

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