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Published on Web 05/23/2006

Ultrafast Excited-State Dynamics of DNA Fluorescent Intercalators: New Insight into the Fluorescence Enhancement Mechanism

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Abstract: The excited-state dynamics of the DNA bisintercalator YOYO-1 and of two derivatives has been investigated using ultrafast fluorescence up-conversion and time-correlated single photon counting. The free dyes in water exist in two forms: nonaggregated dyes and intramolecular H-type aggregates, the latter form being only very weakly fluorescent because of excitonic interaction. The excited-state dynamics of the nonaggregated dyes is dominated by a nonradiative decay with a time constant of the order of 5 ps associated with large amplitude motion around the monomethine bridge of the cyanine chromophores. The strong fluorescence enhancement observed upon binding of the dyes to DNA is due to both the inhibition of this nonradiative deactivation of the nonaggregated dyes and the dissociation of the aggregates and thus to the disruption of the excitonic interaction. However, the interaction between the two chromophoric moieties in DNA is sufficient to enable ultrafast hopping of the excitation energy as revealed by the decay of the fluorescence anisotropy. Finally, these dyes act as solvation probes since a dynamic fluorescence Stokes shift was observed both in bulk water and in DNA. Very similar time scales were found in bulk water and in DNA.

Introduction

Highly sensitive and even sequence-specific detection of DNA became a major issue for researchers when the desire to sequence complete genomes emerged. An important step into the genomic era was enabled by the development of the YOYO and TOTO dye families[1,2] since they allowed for the first time DNA to be detected at sensitivity comparable to that of radioactive probes, but without the danger inherent in radioactivity.[3,4] These dyes are homodimers of oxazole yellow (YO) and thiazole orange (TO) and were soon adapted to cover a large range of emission wavelengths in the visible region of the light spectrum. YO and TO are asymmetric cyanine dyes with a monomethine bridge connecting a benzo-1,3-oxazole and a benzo-1,3-thiazole moiety, respectively, to a quinoline group. As shown in Figure 1 for YOYO-dyes, the homodimers consist of two such chromophores connected with a biscationic linker similar to those of the ethidium homodimer, which is capable of bisintercalating into dsDNA.[5,6]

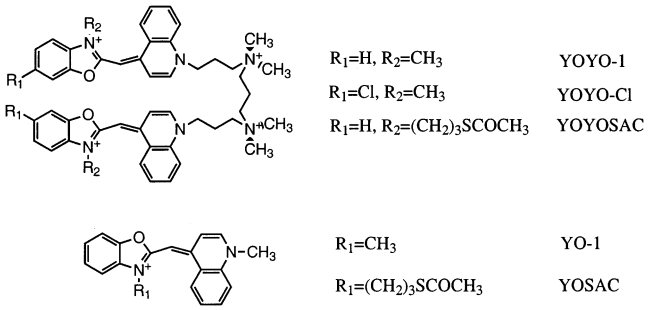

Figure 1. Chemical structures of the YOYO-dyes.

The ability of these dyes to interact with DNA has been addressed through a variety of spectroscopic studies, and two modes of binding have been uncovered.[7] The first one is bisintercalation between the base pairs, which is favored at high DNA base:dye ratio, that is, when the dye concentration is low compared to that of the DNA base pairs. The occurrence of this binding mode is supported by circular dichroism (CD) and fluorescence anisotropy measurements, and linear dichroism spectra of oriented DNA reveal that the bound dyes have a perpendicular orientation relative to the helical axis.[8] Nuclear magnetic resonance (NMR) studies of TOTO-1 and YOYO-1

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indicate that the chromophore lies across the base pairs with the benzothiazole and the benzoxazole ring located between the pyrimidines and the quinoline moiety located between the purines, and that the two chromophores of TOTO-1 do not lie collinearly on top of each other but make a dihedral angle of about 83°.[9,10] Absorption and CD experiments further suggest that, besides intercalation, groove binding is also operative at low DNA base:dye ratios.[8]

The popularity of YOYO-1 and TOTO-1 is due to their large extinction coefficients, high fluorescence quantum yield when bound to DNA, essentially zero fluorescence quantum yield when free in aqueous solution, very high binding constants, and finally the high kinetic stability of the DNA−dye complexes. The huge enhancement of fluorescence quantum yield upon binding to DNA is believed to originate from the loss of mobility around the methine bridge connecting the quinoline and benzoxazole, respectively, and benzothiazole moieties due to the constrictive DNA environment.[11] In the free form, isomerization around this bridge is an important nonradiative decay channel of the photoexcited dye molecule, whereas, upon intercalation, large amplitude motion of the probe is strongly hindered. This nonradiative decay mechanism, which governs the excited-state lifetime of the free dye, is a common feature of cyanine dyes and has been intensively investigated.[12−17] On the basis of the fluorescence quantum yields of the free and bound forms and the radiative lifetime of YOYO-1, this process has been predicted to take place with a time constant of 1−5 ps.[18] However, this ultrashort excited-state lifetime of free YOYO-1 has never been confirmed by direct measurements. Moreover, the question of the nature of the fluorescence of the free dye has until now been widely ignored, even for the best-known intercalators, such as TO.[19,20]

We report here on our investigation of the photophysics of YOYO-1 and its newly synthesized analogues YOYOSAC and YOYO-Cl bound to DNA and free in water using steady-state and time-resolved spectroscopies. We confirm the occurrence of a nonradiative relaxation process on the time scale of a few picoseconds and provide evidence that formation of intramolecular dimeric aggregates also contributes to the reduction of the fluorescence quantum yield of the free form due to excitonic coupling between the two chromophoric units. In DNA, the interchromophoric interaction is substantially reduced, but remains strong enough to enable ultrafast excitation energy hopping, as evidenced by fluorescence anisotropy measurements. Finally, it will be shown that these dyes can be used as local polarity probes to investigate the ultrafast dynamics of their environment, either bulk solvent or DNA.

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Experimental Section

Samples. YOYO-1 and YOYOSAC were synthesized according to literature.[21,22] The synthesis of YOYO-Cl is described in the Supporting Information. Briefly, the dye was synthesized in two steps by condensation of 6-chloro-3-methyl-2-(methylthio)benzo[d]oxazolium methosulfate and 1-(3-iodopropyl)-4-methylquinolinium iodide, and subsequent bisquaternization of the monomeric dye and *N*,*N*,*N*′,*N*′-tetramethyl-1,3-propanediamine with an overall yield of 44%. Methanol (MeOH) was purchased from Fluka, DMSO and 1-heptanol from Acros Organics, phosphate buffer saline (PBS) and double-stranded salmon sperm DNA from Sigma, and EDTA disodium salt dihydrate from AppliChem. All compounds were of the highest commercially available grade and used without further purification. The absence of impurities in the solvents was checked by exciting the pure solvents at 400 nm and looking for any emission not attributable to Raman scattering. One millimolar stock solutions of the DNA binding dyes in DMSO were prepared and stored in the dark. DNA (1 mg/mL) stock solutions in bi-distilled water were stored at −20 °C. All samples were freshly prepared from the stock solutions. For steady-state and time-correlated single photon counting measurements, the dye concentration was of the order of 1−10 μM, and for fluorescence up-conversion experiments, they amounted to about 100 μM. Unless specified, all measurements with the bound dyes were performed with a DNA base:dye ratio of 12.5:1.

Steady-State Measurements. Absorption spectra were recorded on a Cary 50 spectrophotometer, while fluorescence and excitation spectra were measured on a Cary Eclipse fluorimeter (5 nm slit) in a 1 cm quartz cell. Quantum yield measurements were performed against fluorescein in ethanol ($\Phi_{fl} = 0.92$).[23]

Time-Resolved Fluorescence Measurements. Excited-state lifetime measurements in the nanosecond time scale were carried out with the time-correlated single photon counting (TCSPC) technique. Excitation was performed at a repetition rate of 40 MHz with <90 ps pulses generated by laser diodes either at 395 nm (Picoquant model LDH-P-C-400B) or at 469 nm (Picoquant model LDH-P-C-470). Fluorescence was collected at 90° at magic angle with respect to the polarization of the pump pulses. A 420 or 560 nm cutoff filter placed in front of the photomultiplier tube (Hamamatsu, H5783-P-01) ensured that no scattered excitation light could reach the detector, whose output was connected to the input of a TCSPC computer board module (Becker and Hickl, SPC-300-12). The full width at half-maximum (fwhm) of the instrument response function (IRF) was around 200 ps. All measurements were performed in a 1 cm quartz cell. The accuracy on the lifetimes is ca. 0.1 ns.

For the fluorescence up-conversion measurements, excitation was achieved at 400 nm with the frequency-doubled output of a Kerr lens mode-locked Ti:sapphire laser (Tsunami, Spectra-Physics).[24,25] The output pulses centered at 800 nm had duration of 100 fs and a repetition rate of 82 MHz. The polarization of the pump beam was at magic angle relative to that of the gate pulses at 800 nm unless fluorescence anisotropy measurements were done. Experiments were carried out in a 1 mm rotating cell. The fwhm of the IRF was ca. 280 fs. No significant degradation of the samples was observed after the measurements.

Femtosecond-resolved fluorescence anisotropy measurements were carried out with the up-conversion setup by changing the polarization of the pump beam with respect to the gate beam with a half-wave plate and monitoring the fluorescence dynamics at 510 nm. The anisotropy

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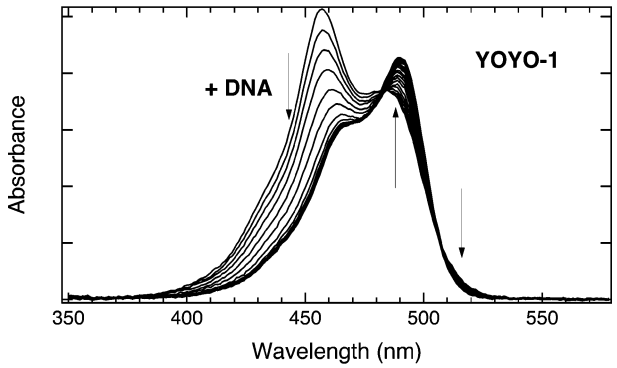

Figure 2. Absorption spectra of YOYO-1 with different DNA concentrations.

decay, $r(t)$, was reconstructed using the standard equation:[26]

$$r(t) = \frac{I_{||}(t) - I_{\perp}(t)}{I_{||}(t) + 2I_{\perp}(t)} \quad (1)$$

where $I_{||}(t)$ and $I_{\perp}(t)$ are the fluorescence intensities recorded with the polarization of the pump beam set parallel and perpendicular to that of the gate beam, respectively.

Fluorescence Data Analysis. Time-resolved fluorescence data were analyzed by iterative reconvolution of the instrument response function with trial functions (sum of exponentials and one Gaussian function) using a nonlinear least-squares fitting procedure (MATLAB, The MathWorks, Inc.). Measurements were carried out at 7−9 detection wavelengths from 480 to 630 nm (except anisotropy experiments, where the detection wavelength was 510 nm) and over 4 to 5 time scales accurately covering the span of the fluorescence decay. The data were then normalized, linearly interpolated to have at all time scales the same time increment as on the shortest time scale, and analyzed globally. To determine the amplitudes of the different decay components, the time profiles, $D(\lambda,t)$, were rescaled with the factor $F(\lambda)$:

$$F(\lambda) = \frac{S(\lambda)}{\int_0^{\infty} D(\lambda,t)\mathrm{d}t} \quad (2)$$

where $S(\lambda)$ is the corrected steady-state fluorescence intensity.[27,28] The accuracy on the lifetimes and on the amplitudes obtained by this method is estimated to be ca. 10%, except on lifetimes shorter than about 500 fs, where the uncertainty is about 100 fs.

Results

Steady-State Spectra and Nanosecond Fluorescence Dynamics. Figure 2 shows the UV−vis absorption spectra of YOYO-1 titrated in aqueous buffer solution with double-stranded DNA up to a DNA base:dye ratio of 15 (see Figure S1 in Supporting Information for YOYOSAC and YOYO-Cl). The absorption spectrum of all three dyes undergoes important changes upon intercalation, as already reported for YOYO-1.[11,29] The spectra of the free dyes in aqueous buffered solutions are characterized by maxima around 460 and 485 nm, the last one appearing as a shoulder for YOYO-1 and YOYO-Cl. The spectra of all three DNA-bound dyes are almost identical with a maximum at about 490 nm and a shoulder at 465 nm. Spectra

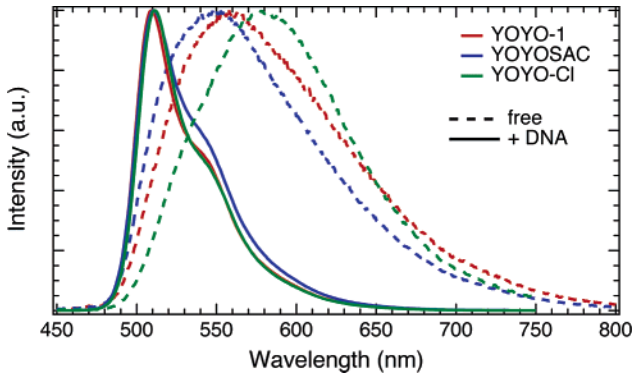

Figure 3. Fluorescence spectra of the YOYO-dyes in aqueous buffer solutions with DNA (solid lines) and without DNA (dashed lines) measured upon excitation at 440 nm.

Table 1. Fluorescence Quantum Yields, Φ_{fl}, of the Dyes in Various Environments Measured upon 440 nm Excitation

dye	environment	Φ_{fl}
YOYO-1	H_2O	0.0011[a]
	DNA	0.45
YOYOSAC	H_2O	0.0016
	DNA	0.40
YOYO-Cl	H_2O	0.0040
	DNA	0.50

[a] Value taken from ref 18.

very similar to those in DNA are measured with the free dyes in organic solvents (Supporting Information, Figure S2).

It should be noted that the spectra of the free dyes in aqueous solutions are substantially broader than those of the bound dyes. This difference manifests itself by the presence of one or two isosbestic points. These spectral changes upon DNA binding have been attributed to the excitonic coupling between the two aggregated chromophoric units in the free form.[11] The 490 nm band measured with DNA is due to the unperturbed chromophore, while the 460 nm band might be ascribed to the transition to the upper excitonic state of the coupled chromophores. Because of the mutual orientation of the transition dipoles in the dimeric aggregate, almost the whole oscillator strength of the two chromophores is associated with this transition. This point will be discussed in more details below (see Discussion).

As shown in Figure 3, the fluorescence spectra of the bound dyes (DNA base:dye ratio ≥12.5) are very similar and are the mirror image of the absorption spectra. Their fluorescence quantum yields lie between 0.4 and 0.5 (see Table 1). On the other hand, the free dyes exhibit very broad emission spectra with a maximum between 550 and 580 nm. This feature has previously been attributed to a large distribution of conformations of the free molecules in solution.[29] The fluorescence quantum yields of all three dyes are less than 0.01 (see Table 1).

Contrary to those of the DNA-bound dyes, the fluorescence excitation spectra of the free dyes were found to depend on the detection wavelength, as illustrated in Figure 4 with YOYO-1. The two features around 460 and 490 nm observed in the absorption spectrum are also present in the excitation spectra. However, their relative intensity varies substantially with the detection wavelength. The 490 nm band associated with the unperturbed "monomeric" absorption is the strongest when the detection is performed at 510 nm, a wavelength corresponding

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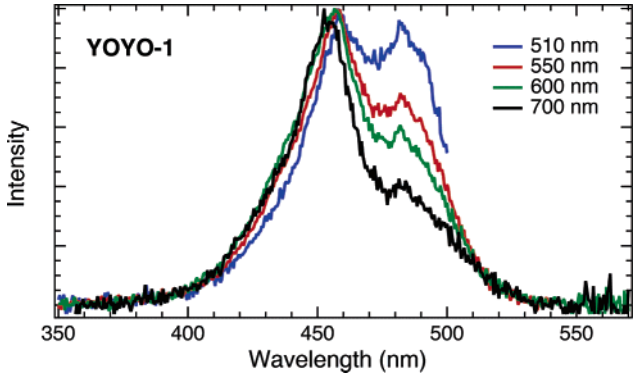

Figure 4. Intensity-normalized fluorescence excitation spectra of free YOYO-1 at different detection wavelengths.

Table 2. Time Constants Obtained from the Analysis of the Fluorescence Time Profiles Measured by TCSPC (τ_6 and τ_7) and Up-Conversion (τ_1−τ_5)

system	τ_1 (fs)	τ_2 (fs)	τ_3 (ps)	τ_4 (ps)	τ_5 (ps)	τ_6[a] (ns)	τ_7 (ns)
YOYO-1	190	180	1.1	3.7	30	1.1	
YOYOSAC	160	150	1.1	5.9	32	1.2	
YOYO-Cl	170	170	1.1	5.7	42	1.6	
YOYO-1/DNA	170	110	1.0		44	1.9	4.1
YOYOSAC/DNA	160	100	1.2		47	1.8	3.9
YOYO-Cl/DNA[b]						2.3	4.4

[a] The relative amplitude of this component is below 2% with the free dyes. [b] Fluorescence up-conversion measurements were not performed with this system.

to the fluorescence maximum of the bound dyes. The relative intensity of this band decreases continuously as the detection wavelength is shifted to the red. The fluorescence excitation spectrum measured at 700 nm is dominated by the 450−460 nm band, ascribed above to the transition to the upper excitonic state of the aggregate. This indicates that the dimeric aggregate contributes significantly to the broad fluorescence of the free dyes especially on the red side of the band.

The fluorescence decay of the bound dyes in the nanosecond time scale could be well reproduced using biexponential functions (Supporting Information, Figure S3). The resulting time constants, τ_6 and τ_7, measured at the fluorescence maximum are listed in Table 2. The relative amplitudes of these components are very similar for all three dyes and amount to 0.3 and 0.7 for the fast and slow components, respectively. The fluorescence lifetime obtained with YOYO-1 agrees with that of the literature.[18,29] The difference in fluorescence quantum yield of the three bound dyes (see Table 1) can be well accounted for by their different average lifetime, indicating a very similar radiative rate constant of the order of $k_{rad} = 1.3 \times 10^8$ s^{-1}. The presence of two decay components might reflect the two ways the dye binds to the DNA double strand, namely, bisintercalation and groove binding. However, even if bisintercalation is the only binding mode, a rather large distribution of local environments can be expected because of the heterogeneous base composition of salmon sperm DNA. As the fluorescence lifetime of bound YOYO-1 has been shown to depend on the base content,[18] the apparent biexponential fluorescence decays measured here are rather due to a distribution of lifetimes.[30,31]

TCSPC measurements with the free dyes indicate that most of the fluorescence decays on a time scale shorter than 200 ps, the IRF of the setup. Only a very minor fraction of the fluorescence time profile is associated with a nanosecond time constant.

Ultrafast Fluorescence Dynamics. The ultrafast fluorescence dynamics of the free YOYO-1, YOYOSAC, and YOYO-Cl, and of YOYO-1 and YOYOSAC with different DNA concentrations was investigated by fluorescence up-conversion and monitored at 7−9 different wavelengths spanning most of the fluorescence spectrum (480 up to 630 nm) (Figure 5). For each DNA concentration, the time profiles were analyzed globally by iterative reconvolution with a sum of a Gaussian and several exponential functions. Not less than 3−4 exponentials were needed to properly reproduce the data in addition to the nanosecond components obtained from TCSPC measurements. The presence in the trial function of a Gaussian component was required to accurately describe the initial rise of the fluorescence intensity. Alternatively, an unrealistically large instrument response function would have had to be assumed. The resulting time constants obtained with the free dyes and with the highest DNA concentration (DNA base:dye ratio of 12.5) are listed in Table 2.

As shown in Figure 6 for YOYO-1, the amplitudes associated with the two fastest components, τ_1 and τ_2, of which the first corresponds to the Gaussian component, have opposite sign,

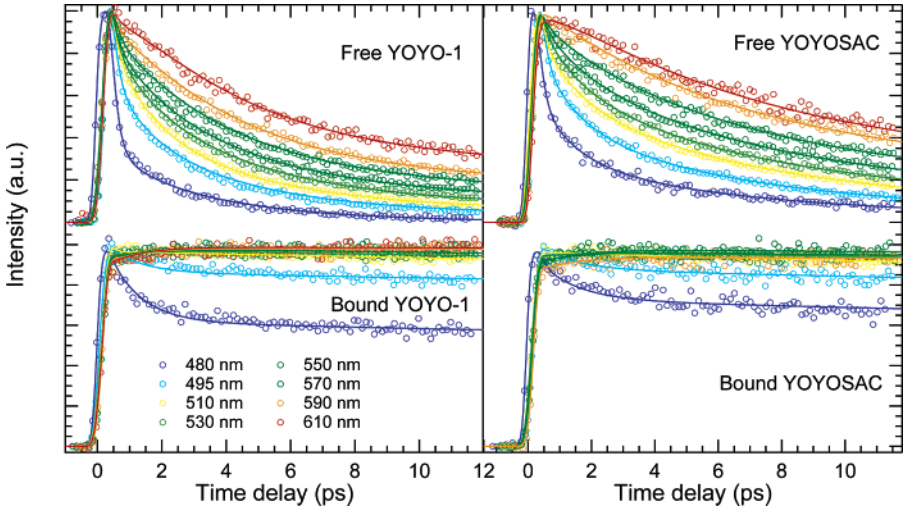

Figure 5. Wavelength dependence of the early fluorescence dynamics of YOYO-1 and YOYOSAC, free in water and bound to DNA.

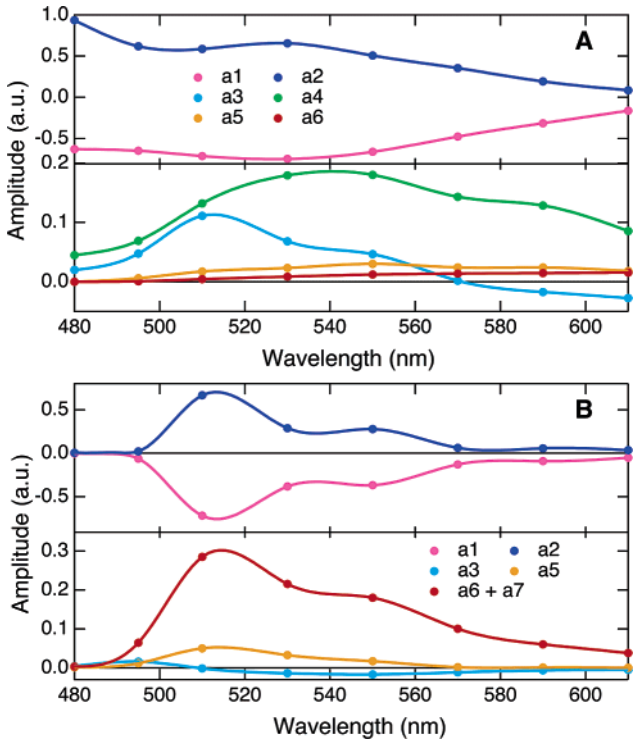

Figure 6. Wavelength dependence of the amplitude factors obtained from the global analysis of the fluorescence dynamics of free (A) and DNA-bound (B) YOYO-1.

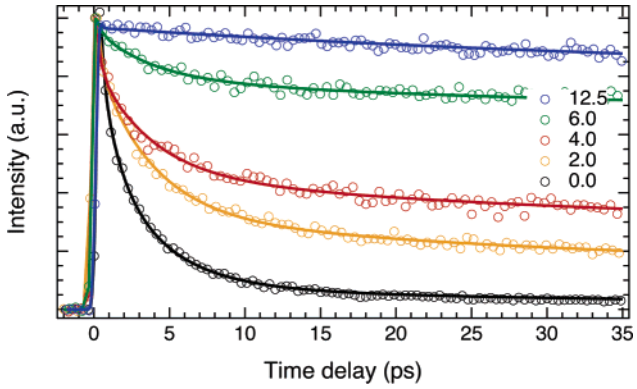

Figure 7. Influence of the DNA base:dye ratio on the early fluorescence dynamics of YOYO-1.

independently of the wavelength. This feature, observed with these two components, is very similar for all investigated dyes in the presence or in the absence of DNA. The amplitude associated with τ_3 is positive at short wavelength and becomes negative when going to the red. This corresponds to a red shift of the fluorescence spectrum and not to a decay of the excited-state population. While the magnitude of the first two components does not vary noticeably upon DNA binding, that of τ_3 is substantially smaller with the bound dyes.

The 3−6 ps component, τ_4, was only found with the free dyes. Its amplitude strongly depends on the DNA concentration: it is the largest component with the free dyes and ultimately drops to zero when all dye molecules are bound to DNA (Figure 7). This amplitude is always positive and matches rather closely the steady-state emission spectrum, indicating that this component is associated with the decay of the excited-state population of the free dyes. The fluorescence dynamics of YOYO-1 and YOYOSAC was also measured in organic solvents

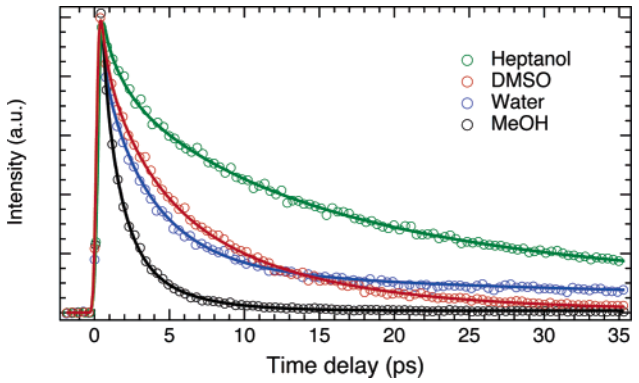

Figure 8. Fluorescence dynamics of YOYO-1 in various solvents measured at the fluorescence maximum (organic solvents, 510 nm; aqueous buffer, 550 nm).

Table 3. Time Constants of the Dominant Decay Component of the Fluorescence of YOYO-1 in Solvents of Various Viscosity, η

solvent	τ (ps)	η (cP)
methanol	1.2	0.54[a]
water	3.7	0.89[a]
DMSO	6.2	1.99[a]
heptanol	10.2	7.42[b]

[a] From ref 32. [b] From ref 33.

Table 4. Best Fit Parameters Obtained from the Analysis of the Fluorescence Anisotropy Decay of YOYO-1 at Various DNA Base:Dye Ratios

DNA:dye	r_0	r_1	τ_{r1} (ps)	r_2	τ_{r2} (ps)	r_∞
0	0.25	0.25	60	0		0
4	0.25	0.14	3.3	0.08	68	0.03
12.5	0.30	0.13	2.9	0.08	55	0.09
25	0.30	0.12	2.6	0.08	51	0.10
50	0.27	0.10	2.3	0.07	40	0.10

at the fluorescence maximum wavelength (Figure 8). It is dominated by a decay component with a time constant varying from 1 to 10 ps depending on the dye and the solvent viscosity (Table 3). Figure 8 shows that, in DMSO and MeOH, the fluorescence decays to zero within 35 ps and does not exhibit any long-lived components, such as in water.

An additional decaying component, τ_5, with a time constant between 30 and 50 ps, was found both in water and inside DNA. Its amplitude is positive at all investigated wavelengths and is similar to the steady-state emission spectrum.

The decay of the fluorescence anisotropy of the free dyes was found to decay exponentially to zero with a time constant of ca. 60 ps (Table 4). Due to the short average fluorescence lifetime of the free dyes, the error on this time constant is quite large.

Although large amplitude motion of the fluorescent probes is strongly hindered in DNA, the decay of the fluorescence anisotropy of DNA-bound YOYO-1 and YOYOSAC was found to have an ultrafast component with a time constant, τ_{r1}, of ca. 3 ps and an absolute amplitude, r_1, of about 0.12 independently of the DNA base:dye ratio, as shown in Figure 9 and Table 4. An additional decay component with a time constant, τ_{r2}, around 50 ps was also observed. However, these two components do

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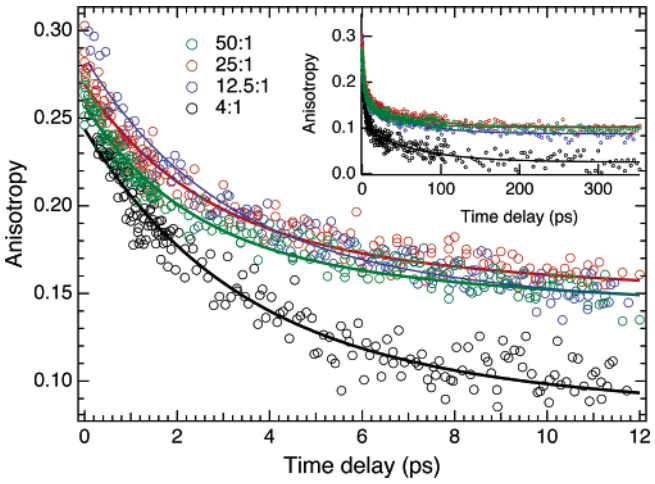

Figure 9. Decays of the fluorescence anisotropy measured with YOYO-1 at different DNA base:dye ratios.

not lead to a total depolarization of the fluorescence, and a residual anisotropy, r_∞, was measured as illustrated in the inset of Figure 9. At low DNA base:dye ratio, its magnitude increases with increasing DNA concentration up to a constant value of about 0.1. Steady-state polarized fluorescence measurements with DNA-bound dyes indicate a similar anisotropy value. As discussed in more detail in the next section, such an ultrafast anisotropy decay is evidence of the occurrence of an intramolecular excitation energy hopping between two dimer subunits intercalated in DNA.

Discussion

Ultrafast Isomerization Dynamics of the Free Dyes. The strong enhancement of the fluorescence intensity upon DNA binding of YOYO-1 has been attributed to the inhibition of nonradiative deactivation of the excited state via isomerization. Knowing the fluorescence quantum yields of the free and bound forms of YOYO-1 in addition to the excited-state lifetime of the latter, Netzel et al. have estimated the excited-state lifetime of free YOYO-1 to be of the order of a few picoseconds.[18] Such an estimation is, however, only valid if the radiative lifetime of the dye, k_{rad}, does not change upon intercalation. The spectacular changes in both the absorption and fluorescence spectra of the dyes upon DNA binding indicate that this assumption may not be rigorously valid. Despite this, our upconversion measurements, performed with the required subpicosecond resolution, indeed confirm that the first singlet excited state of the free dyes is very short-lived. Among the various components observed in the ultrafast fluorescence dynamics of the free dyes, the 4−6 ps component, τ_4 in Table 2, can be safely attributed to the decay of the excited-state population by a process associated with a large amplitude motion. First, the associated amplitude spectrum is similar to the steady-state fluorescence spectrum (Figure 6A), indicating that this component is due to population dynamics and not to solvent or vibrational relaxation. Second, the amplitude of this component decreases with increasing DNA concentration (Figure 7) and vanishes at high DNA base:dye ratio. Third, the time constant increases substantially with solvent viscosity (Table 3) as expected for a process involving large amplitude motion, such as rotation around the monomethine bridge of the dyes.[34−36] The increase of τ_4 by going from YOYO-1 (3.7 ps) to YOYO-Cl (5.7 ps) and YOYOSAC (5.9 ps) could further be attributed to a slowing down of this isomerization dynamics due to the increasing volume of the moving groups. It is interesting to note that these differences are very small, and that all three YOYO-dyes behave very similarly.

Although isomerization times of 60 fs have been reported in the particular environment of a protein,[37] the isomerization process taking place with these YOYO-dyes is one of the fastest measured in a homogeneous condensed environment. It is indeed only slightly slower than the photoisomerization of *cis*-stilbene, which takes place in about 1 ps in *n*-hexane.[38] This indicates that there is essentially no activation barrier for the photoisomerization of these dyes apart from that associated with friction.

Intramolecular Aggregation as an Additional Fluorescence Contrast Mechanism. Figures 2 and S1 show that the absorption spectra of the YOYO-dyes in water substantially change upon binding to DNA. This change cannot be simply ascribed to a solvatochromic shift as the absorption spectra of the free dyes in polar organic solvents, such as methanol, heptanol, and glycerol, are very similar to those of the bound forms in aqueous solutions (Supporting Information, Figure S2). The "anomalous" absorption spectra of the free dyes in water can be ascribed to the formation of aggregates. As the spectra do not vary with dye concentration, the aggregation takes place between the two YO subunits of the dimeric dyes. Aggregation of cyanine dyes is a well documented phenomenon, and two types of aggregates are known.[39−42] J-aggregates are characterized by a intense and red-shifted absorption band, and a head-to-tail arrangement of the chromophores, where the transition dipole moments are collinear, has been suggested.[41,43] On the other hand, H-aggregates exhibit a blue-shifted absorption spectrum, which has been explained in terms of a ladder-type arrangement of the chromophores where the transition dipole moments are parallel.[41,43] Considering the structure of the YOYO-dyes, intramolecular H-aggregation is certainly more probable than J-aggregation. Moreover, the blue shift of their absorption band in water compared to DNA is consistent with H-aggregates. This aggregation is mainly driven by hydrophobic interactions and thus does essentially not take place in organic solvents. As discussed in more detail below, the intercalation of the dye in DNA also prevents aggregation. Whereas other classes of cyanines use DNA as a template for intermolecular aggregation[44−46] or have to be dissolved at relatively high concentrationsto form

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most often J-aggregates,[47,48] the YOYO-dyes do not require such conditions because of the preorganization induced by the linker between the two chromophores, making intramolecular aggregation on one hand easily possible in water (no concentration dependence) and on the other allowing a dihedral angle close to 90° in DNA, thus disrupting the excitonic interaction. The short interchromophoric distance in the aggregate, of the order of 3.5−4 Å, is responsible for a strong excitonic coupling. In the lowest excitonic state, the transition dipole moments of the two chromophores more or less cancel out, resulting in an almost complete loss of oscillator strength for the lower energy transition. Essentially, the whole oscillator strength is associated with the transition to the upper excitonic state, causing the shift of absorption band from 491 to 459 nm upon aggregation. From this shift, an excitonic coupling energy of 1400 cm^{-1} can be deduced. Similar energies have been reported for the coupling in other H-aggregates of cyanine dyes.[19,42,45,49] Divergence of a few hundreds of wavenumbers can be explained by the different chemical structures of the dyes and also by the dissimilar aggregation mechanism. The transition to the lowest excitonic states should, in principle, be characterized by a very weak absorption band red-shifted by the same energy, that is, around 525 nm. This transition might be responsible for the enhanced absorption above 510 nm of the dyes in water compared to DNA (Figures 2 and S1).

The broad fluorescence spectra of the free dyes in water have until now been attributed to the existence of a large distribution of conformations of the free dye in solution.[29] The wavelength dependence of the fluorescence excitation spectrum of YOYO-1 in water shown in Figure 4 indicates that this broad emission band can be ascribed to two forms of the free dyes: (1) the nonaggregated dyes with a spectrum that must be similar to that in DNA and with a similarly large radiative rate constant, and (2) the aggregated dyes with a red-shifted spectrum, mirror image of the absorption band associated with the lowest excitonic state and with a small radiative rate constant. This is further supported by the fact that in organic solvents, where aggregation is not operative, the fluorescence spectrum of the dyes is the mirror image of the absorption spectrum and is thus very similar to the fluorescence spectrum in DNA.

Both these two forms contribute to the fluorescence quantum yield, but the shape of the spectrum indicates that the contribution of the aggregates is larger. Although the nonaggregated dyes have a large radiative rate constant, their contribution to the fluorescence quantum yield is small because of their short excited-state lifetime and also because of their relatively weak concentration. The radiative rate constant of the nonaggregated dyes in water, calculated from that in DNA after correction for the different refractive indices,[50] amounts to about 9.1×10^7 s^{-1}. On the other hand, the larger contribution of the aggregates can be due to their larger relative concentration and to a longer excited-state lifetime that compensates for the small radiative rate constant. Indeed, isomerization that is ultrafast with the nonaggregated dyes can be expected to be sterically slowed down by the close proximity of the two chromophores in the aggregates. The fluorescence of the aggregates might be responsible for the decay component with the time constant τ_6 ranging between 1.1 and 1.6 ns (see Table 2) measured with the free dyes in water. As shown in Figure 7, the spectrum of the amplitude associated with this decay time, a_6, is weak and red-shifted compared to that associated with the isomerization, a_4. In other words, the fluorescence measured at short time delays (<20 ps) with the free dyes in water is essentially due to the nonaggregated dyes. This is further supported by the absence of any long-lived component in the fluorescence decay of the dyes in organics solvents (Figure 8).

The enhancement of the fluorescence quantum yield upon DNA binding is thus due to two phenomena: (1) the inhibition of the ultrafast isomerization of the nonaggregated dyes, and (2) the disruption of the excitonic coupling upon dissociation of the aggregates. Excitonic interaction in YOYO-1 is widely accepted in the literature,[11,29,51] but with one exception,[19] it has not been considered as a mechanism which reduces the emission of the free form of the dye. It is interesting to note that, if aggregation was not taking place, the fluorescence contrast between free and DNA-bound dyes would be even larger than that observed. Indeed, multiplying the radiative rate constant of 9.1×10^7 s^{-1} by τ_4 results to a theoretical fluorescence quantum yield smaller by a factor 3−7 than those listed in Table 1. In other words, aggregation is not as efficient as isomerization as a fluorescence contrast mechanism, at least for the dyes investigated here.

Origin of the Ultrafast Fluorescence Dynamic Components. Several ultrafast components are observed in the fluorescence dynamics of the dyes, both free and bound to DNA, additionally to that associated with isomerization. The two fastest components (100−200 fs) have time constants smaller than the response function of our apparatus. However, they have to be included to properly reproduce the collected data, independently on the dye and the presence of DNA. The Gaussian component, which has negative amplitudes at all wavelengths, is very important to describe the initial rise of the fluorescence. This rise, which introduces an initial delay between excitation and the start of the emission, might be associated with vibrational relaxation as excitation is performed at 400 nm, that is, in the far blue edge of the S_0-S_1 absorption band. Indeed, some intramolecular relaxation might have to take place before the Franck−Condon factor for the S_1-S_0 emission is sufficiently large. The second ultrafast component with the time constant, τ_2, might also be due to vibrational relaxation. Its amplitude is positive at all wavelengths and seems to match the steady-state fluorescence spectrum quite well. However, as both τ_1 and τ_2 are very short and have similar magnitude but opposite amplitude, the error on a_1 and a_2 is very large. Therefore, given this uncertainty on both the lifetimes and the amplitudes, it is not possible to give a precise assignment of the origin of these components. It is, however, important to note that these components are essentially unchanged upon DNA binding.

As shown in Table 2, the component with a time constant of ca. 1 ps, τ_3, was observed with all three dyes. The spectrum of the associated amplitude, a_3, indicates that this process corresponds to a red shift of the fluorescence spectrum and not to a

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population decay. Therefore, this time constant can be ascribed to solvation. The solvation dynamics of water has been shown to be essentially biphasic, with inertial motion occurring within a few tens of femtoseconds and diffusive motion in the 1 ps time scale.[27,28,52,53] In the case of the free dyes in water, this 1 ps component can thus be safely ascribed to the diffusive solvation of water.

Interestingly, this 1 ps dynamic Stokes shift of the fluorescence is still observed with the DNA-bound dyes, although the chromophores could be expected to be in a hydrophobic environment. Its relative magnitude is, however, substantially smaller than that for the free dyes. The origin of this component is not absolutely clear. As the time constant is the same as that in bulk water, it is very tempting to attribute it to the diffusive motion of water molecules surrounding DNA. Intercalation of the YOYO-dyes is known to partially unwind the DNA helix[10] and might favor the access of water near the chromophores. The absence of any other Stokes shift component seems to point out that there is no significant reorganization of the DNA interior upon excitation of the dyes, unless it takes place on a time scale very similar to that of water. Zewail and co-workers have reported a biphasic dynamic fluorescence Stokes shift with a dye bound in the minor groove of DNA. The major component, with a time constant of 1.1−1.4 ps, was ascribed to bulk water, while the smaller component with a time constant around 20 ps was assigned to water molecules "ordered" at the DNA surface.[54,55] On the other hand, a recent report on the dynamic fluorescence Stokes shift measured with a coumarin replacing a base pair in DNA indicates a solvation dynamics following a power-law kinetics over six decades in time.[56] Moreover, no distinct component that could be attributed to bulk water was observed. The difference between all these results may originate from the nature of the solvation probe. The YOYO-dyes investigated here are certainly much less sensitive polar probes than coumarin and than that used in ref 54, and may thus only reveal the main solvation component. Moreover, the exposure of the probe to water might also depend very strongly on its location in DNA. Indeed, the coumarin probe investigated in ref 56 can be expected to be less exposed to water than the YOYO-dyes.

Finally, the origin of the 30−50 ps component is unclear. This component, whose amplitude, a_5, is very weak, is present in both free and DNA-bound dye emissions, but the similarity of the time constants and amplitudes might be coincidental. The spectral dependence of the amplitude suggests a process associated with population dynamics. Furthermore, DNA breathing takes place on much slower time scales.[57] This component might be due to the dyes attached to DNA by groove binding and for which photoisomerization is less inhibited than for the intercalated dyes. For the free dye, this component might be associated with aggregates. A distribution of aggregate structures should, in principle, lead to a distribution of fluorescence decays, which, as discussed above, can be well reproduced by a biexponential function. The decay components with time constant τ_5 and τ_6 might thus correspond to such a distribution. However, other hypotheses cannot be excluded.

Decay of the Polarization Anisotropy. Keeping in mind that essentially only the nonaggregated dyes are monitored in time-resolved fluorescence experiments, the 60 ps decay of the fluorescence anisotropy measured with the free dyes in water can be reasonably assigned to the reorientational motion of the excited chromophore. Indeed, quasi-free rotation of the YO moieties in the nonaggregated dyes should be enabled by the flexible linker. The reorientational time, τ_{or}, can be estimated using the Stokes−Einstein−Debye equation, $\tau_{or} = V\eta/k_BT$,[58,59] where V is the volume of the rotating body and η the solvent viscosity. With a molecular volume of 262 Å^3 for YO estimated from the van der Waals increment method,[60] this equation results in a reorientational time of 65 ps at 20 °C in water, in very good agreement with the experimental value. If these dimeric dyes were rigid, rotational motion in water would occur with a time constant of about 200 ps.

The decay of the fluorescence anisotropy measured in DNA-bound dyes is clearly not due to rotational motion, but can be ascribed to excitation energy hopping between the two chromophores of the dimeric dye. Upon DNA intercalation, the strong excitonic interaction present in the intramolecular aggregates vanishes. The NMR structure of the structurally related dye TOTO-1 intercalated in DNA (available in the protein databank, file 108D)[9] shows that the two chromophores of the dye are coplanar and at a distance of about 9.5 Å. The length of the linker is such that, upon intercalation into DNA, two base pairs are "sandwiched" between both chromophores. The coupling between the chromophores is now too weak to give rise to two excitonic states, but is however large enough to enable energy hopping. Using the point dipole approximation, which can be reasonably assumed at this distance, the rate constant of energy hopping, k_{EH}, is[61]

$$k_{EH} = 1.18 \times V^2\Theta \tag{3}$$

where k_{EH} is expressed in ps^{-1}, Θ is the overlap integral between the donor emission and acceptor absorption spectra with the area normalized to 1 on the cm^{-1} scale, and V is the dipole−dipole coupling energy in cm^{-1}. The latter is given by[62]

$$V = \frac{5.04 \times |\mu|^2 f_L^2 \kappa}{\epsilon_{op} d^3} \tag{4}$$

where $|\mu|$ is the magnitude of the transition dipole moment in D, κ is the orientational factor, d is the distance in nm, $f_L = (\epsilon_{op} + 2)/3$ is the Lorentz local field correction factor, and ϵ_{op} is the dielectric constant at optical frequencies, $\epsilon_{op} \approx n^2$, n being the refractive index of the surrounding medium.

To estimate V and k_{EH} for the YOYO-dyes in DNA, the relative orientation of the two chromophores in DNA has to be

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known. As this is not the case for YOYO-1, the parameters reported for TOTO-1 were used.[9] From this structure, the orientational factor amounts to $\kappa = 0.12$ only. This very small value is due the fact that, although coplanar, the two chromophoric units make a dihedral angle of ca. 83°.[9] Considering the natural pitch of DNA in the B-form of one turn every 3.5 nm,[63] a dihedral angle of about 98° could have been expected. The smaller angle measured experimentally is evidence of the unwinding effect of intercalation. With $|\mu| = 7.75$ D for YO,[11] and a refractive index of 1.5, the dipole−dipole coupling energy amounts to $V = 38$ cm^{-1}. This coupling is of similar magnitude to that found between the bacteriochlorophyll pigments B800 and B850 of the light harvesting antenna of photosynthetic bacteria and where the B800 to B850 excitation energy transfer occurs with a time constant of 1.2 ps.[64] This V value also confirms that the excitation is indeed localized on a single YO chromophore. With these parameters and a spectral overlap integral, Θ, of ca. 6×10^{-5}, eq 3 predicts an energy hopping rate constant of $k_{EH} = 0.1$ ps^{-1}. This value cannot be directly compared to the rate constant of anisotropy decay, τ_r^{-1}. Indeed, if the decay of the anisotropy is due to a reversible hopping of the excitation energy between the two chromophores, the relationship between the anisotropy decay time, τ_r, and the rate constant of energy hopping, k_{EH}, is $\tau_r^{-1} = 2k_{EH}$. Thus the anisotropy data indicate an energy hopping time, τ_{EH}, of about 6 ps, while Förster theory predicts 10 ps.

As the transition dipoles are close to orthogonal, a small deviation from the value of 83° used in the calculation has a strong repercussion on τ_{EH}. Indeed, the calculated τ_{EH} goes from 4.5 to 470 ps by varying this angle from 80 to 89°, a value of 6 ps being found with an angle of 81°.

The slower component of the anisotropy listed in Table 4 has a decay time τ_{r2} close to that measured with the free dye and thus could, in principle, be ascribed to dyes where only one chromophore is intercalated in DNA. However, this assignment can be ruled out, first because the amplitude of this component is independent of the DNA base:dye ratio and second because ultrafast photoisomerization would not be totally suppressed and thus the fluorescence quantum yield would be much smaller than that measured. Therefore, this slower decay component is most probably also due to energy hopping. Because of the strong dependence of τ_{EH} on the relative orientation of the chromophores, the biphasic decay of the fluorescence anisotropy to a constant value of about 0.1 could be due to a distribution of the relative orientations of the chromophores in DNA. Simulation of the fluorescence anisotropy decay, assuming a Gaussian distribution of angles centered at 83° with a full width at half-maximum of 10°, results in a decay that can be perfectly reproduced with a biexponential function with time constants of 3.8 and 42 ps. Such a distribution of angle is reasonable as intercalation does not involve any bond between the dye and DNA.

The initial value of the anisotropy, r_0, is substantially smaller than the expected value of 0.4. This difference is most probably due to the fact that excitation was carried out at 400 nm, that is, rather far from the maximum of the S_0-S_1 absorption band. Vibronic coupling might introduce some admixture from upper excited states that could affect the orientation of the dipole moment of vibronic transitions.[65,66]

Finally, the residual anisotropy value indicates that energy hopping does not result in a total depolarization of the fluorescence. After a single energy hopping from the originally excited chromophore to the other one, the polarization anisotropy should, in principle, decay from 0.4 to −0.19. However, as the energy hopping is reversible, a final anisotropy value of ca. 0.1 should be achieved upon randomization of the excitation. This value agrees well with that found experimentally.

Conclusion

The investigation presented here gives a much clearer picture of the origin of the remarkable fluorescence enhancement of YOYO-dyes upon interaction with DNA. The excited-state dynamics of the free dyes in water could be resolved for the first time, and the time scale of the ultrafast nonradiative deactivation process could be firmly established. However, the present study shows that this ultrafast process is not the main reason for the strong fluorescence enhancement of the dyes upon DNA binding in water. Indeed, most of the dyes in water form intramolecular H-type aggregates that are essentially nonfluorescent because of a strong excitonic interaction, which is clearly visible in the absorption and emission spectra. Intercalation of the dyes in DNA results in both the inhibition of the ultrafast nonradiative deactivation of the chromophores and the disruption of the excitonic interaction in the aggregates. Interestingly, the suppression of the excitonic interaction is not really caused by the distance between the chromophores in DNA but rather by their quasi-orthogonal relative orientation. Indeed, if both chromophores were parallel, the dipole−dipole interaction energy V would be sufficiently large, $V \approx 300$ cm^{-1} according to eq 4, to enable the presence of two excitonic states. Although intercalated into DNA, the YOYO-dyes would in this case be essentially nonfluorescent.

Acknowledgment. We thank Cyril Nicolas for his assistance with the NMR experiments. This work was supported by the Fonds National Suisse de la Recherche Scientifique through Project No. 200020-107466/1.

Supporting Information Available: Details of YOYO-Cl synthesis, absorption spectra of YOYO-dyes in various solvents and with DNA, and time-correlated single photon counting traces. This material is available free of charge via the Internet at http://pubs.acs.org.

JA0609001

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