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ORIGINAL PAPER

Is photocleavage of DNA by YOYO-1 using a synchrotron radiation light source sequence dependent?

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Abstract The photocleavage of double-stranded and single-stranded DNA by the fluorescent dye YOYO-1 was investigated in real time by using the synchrotron radiation light source ASTRID (ISA, Denmark) both to initiate the reaction and to monitor its progress using Couette flow linear dichroism (*LD*) throughout the irradiation period. The dependence of *LD* signals on DNA sequences and on time in the intense light beam was explored and quantified for single-stranded poly(dA), poly[(dA-dT)$_2$], calf thymus DNA (ctDNA) and *Micrococcus luteus* DNA (mlDNA). The DNA and ligand regions of the spectrum showed different *LD* kinetic behaviors, and there was significant sequence dependence of the kinetics. However, in contrast to expectations from the literature, we found that poly(dA), mlDNA, low salt ctDNA and low salt poly[(dA-dT)$_2$] all had significant populations of groove-bound YOYO. It seems that this mode was predominantly responsible for the catalysis of DNA cleavage. In homopolymeric DNAs, intercalated YOYO was unable to cleave DNA. In mixed-sequence DNAs the data suggest that YOYO in some but not all intercalated binding sites can cause cleavage. It is also likely that cleavage occurs at transient single-stranded regions. The reaction rates for a 100 mA beam current of 0.5-μW power varied from 0.6 h^{-1} for single-stranded poly(dA) to essentially zero for low salt poly[(dG-dC)$_2$] and high salt poly[(dA-dT)$_2$]. At the conclusion of the experiments with each kind of DNA, uncleaved DNA with intercalated YOYO remained.

Keywords YOYO-1 · Linear dichroism · Synchrotron radiation · DNA cleavage · Sequence dependence

Abbreviations

A	Adenine
ctDNA	Calf thymus DNA
C	Cytosine
DMSO	Dimethyl sulfoxide
G	Guanine
LD	Linear dichroism
mlDNA	*Micrococcus luteus* DNA
T	Thymine

Introduction

YOYO-1 (Fig. 1, hereafter referred to as YOYO) is a dimer of oxazole yellow (YO) that has proved to be a very effective DNA stain since oxazole yellow only fluoresces when bound to DNA, and the dimer has a very high binding constant of the order of 10^{10}–10^{12} M^{-1} (Larsson et al. 1994). The oxazole yellow monomer consists of a quinoline and an oxazole ring linked in a conjugated fashion; two monomers are linked to make the dimer YOYO via a biscationic linker (Larsson et al. 1994; Eriksson et al. 2003). In addition to being an effective DNA stain, YOYO is also a DNA cleavage agent when it is irradiated with UV light (Li et al. 2003).

It has been established that YOYO has two modes of binding to DNA: bisintercalation and groove binding

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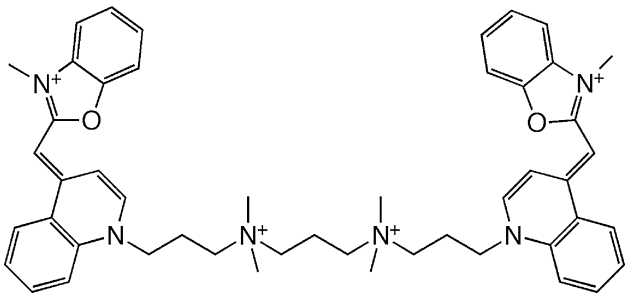

Fig. 1 YOYO-1 dye

(Larsson et al. 1994). The bisintercalation mode is operative at dye loadings in mixed sequence DNAs of less than 8 DNA bases:1 dye molecule. Due to the two intercalating moieties, YOYO intercalative binding results in a binding motif resembling a clamp structure. This geometry, together with its tetravalent positive charge, causes a reduction in the persistence length of DNA when YOYO intercalates (Sischka et al. 2005). This contrasts with normal intercalators, which increase the persistence length by unwinding and stiffening the DNA (Nordén et al. 2010). Although bisintercalation is the more stable binding mode for YOYO, the groove-binding mode has been shown to be more effective at cleaving the DNA, especially in an oxygenated environment. In a reduced-oxygen environment, the bisintercalation mode becomes more significant and perhaps dominates the photocleavage (Akerman and Tuite 1996).

It has also been found that YOYO shows only weak sequence selectivity in its DNA binding, so its DNA binding is considered to be random (Flyvbjerg et al. 2006; Keatch et al. 2004); however, it has been suggested that the type of sequence of the DNA affects the type of binding that is exhibited by the YOYO dye. It has been speculated that the torsion about the methine chain that links the quinoline and the oxazole ring can be changed depending on the DNA sequence to which it binds, which could lead to stiffening of the dye (Keatch et al. 2004). It has also been suggested that oxygen may have a role in binding, and, with guanine being easier to oxidise than adenine, this could affect the type and strength of binding to the different sequences (Netzel et al. 1995).

The main application of YOYO dye is as a non-sequence specific fluorescent DNA stain, for example, for visualizing DNA in optical microscopy (Geron-Landre et al. 2003). YOYO has a different fluorescence emission wavelength maxima when bound to single-stranded (518 nm) and double-stranded DNAs (516 nm), which may be used to identify how much of the DNA is single-stranded (Cosa et al. 2001). For this application, it is unfortunate that cleavage of the DNA in the DNA-YOYO complex occurs in the presence of intense light. Some work has been done on the mechanism of the cleavage process. The YO monomer causes single-strand breaks, whereas the YOYO dimer produces what appears to be double-strand breaks (Cosa et al. 2001). However, this has been concluded to be the result of two monomers being in close proximity, so that single-strand breaks are most likely to occur opposite one another, thus breaking both strands (Akerman and Tuite 1996). These experiments were conducted by diluting the YOYO (1 mM solution in DMSO) in water, thus keeping the ionic strength of the solution down. It is known that the dissociation rate of the YOYO dye from DNA is dependent on ionic strength and occurs faster in high salt concentrations (Eriksson et al. 2003). Therefore, to simplify any kinetic analysis, in this work it has been deemed best to use as low an ionic strength as possible.

Despite the literature available on DNA-YOYO complexes, kinetic analysis has been limited to the extensive study of Åkerman and Tuite (1996) who used supercoiled and nicked circular DNA from ΦX174 DNA, a xenon lamp, and monitored the photocleavage products using gel electrophoresis. The gel methodology with supercoiled DNA has the advantage of enabling differently cleaved species to be identified, but has the significant disadvantage of being extremely labor intensive and time consuming, requiring sampling at appropriate time points and loading onto a gel. In this work we chose to monitor the photocleavage of DNA by YOYO in real time by using a synchrotron radiation light source to irradiate the samples and simultaneously to measure the linear dichroism (*LD*) of the samples.

Linear dichroism is the differential absorbance of light polarized parallel and perpendicular to an orientation direction.

$$LD = A_{//} - A_{\perp} \quad (1)$$

By flow orienting the DNA-YOYO samples in a micro-Couette flow cell in the light beam, one provides sufficient orientation to give a good *LD* signal using 50–60 μl of sample (Marrington et al. 2004). When linear DNA is cleaved, the *LD* signal reduces in magnitude (Hicks et al. 2006), which enables one to monitor the progress of the cleavage. Given the assumption that YOYO binding is not sequence selective, our basic questions were

How quickly does the cleavage take place? And

Is there a sequence preference to the cleavage?

The different DNA types used in this work were (in order of decreasing G-C content): double-stranded poly[(dG-dC)$_2$], double-stranded *Micrococcus luteus* DNA (mlDNA) with 72% GC content, double-stranded calf thymus DNA

(ctDNA) with 42% GC content, double-stranded poly[(dA-dT)$_2$] and single-stranded poly(dA), both of which have 0% GC. Our aim was to determine how different GC contents affect the kinetics of the photocleavage of DNA by intercalated YOYO.

Materials and methods

Alignment of the DNA is achieved via shear flow in a microvolume Couette *LD* cell (Marrington et al. 2004, 2005) manufactured by Dioptica Scientific Ltd., Rugby, UK (now available via Kromatek UK Ltd.). In this case the sample is placed in a quartz capillary into which a quartz rod is inserted. The capillary is rotated at ~3,000 rpm, creating a gradient of flow in the fluid sample. The incorporation of synchrotron radiation to create a higher intensity light source is a relatively new improvement (Marshall et al. 2010; Dicko et al. 2008; Hicks et al. 2009; Rittman and 2010). The intense light source created by the synchrotron was the required element to allow for real-time *LD* measurement of the photocleavage to be recorded. Measurements were performed using the ASTRID light source linear dichroism instrument at 30°C. The instrument is described in detail in the following references: Dicko et al. 2008; Hicks et al. 2009. We have chosen to plot the *LD* intensity in the units of mV output from the instrument as it provides convenient numbers for easy comparison. The factor of 3.26×10^{-4} arises because the conversion from mV (the output units from ASTRID) to Δ(Absorbance) is 1 mV corresponds to $\Delta A = 3.26 \times 10^{-4}$; 6.5×10^{11} photons/s (at 256 nm = 4.84 eV) and is dependent on the sample. This corresponds to $6.5 \times 10^{11} \times 4.84 \times 1.602 \times 10^{-19}$ J/s, i.e., a power of 0.5 μW. As the beam intensity is to a good approximation linearly dependent on the ring current, the rates determined have also been normalized to a ring current of 100 mA to enable comparison between different experiments at different beam intensities. Baselines of non-spinning samples were subtracted from all spectra. Spectra were further corrected by ensuring the signal was zero at long wavelength, and between 325 and 400 nm by subtracting a straight line.

The samples were obtained from the following places: mlDNA, ctDNA and poly(dA-dT)$_2$ were purchased from Sigma-Aldrich, poly(dA) was purchased from Amersham, and YOYO-1 was purchased from Invitrogen. Distilled water and UV-treated water of 18.2 MΩ.cm purity was obtained from a Elga PURELAB Ultra Genetic System. The DNA stock solutions were ~1 mg/ml, and the concentrations used in experiments were calculated using UV absorption and the Beer-Lambert law using $\varepsilon = 6{,}600$ mol^{-1} cm^{-1} dm^3 for ctDNA, and poly(dA-dT)$_2$, $\varepsilon = 8{,}600$ mol^{-1} cm^{-1} dm^3 for poly(dA) and poly[(dG-dC)$_2$], and $\varepsilon = 6{,}860$ mol^{-1} cm^{-1} dm^3 for mlDNA.

Results and discussion

Mode of YOYO binding to DNA probed by *LD*

In this section the discussion is on what can be deduced from the wavelength dependence of the *LD* spectra. The changes as a function of time are discussed in the next section.

DNA LD (below 300 nm)

Typical data sets of *LD* spectra of the DNA YOYO systems are given in Figs. 2, 3, 4 and 5 for each DNA, with the DNA base:ligand ratio at an average loading of one dye per turn of the DNA helix (nucleotide: YOYO ratio 20:1). We chose 20:1 DNA base:YOYO ratios based on the fact that the literature suggested all YOYO present in the solution would be bound intercalatively under these conditions (Akerman and Tuite 1996). We return to this issue below.

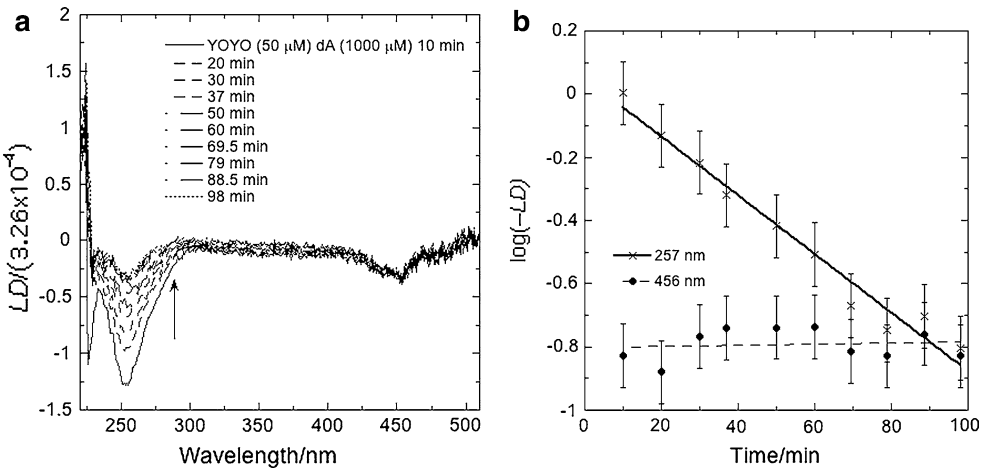

Fig. 2 *LD* of poly(dA) (1,000 μM) with YOYO (50 μM) in 1 mM buffer. **a** As a function of wavelength and **b** logarithm of the minus *LD* (averaged over a 20 nm data interval) as a function of time at a wavelength in the DNA region and a wavelength in the ligand region of the spectrum. Measurements were taken consecutively with the times at the end of each scan shown in the legends on each graph. Beam current was 127 mA

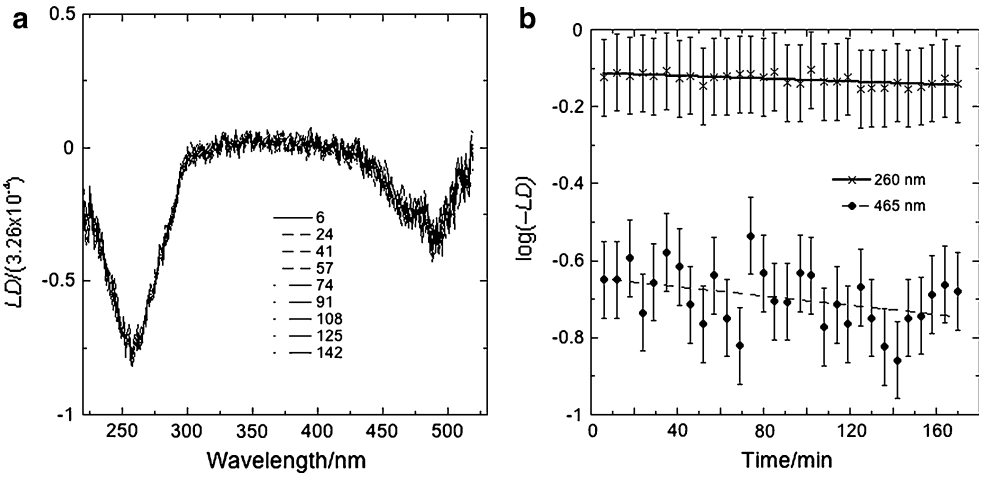

Fig. 3 *LD* spectra of poly[(dG-dC)$_2$] (100 μM in 20 mM NaCl, 1 mM buffer) with YOYO (5 μM). **a** As a function of wavelength and **b** logarithm of the minus *LD* (averaged over 20-nm data interval) as a function of time at a wavelength in the DNA region and a wavelength in the ligand region of the spectrum. Measurements were taken consecutively with the times at the end of each scan shown in the legends on each graph. Beam current 120 mA

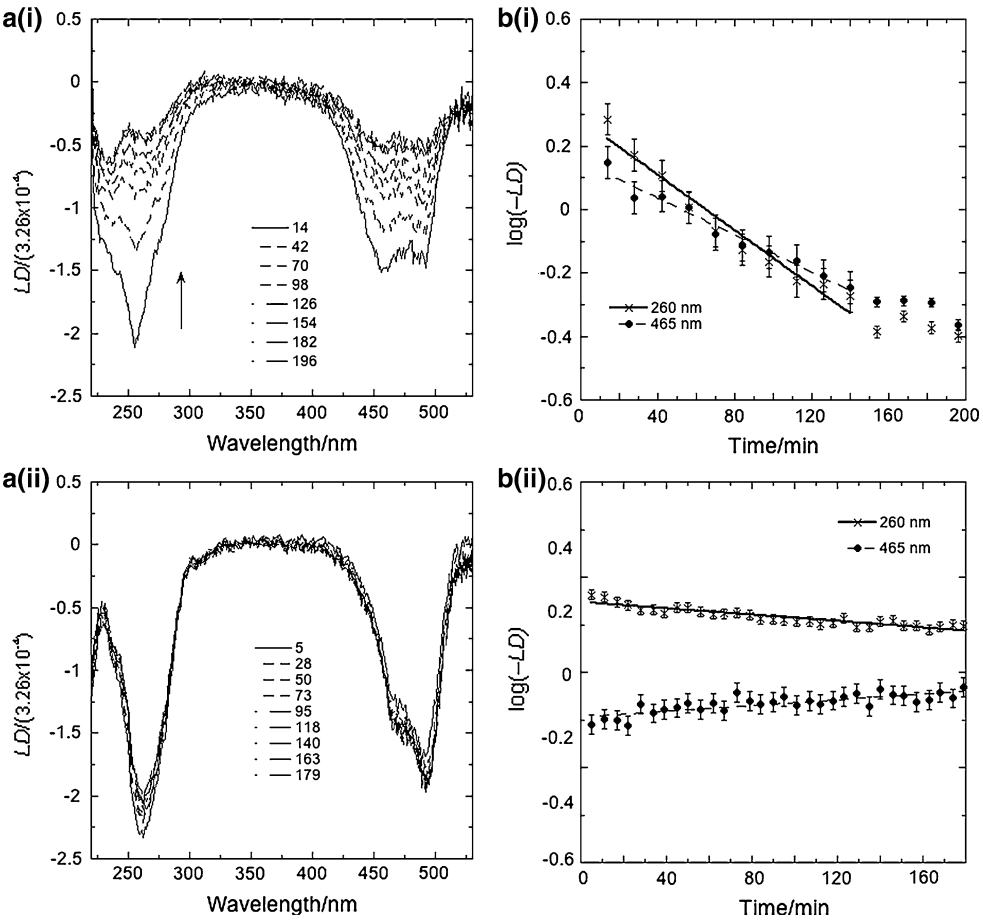

Fig. 4 *LD* spectra of poly[(dA-dT)$_2$] with YOYO at ratios of 20:1 DNA base:YOYO. **a** As a function of wavelength and **b** logarithm of the minus *LD* (averaged over 20-nm data interval) as a function of time at a wavelength in the DNA region and a wavelength in the ligand region of the spectrum. (*i*) poly[(dA-dT)$_2$] (200 μM), beam current 120 mA; (*ii*) poly[(dA-dT)$_2$] (42 μM in 1 mM buffer, 20 mM NaCl), beam current 127 mA. Measurements were taken consecutively with the times at the end of each scan shown in the legends on each graph

The *LD* signal below 300 nm is mainly due to DNA. In each case the sign of this *LD* is negative, indicating that the base pairs are predominantly perpendicular to the orientation (helix) axis even for the single-stranded DNA. The naturally occurring double-stranded DNA samples have larger *LD* signals than the synthetic homopolymers, consistent with their greater lengths. The single-stranded poly(dA) *LD* signal is particularly small (its concentration is at least five times as large as the other DNAs), reflecting the flexible nature of single-stranded DNA. We did also collect data at ∼0 mM ionic strength for poly(dA); however, under these conditions, poly(dA) showed an *LD* signal larger than the wild-type duplex DNAs, indicating some kind of assembled structure, presumably involving protonation of adenines.

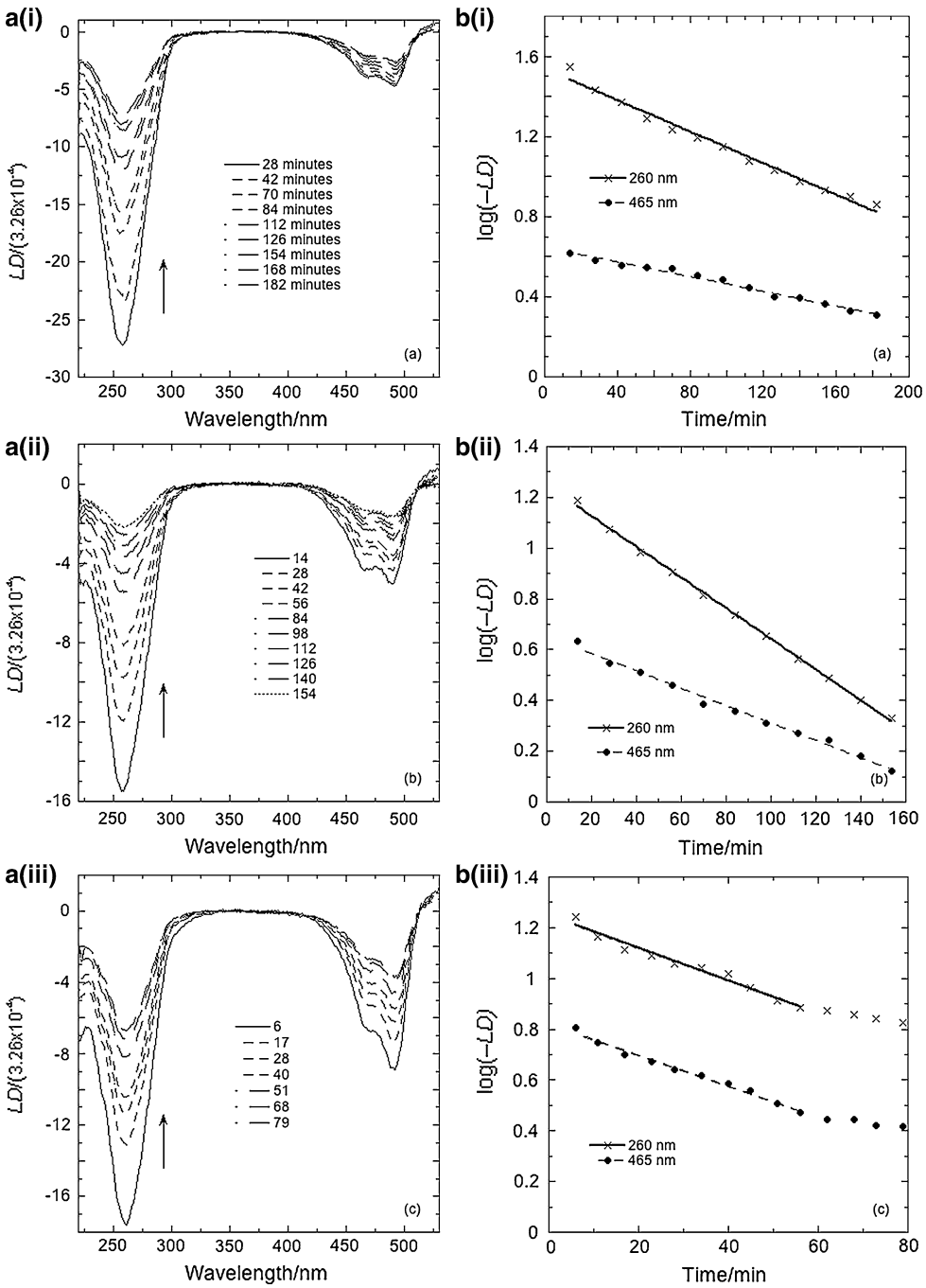

Fig. 5 *LD* spectra of mixed sequence DNAs with YOYO at ratios of 20:1 DNA base:YOYO. **a** As a function of wavelength and **b** logarithm of the minus *LD* (averaged over 5 nm data interval) as a function of time at a wavelength in the DNA region and a wavelength in the ligand region of the spectrum. (*i*) mlDNA (200 μM), beam current 128 mA; (*ii*) ctDNA (200 μM), beam current 102 mA; (*iii*) ctDNA (100 μM, 20 mM NaCl, 1 mM buffer), beam current 123 mA. Measurements were taken consecutively with the times at the end of each scan shown in the legends on each graph

YOYO LD (above 400 nm)

In all cases the YOYO *LD* at ~465 and 490 nm gives two negative overlapping components, indicating that on average the YOYO is more perpendicular than parallel to the DNA helix axis, consistent with a predominantly intercalative binding mode. The fact that the longest wavelength peak is closer to 490 nm than 480 nm is indicative of significant intercalation (Larsson et al. 1994) [except for poly(dA) where this band is hardly present and a very small positive signal is detectable at about 500 nm]. In all cases, again bar poly(dA) and the initial low salt poly[(dA-dT)$_2$], the 490 nm peak is larger (more negative) than 465 nm. The 465 nm transition is deemed to be enhanced and to occur at lower wavelength when YOs are able to interact with one another (Larsson et al. 1994; Furstenberg et al. 2007). This means poly(dA) and the low salt poly[(dA-dT)$_2$] have at least some YOs interacting, which in turn means that they cannot all be intercalated. Thus, our starting assumption, based on literature experiments with

Table 1 Rates of cleavage for different DNAs determined from the gradients of the linear plots in Figs. 2, 3, 4, 5

	λ (nm)	Initial ratio of 260 nm: 482 nm intensity	Final ratio of 260 nm: 482 nm intensity	Rate (h^{-1})	Error (h^{-1})	Beam intensity mA	Normalised current rate h/100 mA
Poly(dA) (1,000 μM) 1 mM buffer	257	3.9	0.97	0.56	0.04	127	0.44
	465			0.011*	0.035	127	0.009
Poly(dG-dC)$_2$ (100 μM)	260	2.4	2.4	0.011	0.003	120	0.009
	465			0.036	0.016	120	0.03
Poly(dA-dT)$_2$ (200 μM)	260	1.5	1.1	0.26	0.020	120	0.22
	465			0.18	0.011	120	0.15
Poly(dA-dT)$_2$ (42 μM) 1 mM buffer; 20 mM NaCl	260	1.4	1.2	0.020	0.0015	127	0.16
	465			0.018*	0.0025	127	0.14
mlDNA (200 μM)	260	6.3	2.8	0.20	0.007	128	0.16
	465			0.13	0.004	128	0.10
ctDNA (200 μM)	260	3.3	1.4	0.37	0.004	102	0.36
	465			0.23	0.010	102	0.22
ctDNA (100 μM) 1 mM buffer; 20 mM NaCl	260	2.3	2.0	0.38	0.016	123	0.26
	465			0.37	0.012		0.24

The DNA base:YOYO ratio is 20:1 in each case. The quoted error is the standard deviation (2σ is a 95% confidence interval for Gaussian distributions) given by the Kaleidagraph curve-fitting routine used. The rates with the beam current normalized to 100 mA (a power of 0.5 μW) are given in the final column. The average flux to which the sample is exposed is assumed to be approximately the average of the flux incident on the 'No capillary/No rod/No sample' configuration and the 'Capillary filled with water/rod' configuration, so at 256 nm the flux is $0.5 \times (8 \times 10^{11} + 5 \times 10^{11}) \sim 6.5 \times 10^{11}$ photons/s/100 mA ring current

* Denotes a trace with an increase in *LD* magnitude, all others are decreases

the mixed sequence DNA supercoiled and nicked circular form ΦX174 DNA (Akerman and Tuite 1996) and T2 DNA (Larsson et al. 1994), that all YOYOs would be intercalatively bound in our experiments was incorrect.

Comparison of DNA and YOYO LD signals

We can gain a further qualitative indication of how important intercalation is to the binding in our experiments by considering the ratios of the *LD* signal at ~260 and ~482 nm. As the binding constant of YOYO with DNA is extremely high, we can assume that all available YOYO is bound in some mode. More intercalation will result in a smaller 260 nm:482 nm ratio (due to more negative *LD* at 482 nm). If 100% of the YOYO is bound intercalatively, at a DNA base:YOYO ratio of 20:1, given that the ratio of YOYO and DNA extinction coefficients is ~15:1 (before any hypochromic effect on YOYO is accounted for), then (assuming also no additional stiffening of the DNA about the intercalator) an *LD* 260 nm:482 nm ratio of ~1.3 is to be expected. Hypochromism could increase this to at most 2.6. From the ratios summarized in Table 1 and the discussion of the kinetics given below, we infer that the ratio for ~100% intercalation is expected to be ~1 for AT DNAs and >2 for GC-rich DNAs. In any case, a 260 nm:482 nm *LD* ratio of higher than 2.6 indicates less than 100% intercalation. Thus, poly(dA), mlDNA, low salt ctDNA and (from above) low salt poly[(dA-dT)$_2$] initially have a significant population of groove-bound YOYO, and at the end of the cleavage reactions have mainly intercalated YOYO. This is further supported by reduced *LD* data for ctDNA-YOYO complexes (collected on a Jasco J-815 spectropolarimeter) shown in (Fig. 6). Low salt ctDNA-YOYO has a lower YOYO LD^r than the DNA region, and the 460-nm signal is less negative than the 490-nm region—which means a significant part of the YOYO population is groove bound. The high salt ctDNA is flat across the YOYO region, which is larger than the DNA signal, consistent with intercalation of YOYO and consequent local stiffening of the DNA about the dye (Nordén et al. 2010).

Kinetics of DNA with YOYO in the presence of an intense light beam

The overall observation from the time dependence of the *LD* spectra is that the ASTRID light beam is intense enough to cause cleavage of the DNA. However, in no case does the DNA completely degrade (the *LD* signal does not completely disappear for any system). In addition, in contrast to the literature conclusion of no sequence dependence of YOYO's DNA binding, there are significant differences in the sequence dependence of the cleavage kinetics. The rate of loss of *LD*, where it occurs, is first order within experimental error as the plots of log(−*LD*) as

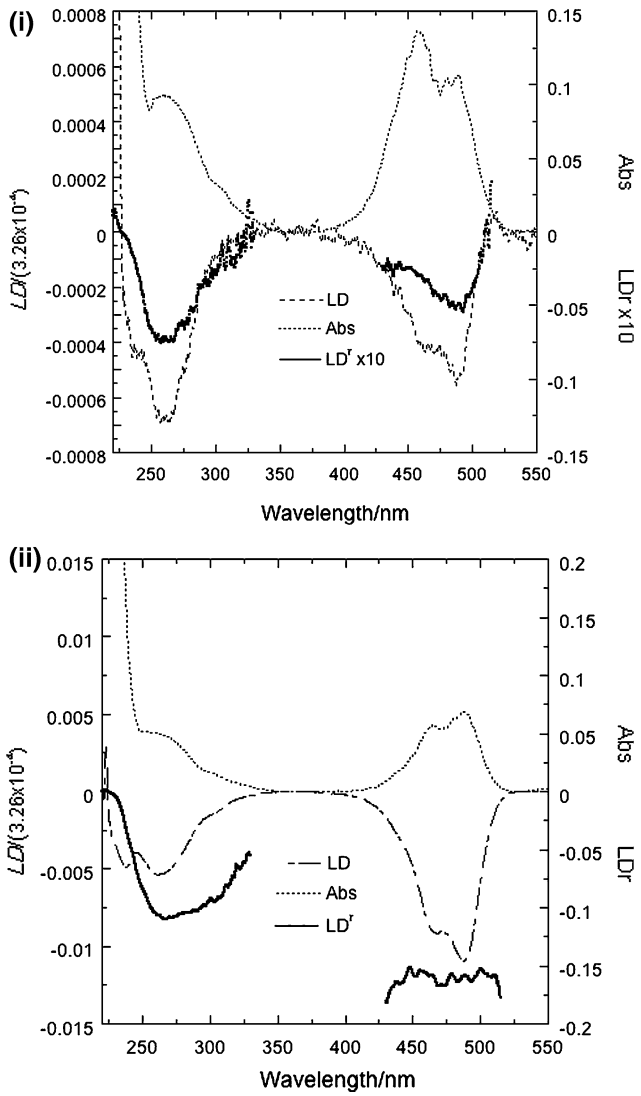

Fig. 6 *LD*, absorbance and LD^r of (*i*) ctDNA (200 μM) and (*ii*) ctDNA (200 μM, 20 mM) with YOYO at ratios of 20:1 DNA base:YOYO. The spectra were measured on a J-815 spectropolarimeter

a function of time indicate (Figs. 2, 3, 4, 5). The rate constants, assuming first order kinetics and that the decrease in *LD* signal is proportional to cleavage, are given in Table 1. For a recirculating system such as the Couette cell, the entire sample is irradiated. The rate will depend on beam intensity so the final column in Table 1 contains the rate adjusted for beam intensity. The 260:482 ratios (see above) for the first and last scan are also given in Table 1.

Kinetics of YOYO-poly(dA)

The *LD* spectra of single-stranded poly(dA) with YOYO in 1 mM pH 7 buffer are shown in Fig. 2a. Initially, the DNA region of the spectrum is approximately four times as intense as the ligand region. Over time the DNA region decreases in a manner suggestive of a first order reaction mechanism (Fig. 2b) with a rate constant 0.6 h^{-1}. The YOYO *LD* signal, by way of contrast, within experimental error, does not change with time. The end point of the DNA reaction is of similar magnitude to the YOYO *LD*, suggesting only ~¾ of the DNA is cleaved. The unchanging nature of the YOYO region *LD* suggests that whatever binding mode results in YOYO being sufficiently oriented to give an *LD* signal does not cleave the DNA backbone and in fact protects the DNA from cleavage. This is intriguing as there is nothing previously in the literature to suggest that YOYO has a binding mode that precludes cleavage. Rather the suggestion has been that there is a faster groove-bound cleavage mode and a slower intercalatively bound cleavage mode (Akerman and Tuite 1996). The *LD* spectrum for poly(dA) with YOYO suggests that a mode with orientation approximately parallel to the bases protects the DNA from the cleavage action of the YOYO.

Kinetics YOYO-alternating homopolymeric DNAs

When YOYO and poly[(dG-dC)$_2$] were mixed at a 20:1 base:YOYO ratio and the *LD* measured in the synchrotron beam line very little change occurred. The kinetics of this experiment suggest a very slow cleavage of the DNA (0.001 h^{-1} in the DNA region), hardly above the noise level. The YOYO region's rate may be slightly larger than the DNA region; however, both rates are almost zero within the errors of the experiment.

We chose to do two experiments with poly[(dA-dT)$_2$] as we had evidence from other work (Rittman and 2010) with poly[(dA-dT)$_2$] that single-stranded regions are present in this DNA at low ionic strengths. The low salt experiment (Fig. 4i) shows a YOYO *LD* of similar magnitude to that of the DNA, both of which decreased at similar rates though the DNA region rate is 30% faster. With this DNA, the 465 nm peak the larger, so a population of YOs are groove bound in this case. This reaction goes to completion, and yet not all the DNA is degraded, indicating again that some of the DNA is protected from cleavage. The higher ionic strength experiment showed no DNA degradation.

By way of contrast, the high salt fully duplex poly[(A-dT)$_2$]/YOYO system (Fig. 4ii) showed similar magnitude *LD* signals for the DNA and the YOYO, neither of which change much with time—though the change that does occur is a decrease of the DNA in accord with cleavage and an *increase* for YOYO, presumably correlating with loss of a small population of groove-bound YOYO and/or increase of intercalated YOYO.

Mixed sequence DNAs with YOYO

The cleavage of the mixed sequence DNAs by YOYO in the light beam have comparable rates (0.20–0.37 h^{-1}) in all

cases, with the DNA region losing orientation faster than the YOYO region, especially for the low ionic strength experiments. The difference is hardly significant for the higher ionic strength experiments, which, as noted above, have a very low groove-bound population. This difference in loss of orientation of the DNA chromophores and the YOYO is therefore likely to be due to groove-bound molecules no longer contributing a positive *LD* once cleavage has occurred. The mlDNA kinetics are fitted as if they are first order, but this is not entirely convincing, and it and the high salt ctDNA almost certainly have uncleaved DNA with intercalated YOYO when the reaction stops.

Conclusion

We have shown that the ASTRID synchrotron light beam may be used simultaneously to initiate DNA photocleavage by the dye YOYO and to monitor the kinetics of the reaction by *LD*. From the *LD* spectra collected during the kinetics, we concluded that the predominant mode of YOYO binding was by intercalation in all sequences in accord with literature expectations at the low loading used in our experiments. However, in contrast to expectations from the literature, we found that poly(dA), mlDNA, low salt ctDNA and low salt poly[(dA-dT)$_2$] all had significant populations of groove-bound YOYO. This changed during the course of the experiments.

In addition, in contrast to the literature's evidence for no sequence-dependence of YOYO's DNA binding, we found significant differences in the sequence dependence of the cleavage kinetics. The rates for a 100-mA beam current (about 6.5×10^{11} photons per second) varied from 0.6 h^{-1} for low salt ctDNA to essentially zero for poly[(dG-dC)$_2$] and high salt poly[(dA-dT)$_2$]. The main mechanistic conclusion from the analysis of the *LD* data is that, even where intercalation is the predominant binding mode, the DNA cleavage is dominated by groove-bound YOYO, not by intercalated YOYO. In homopolymeric DNA only the groove-bound YOYO seems able to catalyze cleavage. At the conclusion of the experiments with each kind of DNA, uncleaved DNA with intercalated YOYO remained. The data suggest that in mixed sequence DNAs, YOYO in some, but not all, intercalated binding sites can cause cleavage. Thus, the low salt poly[(dG-dC)$_2$] and high salt poly[(dA-dT)$_2$] where there was essentially no DNA cleavage occurring were concluded to have little or no groove-bound YOYO. It may be that YOYO has more than one type of intercalative binding, one of which protects the DNA from cleavage.

We also observed reasonable orientation of single-stranded poly(dA). The only YOYO molecules that were sufficiently oriented to give an *LD* signal with this DNA adopted some kind of geometry oriented more parallel to the bases than perpendicular, which induced no cleavage. YOYO molecules that are invisible in the *LD* did, however, induce poly(dA) cleavage. This observation and also the fact that cleavage occurs with low salt poly[(dA-dT)$_2$], where we know there is significant single-stranded DNA, suggests that it is the (transient in the case of B-DNA) single-stranded regions that are cleaved. The mixed sequence cleavage sites in, e.g., calf thymus and *Micrococcus luteus* DNA may well be those most prone to adopting transient single-stranded structures.

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