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DNA in a Polyvinyl Alcohol Matrix and Interactions with Three Intercalating Cyanine Dyes

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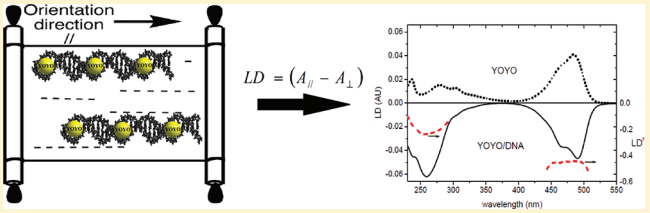
Supporting Information

ABSTRACT: We investigate how DNA interacts with drugs in humid polyvinyl alcohol (PVA) films by using a homologous set of cyanine dyes (YO^+, $YO\text{-}PRO^{2+}$, and $YOYO^{4+}$) known to intercalate into DNA with increasing affinity with increasing charge. UV−vis spectroscopy shows that the PVA matrix destabilizes all three DNA−dye complexes compared to aqueous solution but to a lesser degree as the dye charge increases. The monovalent YO is fully dissociated from DNA within minutes, whereas the dissociation of the divalent YO-PRO takes about one hour and occurs by a two-step mechanism. The tetravalent homodimer YOYO is even less affected by the PVA environment and remains intercalated in the B-form DNA also in the PVA films. The reduced stability of the DNA−dye complexes is discussed in terms of steric and dielectric properties of the PVA matrix. After being kept in dry PVA films for 48 h the DNA−YOYO complexes can be reformed reversibly by rehumidifying the films for 30 min. The ability to store aligned and confined DNA intercalated with ligand complexes may be useful in studies on structural properties of nucleic acids.



INTRODUCTION

Deoxyribonucleic acid (DNA), besides its uncontested importance for life as genetic information carrier, has found applications in many new developing fields including photonics,[1−5] molecular bioengineering,[6−9] or biosensing.[10−14] Its unique properties, in particular how the double helix structure is self-recognizing, chiral, and sensitive to environmental conditions,[15,16] makes DNA a smart biopolymer that may be tuned for various applications.[17] However, its unique molecular properties rely on a rich presence of water, which makes DNA structure difficult to control. One approach is then to form a composite where the DNA is incorporated in a biocompatible matrix of polyvinyl alcohol (PVA), a water-soluble synthetic polymer which is known as an excellent host matrix for native DNA.[18−21] The compatibility between PVA and native DNA allows for exploring DNA−drug interactions with an insight into dynamic processes that occur while changing the external conditions. It is also a valuable system to study structure and conformation when nucleic acids are doped with various ligands.

One important step is then to investigate how DNA and its complexes with ligands behave in the environment of a PVA matrix. An ideal system for this purpose is humidified films of PVA because their water-content can be as high as 50%,[22] keeping in mind that the films differ from a purely aqueous solution as revealed by a change in DNA secondary structure to A-form if the PVA films are too dry.[23] Regarding the behavior of DNA−ligand complexes in the PVA environment we only know about a few studies, based on either the fluorescence from nanoparticles formed by PVA and DNA[24,25] or by laser irradiation of DNA−dye complexes.[26,27] PVA has also been used as a template for aligning DNA−dye complexes in a surface layer.[28] In the present study we use the cyanine dye oxazole yellow (YO) and its two derivatives YO-PRO and YOYO (Figure 1B).

The complexes between these dyes and DNA in an aqueous solution have been characterized in detail. The YO-based dyes bind to DNA by intercalation between the base pairs.[29] The divalent YO-PRO has a higher DNA-affinity than the monovalent YO due to a stronger electrostatic interaction, and the YOYO dimer binds even stronger than YO-PRO most likely because the two YO-monomers are connected by a biscationic linker[30] similar to the linker that was originally used to enhance the DNA-affinity of ethidium bromide by forming its homodimer.[31] The binding constants of the YO-type of dyes to DNA are summarized in Table 1.

Furthermore, as expected for the cationic YO-based dyes the DNA affinity decreases[32] and the rate of dissociation increases[33] when the ionic strength is increased. Third the YO type of dyes exhibit a strongly enhanced fluorescence upon binding to DNA (by a factor as large as 3200[30]), because the intramolecular rotation between the quinoline and the benzo-oxazole moieties is suppressed when the YO chromophores intercalated in DNA.[34] Finally, the absorption spectra of the YO-based dyes are sensitive to the environment as demonstrated by the red shift they exhibit when binding to DNA.[29,34] YOYO is a particularly useful probe in this respect because also the shape of its absorption spectrum is sensitive to the polarity and viscosity of the environment.[34]

Here we exploit these well-established properties of the homologous set of YO-based dyes in Figure 1B and their complexes with

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DNA to study DNA−dye interactions inside a humid PVA matrix using spectroscopic methods.

MATERIALS AND METHODS

Materials. PVA with an average molecular weight of 80,000 was purchased from Du Pont (Sweden) under the commercial name Elvanol 71−30. Double-stranded calf thymus DNA (type I) was obtained from Sigma Aldrich. The dyes YOYO-1 (here denoted YOYO) and YO-PRO-1 (YO-PRO) were purchased from Molecular Probes and used as received. The monomer YO was kindly provided by Per Lincoln at Chalmers University of Technology. The dye we denote YO-PRO (Figure 1B) is referred to as YO other works.[29,34]

Film Sample Preparation. First PVA powder was dissolved to 10% (w/v) in a 2 mM NaCl solution by stirring for 5 min at room temperature. The PVA solution was then incubated at 80 °C for 30 min while stirring until a transparent viscous solution was obtained. Samples containing PVA, DNA, and dyes were prepared by mixing the three constituents at a volume ratio of 100:10:0.5, using stock solutions with the following concentrations: 10% w/v (0.1 g/mL) PVA, 1 mg/mL DNA (3.03 mM base), and dye solutions that were 1 mM for YOYO and YO-PRO, but 1.14 mM for YO. The final sample composition was 0.090 g/mL PVA, 270 μM DNA base, and 4.5 μM YO-PRO or YOYO (5.1 μM YO) in 2 mM NaCl. The final mixing ratios (r = [dye]/[DNA base]) was r = 0.017 for YO-PRO and YOYO and r = 0.019 for YO. In a few cases the ionic strength of the DNA−dye sample was increased to 150 mM NaCl and then mixed with PVA dissolved in 2 mM NaCl from the start. The estimated final salt concentration in the film was then 17 mM NaCl. Attempts to increase the ionic strength further resulted in PVA films too turbid for spectroscopic measurements.

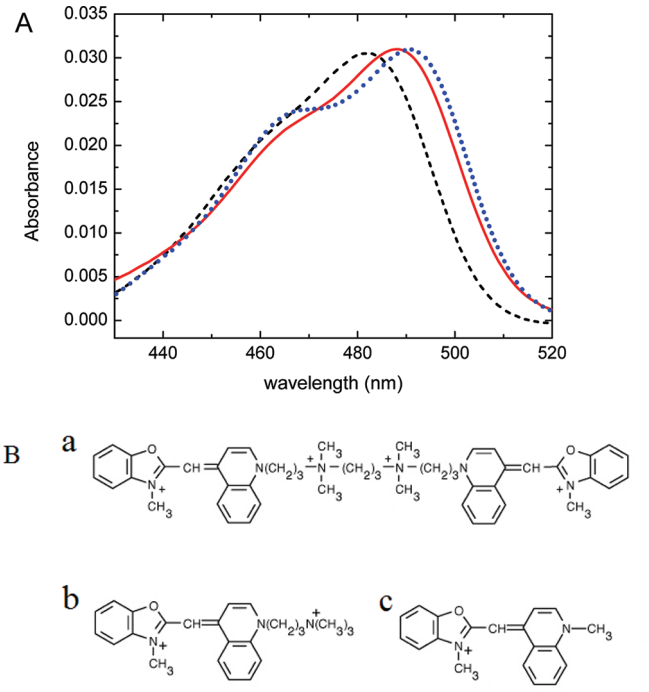

Figure 1. (A) Absorbance spectra of DNA−dye complexes with YO (dashed), YO-PRO (solid), and YOYO (dotted) in fully hydrated PVA films (equilibrated for 4 h) at 2 mM NaCl. For the sake of comparison the spectra were normalized to the same peak height. (B) The oxazole yellow dyes used in this study. (a) YOYO, (b) YO-PRO, (c) YO. Table 1 summarizes the DNA binding properties.

PVA films containing the DNA−dye mixtures were prepared following established procedures for PVA−DNA films.[35] Briefly, the PVA−DNA−dye samples (typically 1 mL) were poured on a glass slide and were allowed to dry form dehydrated PVA sheets (2 days at room temperature and in a dust-free environment). The sheets were then assembled into a stretching device and hydrated into humid films by exposing them (for 15 min if not otherwise stated) to distilled water in a closed chamber holding 100% relative humidity at room temperature. Finally the hydrated films were stretched under the same controlled humidity conditions to a predetermined stretching ratio $R_s = D_\parallel/D_\perp$ between the lengths of the long and short axis of the stretched film. The time dependence of the DNA−dye complex behavior in humid films were studied by two methods. One approach was to perform repeated spectral measurements on a stretched film (after it was humidified for 15 min). Since the stretching of the DNA may perturb the dye binding equilibrium a second approach was to equilibrate different unstretched films for different times in the humid chamber and record spectra directly after stretching them.

UV−Vis Spectroscopy. Absorption spectra were recorded on a CARY-5000 spectrophotometer, using as a reference PVA films prepared as above but lacking the DNA−dye complexes.

Linear Dichroism (LD). LD is defined as the difference in absorbance of light linearly polarized parallel ($A_\parallel$) and perpendicular ($A_\perp$) to the macroscopic axis of orientation (in this study the stretching direction of the film).

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

Table 1. Properties of the Cyanine Dyes (n.m. = Not Measured; n.a. = Linear Dichroism = 0 for Free Dye)

			λ_{max}[c] (n.m.) for absorption and linear dichroism				
	charge	K_a[b] (M^{-1})	with DNA in water	dye itself in water	with DNA in PVA film[e]	dye itself in PVA film[e]	dye itself in PVA solution[e]
YO	+1	1.0×10^6	486[e] (n.m.)	476[e] (n.a.)	481 (481 +)	481 (481 +)	477 (n.a.)
YO-PRO[a]	+2	1.6×10^7	490[d] (490 −)	481[d] (n.a.)	488 (495 −)	488 (488 +)	n.m. (n.a.)
YOYO	+4	$\sim 1 \times 10^{10}$	490/460[d] (490 −)	457/481[d] (n.a.)	491/465 (491/465 −)	486/465 (484/464 +)	457/485 (n.a.)

[a] YO-PRO is referred to as YO in refs 36 and 39. [b] Affinity constant for mixed sequence double-stranded DNA in aqueous solution at 20 mM NaCl. YO-PRO from ref 40. YO calculated by comparing with thiazoleorange (TO)[41] and TO-PRO.[40] YOYO estimated by comparing with ethidium bromide and its homodimer.[38] [c] Wavelength of peak position in absorption and in linear dichroism (LD, in paranthesis; the ± indicates the sign of the LD). From left to right: DNA-bound and free dye in aqueous solution, DNA-bound and free dye in humid PVA film, and free dye in a 10% aqueous solution of PVA. For YOYO the wavelength after the slash refers to the shoulder or secondary peak in the spectra. In PVA films the absorption spectra were measured after 4 h of equilibration, whereas the LD data correspond to the spectrum at the start of the equilibration. [d] References 36 and 39. [e] This work.

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The LD spectra of DNA−dye complexes oriented in such stretched films can provide information on the orientation of the bound dye relative to the DNA helix axis,[36] while a nonstretched film should exhibit zero LD. LD spectra were recorded on a Chirascan CD spectrophotometer equipped with an LD accessory unit.

The reduced LD (LD^r) is obtained by dividing the LD by the absorbance of the corresponding isotropic sample (A_{iso}), which for an anisotropic sample can be obtained as $A_{iso} = (A_{\parallel} + 2A_{\perp})/3$,[35] so that

$$LD^r = \frac{LD}{A_{iso}} = \frac{3LD}{A_{\parallel} + 2A_{\perp}} \quad (2)$$

For molecules with a uniaxial orientation distribution, as can be expected for DNA in PVA matrix since the films are thick (40 μm for dry film) compared to the helix diameter (2 nm), LD^r is a product of an orientation factor S and an optical factor O.

$$LD^r = SO = \frac{3}{2}S(3\cos^2\alpha - 1) \quad (3)$$

The optical factor O is related to the angle α between the helix axis and the light absorbing transition moment of the actual chromophore, which could be a DNA base or a DNA-bound ligand. The orientation factor $S = {}^1/_2(3\langle\cos^2\theta\rangle - 1)$ corresponds to the average orientation of the DNA helix in the PVA film, where θ is the angle between the macroscopic stretching direction and the local helix axis of a particular molecule and where the average runs over all DNA molecules in the sample. The degree of helix orientation S was calculated from the LD^r values at 260 nm using the average angle $\alpha_{DNA} = 86°$ for the DNA bases.[23] The binding angle α_{dye} for a dye with respect to the DNA helix axis can then be calculated from the LD^r value in the visible region of its absorption spectra.

Fluorescence Imaging of PVA Films. PVA films containing YOYO or YOYO/DNA complexes were scanned at a 100 μm resolution using a Typhoon 9401 gel scanner with excitation at 488 nm and a 40-nm emission band-pass filter centered 520 nm.

RESULTS

Absorption Spectroscopy. Figure 1A shows the absorption spectra of DNA complexes with YO, YO-PRO, and YOYO, respectively, after they were equilibrated for 4 h in a humid PVA film. With YO the absorption maximum is at 481 nm (dashed), while for the DNA/YO-PRO sample the maximum is at 488 nm (solid). The dimer YOYO gives an absorption maximum at 491 nm, but there is also a distinct shoulder (at about 465 nm) on the blue side of the main peak (dotted).

When the YO type of dyes (Figure 1B) bind to DNA in aqueous solution their absorption spectra are red-shifted compared to nonbound dye. As summarized in Table 1, this effect has been shown previously for YO-PRO and YOYO[29] and is confirmed here for YO (Figure S1 in Supporting Information). Our first tool to investigate if the YO type of dyes interacts with DNA in PVA films was then to compare the absorption spectra of the DNA−dye complexes in Figure 1A with the spectra of nonbound YO dyes in the same PVA film. Figure 2 shows the absorption spectra of YO and YOYO themselves in PVA films and also includes their absorption spectra in a 10% aqueous solution of PVA intended as an intermediate PVA environment between water and the humid PVA films.

Starting with YO (Figure 2A) in the 10% PVA solution the maximum is at 477 nm (solid), close to the 476 nm of YO itself in water (Figure S1 of Supporting Information), but in a humid PVA film the YO absorption spectrum is red-shifted by 5 nm to give a maximum at 481 nm (dotted). YO-PRO exhibits a similar 7 nm red-shift, from 481 nm in aqueous solution[34] to 488 in a humid PVA film (results not shown). The absorption spectrum of YOYO (Figure 2B) is more strongly affected by the PVA matrix. In the 10% PVA solution the main maximum is at 457 nm with a secondary maximum at 485 nm on the red side of the main peak (solid), similar to the spectrum of YOYO dissolved water.[34] By contrast, in the humid PVA film theYOYO spectrum is seen to have a reversed spectral shape with the maximum at 486 nm and the secondary peak on its blue side at 465 nm (dotted).

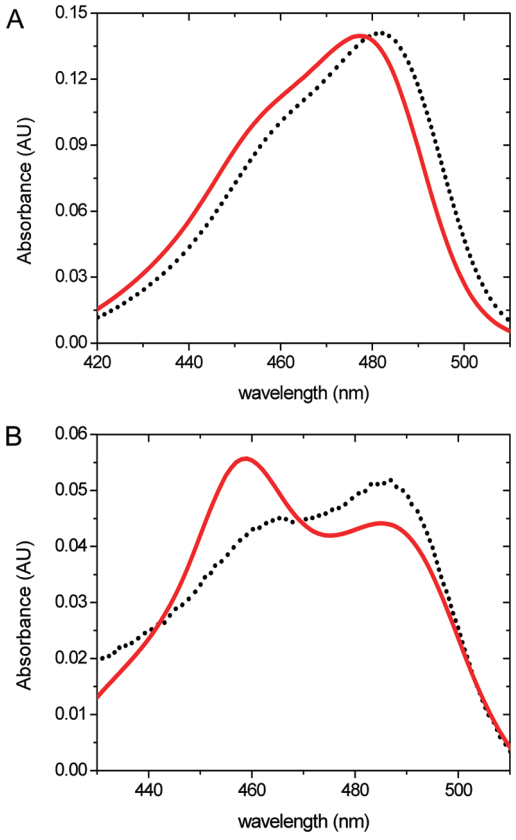

Figure 2. Absorbance spectra of YO (A) and YOYO (B) themselves (no DNA) in a humid PVA film (dotted) or in a 10% w/v PVA solution (solid). For the sake of comparison the spectra are normalized to the same peak height.

Comparing Figures 1A and 2 (cf. Table 1) shows that the presence of DNA in the PVA matrix causes no red-shift of the absorption in the case of YO and YO-PRO, which suggests theses dyes do not interact with DNA in humid PVA films (at least after 4 h). YOYO on the other hand exhibits a marked red-shift and altered spectral shape in the presence of DNA, indicating a DNA interaction also after 4 h. LD was used to further investigate the binding mode and the dynamics of the DNA−dye complexes.

LD Spectroscopy. *DNA without Dyes.* First we investigated the alignment of DNA itself without dye by using the DNA absorption around 260 nm. Figure 3A shows how the corresponding LD spectrum evolves over time after the humidified PVA film was stretched to $R_s = 1.5$ (at 2 mM NaCl). That the LD is negative shows that the DNA helix axis is more or less aligned with the stretching direction of film, in agreement with previous

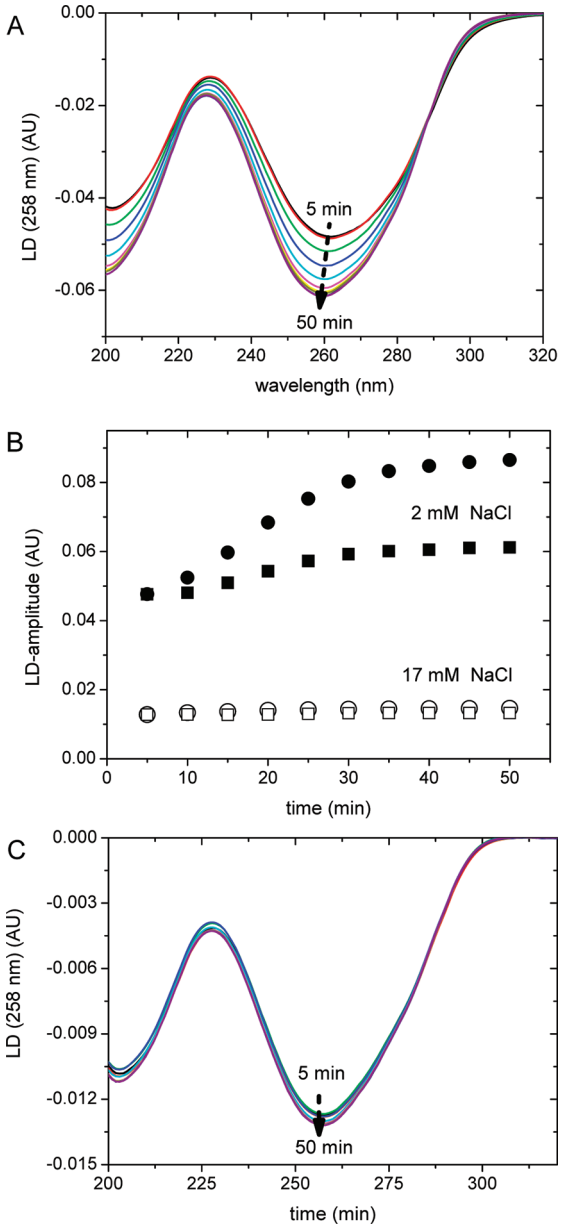

Figure 3. Time dependence of the DNA alignment in stretched PVA films. The initially dry films were humdified for 15 min and then stretched to R_s = 1.5 at time zero. LD spectra were recorded at indicated times in films that contained 2 mM (A) or 17 mM NaCl (C). Panel B shows LD amplitudes vs time in PVA−DNA films containing 2 mM (solid symbols) or 17 mM NaCl (open symbols). Squares show the amplitude of the (negative) DNA LD at 258 nm (corrected for the LD contribution from PVA), and circles show the amplitude of the (positive) LD from PVA at 350 nm (where DNA does not absorb) scaled to coincide with the DNA LD at time zero.

reports.[35] Figure 3A also shows a slight shift in the position of the LD minimum, starting at 261 nm after 5 min and reaching a final position at 258 nm at the end of the equilibration. This observation confirms that the DNA secondary structure turns from an A form in the partially hydrated PVA film at early times to the B form DNA in the fully humidified films.[23] The PVA itself exhibits a positive LD as the films are stretched (Figure S2 in Supporting Information), and the DNA spectra in Figure 3A were obtained by subtracting this LD contribution from the aligned PVA matrix.

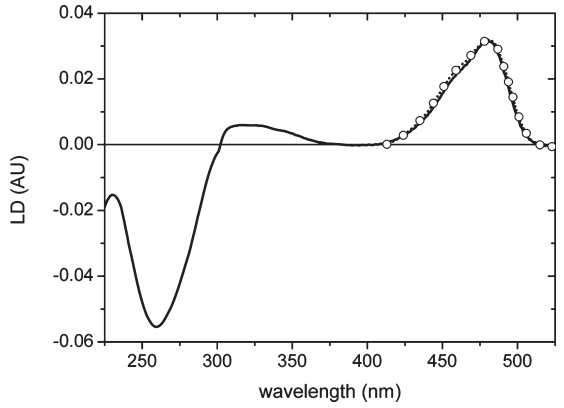

Figure 4. LD spectra of YO/DNA (solid) and YO itself (dotted) in a humid PVA film stretched to R_s = 1.5 in 2 mM NaCl.

In Figure 3B the solid circles show how the amplitude of the PVA-LD (at 350 nm where DNA does not absorb) increases over time after stretching, and the solid squares show how the amplitude of the (negative) DNA LD at 258 nm evolves in the same time span. The PVA and DNA alignment are seen to increase essentially in paralell over tens of minutes, and reach their final level about 40 min after the film was stretched.

Figure 3C shows the corresponding LD spectra of DNA when the PVA film contains 17 mM NaCl. The spectra are seen to be virtually time-independent when compared to the changes seen at 2 mM NaCl (Figure 3A) including that the LD minimum has reached 258 nm already after a few minutes. A plot of the LD amplitude at 258 nm vs time (open squares in Figure 3B) confirms this time constancy, and a plot of the LD from the PVA matrix (open circles) is seen to be as constant as the DNA LD. Finally we note that the DNA-LD at 17 mM NaCl is about five times lower than the final DNA-LD at 2 mM NaCl.

DNA−Dye Complexes. YO. Figure 4 (solid) shows the LD spectrum of the YO/DNA complex in a humid PVA film streched to R_s = 1.5.

The negative LD at 258 nm confirms that the DNA helix is still preferentially oriented in the stretching direction of the film (cf Figure 4). The LD contributions above 300 nm stems from the YO dye, and the positive LD sign in its main absorption band (maximum at 481 nm) shows that the long axis of the YO chromophore is aligned with the stretching direction. That the long axis of the dye is more or less paralell to the DNA helix axis strongly indicates that YO is no longer bound to DNA in the PVA; if YO was intercalated between the base pairs its long axis would be perpendicular to the helix axis.[34] Indeed, YO by itself (no DNA) in a humid PVA film stretched to the same R_s = 1.5 also exhibits a positive LD in the dye absorption band (Figure 4; dotted), and the spectral shape is identical to that observed for the DNA−YO sample. Repeated measurements showed that there was no change in the LD spectrum of the YO−DNA sample over time.

YO-PRO. In contrast to YO/DNA (Figure 4), the LD spectrum of YO-PRO did change over time. Figure 5 shows LD spectra recorded after identical YO-PRO/DNA samples had been equilibrated in three separate PVA films for 15 min, 1 h, or 24 h and then stretched to R_s = 1.5. The spectrum recorded after 15 min (Figure 5; red solid) shows a negative LD in the main dye absorption band, which indicates that a majority of the YO-PRO molecules remain bound to DNA at this stage (see Discussion).

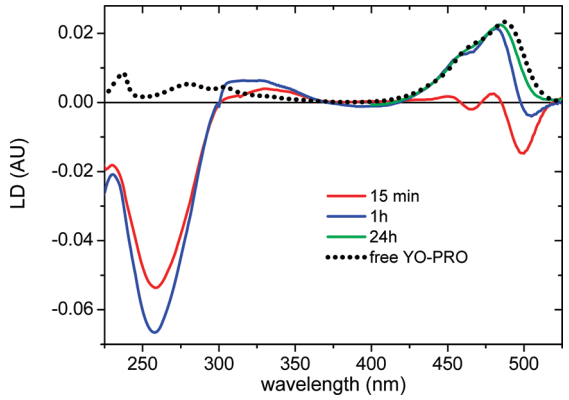

Figure 5. LD spectra of DNA/YO-PRO after the indicated times (solid) and of YO-PRO itself (dotted) in humid PVA film stretched to R_s = 1.5 in 2 mM NaCl.

However, when the same YO-PRO/DNA sample was allowed to equilibrate for one hour before the film was stretched (Figure 5; blue solid) the dye LD has turned mostly positive, indicating that most YO-PRO molecules now have dissociated from the DNA in analogy with YO (Figure 4). After 24 h the dye LD has turned fully positive suggesting complete dissociation, but it should be noted that the dye part of the spectrum still differs slightly from that of YO-PRO itself in a stretched PVA film (dotted line in Figure 5). Figure 5 also shows that the (negative) LD signal of DNA at 258 nm has increased in amplitude by about 25% after 1 h.

The kinetics of the YO-PRO dissociation was studied by recording repeated LD spectra on one and the same humid PVA film kept stretched at R_s = 1.5. Figure 6A shows the change in the dye absorption band over about 1 h. The first spectrum (18 min) has negative dye LD with a peak at 497 nm, which gradually decreases in amplitude and is replaced by a positive band that has a maximum at 481 nm after 45 min, although it should be noted that both peak positions exhibit a steady red shift over time.

Figure 6B (main panel) shows the fraction α of free YO-PRO vs time, as obtained by resolving the measured LD spectra in Figure 6A into the contributions from bound and free dye spectra shown in the same figure. (See Figure S3 in Supporting Information for details). The amount of free YO-PRO clearly increases with time, but it is also seen that the time delay in our present protocol (about 15 min before the first spectrum) prevents us from monitoring the early stages of the dissociation. The inset of Figure 6B shows that a first order dissociation model ($\alpha = 1 - e^{-t/\tau}$) failed to describe the data, since the ratio of free and bound dye concentrations form a straight line that fail to pass through the origin. A two-step process

$$\alpha = \alpha_1[1 - e^{-t/\tau 1}] + \alpha_2[1 - e^{-t/\tau 2}] \quad (4)$$

described the data well, but the fit was insensitive to the parameter τ_1 due to the time gap in the experimental data. The best fit (curve in Figure 6B) was obtained by replacing the first term in eq 4 by a step of amplitude α_1 that occurs faster than the experimental delay. Notably, the need for the term α_1 to get a satisfactory fit does not stem from free dye being present at time zero. The high binding constant for YO-PRO to DNA means that more than 99% of the dye is bound in the original dye/DNA mixture under our conditions (see Supporting Information), so it is reasonable to assume α = 0 at the start of the experiment in Figure 6. The fitted parameter values (see caption of Figure 6) shows that the second step has about twice the amplitude of the first one and occurs with a time constant of about 30 min.

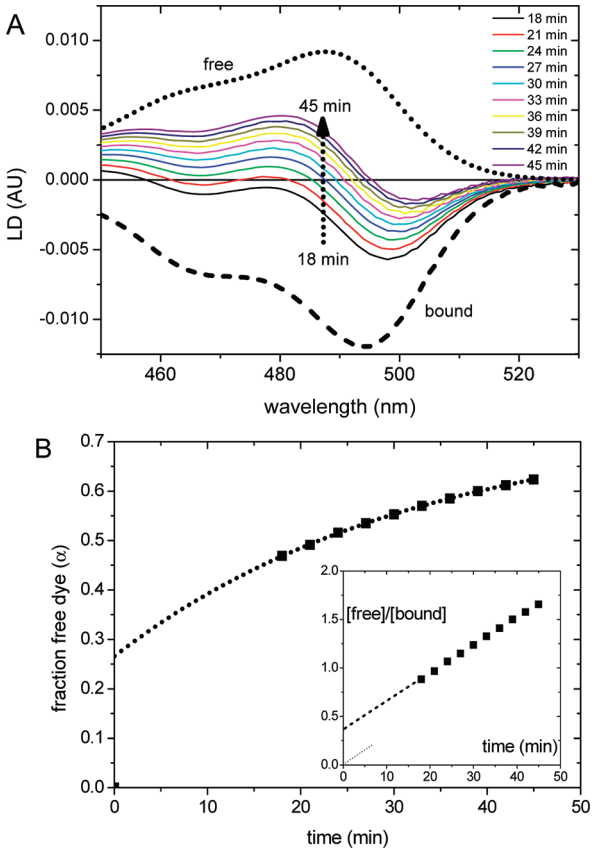

Figure 6. Time dependence of LD spectrum of YO-PRO/DNA in humid PVA film. (A) LD spectra in the visible region measured at indicated times after the film was humidified and stretched to R_s = 1.5. Also included are the measured LD spectrum of free YO-PRO and the derived LD spectrum for DNA-bound YO-PRO in the PVA film (see Discussion). (B) Main panel: Fraction α of free YO-PRO vs time, obtained by resolving the spectra in A into the spectral contributions from bound and free form also shown in part A. The curve is the least-squares fit to $\alpha = \alpha_1 + \alpha_1[1 - e^{-t/\tau 2}]$ with α_1 = 0.27 ± 0.01, α_2 = 0.48 ± 0.01, and τ_2 = 33 ± 2 min. Inset: Ratio of concentrations of free and bound YO-PRO vs time. Line is least-squares fit to a straight line.

Together the two processes contribute a total amplitude of 0.75 to the fraction of free dye, suggesting that a non-negligible fraction of YO-PRO remains bound to DNA also after long times.

YOYO. Figure 7 shows the LD spectrum of YOYO/DNA and of YOYO itself in a humid PVA film stretched to R_s = 1.5.

Free YOYO has a positive LD in the dye absorption band (maximum at 486 nm), in agreement with free YO (Figure 4) and YO-PRO (Figure 5). When DNA is present the YOYO dye is seen to exhibit a negative LD in the visible region with a minimum at 491 nm, red-shifted by 5 nm compared to the (positive) LD of the free dye. Figure 7 also includes the LD^r spectrum of the YOYO/DNA sample (dashed), and it is seen that LD^r varies with wavelength in the DNA band (around 260 nm) but is essentially constant in the dye absorption band. The overall LD^r level in the DNA band is clearly less negative than the LD^r of the dye, as previously observed for the YOYO/DNA complex in aqueous solution.[29] As discussed below these observations support that YOYO is intercalated in DNA in the PVA matrix.

In contrast to YO-PRO (Figure 5) the LD spectrum of YOYO/DNA in Figure 7 was essentially constant over an hour.

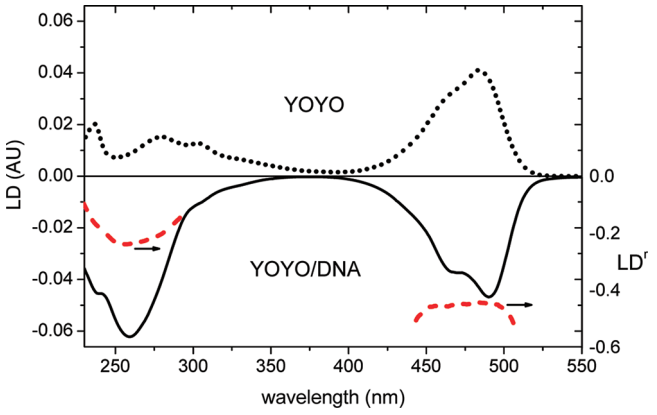

Figure 7. (A) LD spectrum of YOYO/DNA (solid line) and YOYO self (dotted line) in humid PVA film stretched to R_s = 1.5. Dashed line (and right-hand scale): LD^r spectra in the main dye and DNA absorption bands (dashed).

The behavior of the YOYO/DNA complex over 20 h was studied by recording LD spectra on a PVA film kept at R_s = 1.5, and Figure 8A shows the resulting spectra in the dye absorption band at indicated times. The decrease in LD amplitude over several hours suggests that YOYO eventually dissociates from DNA, but the dissociation is slow compared to YO-PRO: even after 18 h there are no signs of the positive LD of free dye seen with YO-PRO already after 1 h (Figure 5). Figure 8B shows the relative LD amplitudes (compared to time zero) vs time, at 258 nm for DNA (spectra not shown) and at 491 nm for YOYO. The DNA LD is seen to be essentially constant over time, showing that the helix alignment conferred by the stretched PVA matrix does not change appreciably over 20 h. By contrast, there is a clear decrease in the LD amplitude of YOYO (491 nm) in the same time span. Since there is no change in the alignment of the DNA helix YOYO binds to, the decrease in LD at 491 nm can be ascribed to loss of bound dye. The curve in Figure 8B shows the best fit of the dissociation to a biexponential decay with time constants of 0.9 and 9.2 h (see legend of Figure 8 for fitted parameters). The fitted amplitudes show that a major fraction of the initial LD signal (about 75%) remains at long times.

The high stability of the YOYO/DNA complex allowed us to study how the binding of YOYO to DNA depends on the degree of film stretching. Figure 9 shows the LD spectra of YOYO/DNA when R_s is increased stepwise from 1 to 2. The amplitude of both the DNA band (around 260 nm) and YOYO (centered at 491 nm) is seen to increase as the stretching of the film increases, as expected since both the DNA helix and its complex with YOYO should become better aligned. The peak position of the DNA band stayed at 258 nm throughout the stretching experiment, so the degree of helix alignment (S) could be calculated from the LD^r values measured at 258 nm, using α(DNA) = 86° in eq 3. The inset of Figure 9 (open symbols) shows how the obtained S values increase and level off as the film is increasingly stretched.

Also included in the inset of Figure 9 (solid symbols) is the ratio LD(491)/LD(258), which is seen to increase and level off with increasing R_s in paralell with the DNA orientation (S, open symbols).The LD signal at 491 nm depends on how many dye molecules are bound to the DNA as well as on how well the target DNA helix is aligned. Dividing by the LD at 258 nm compensates for the latter effect, so the LD ratio can be taken to reflect the amount of bound YOYO. The observation that the LD ratio

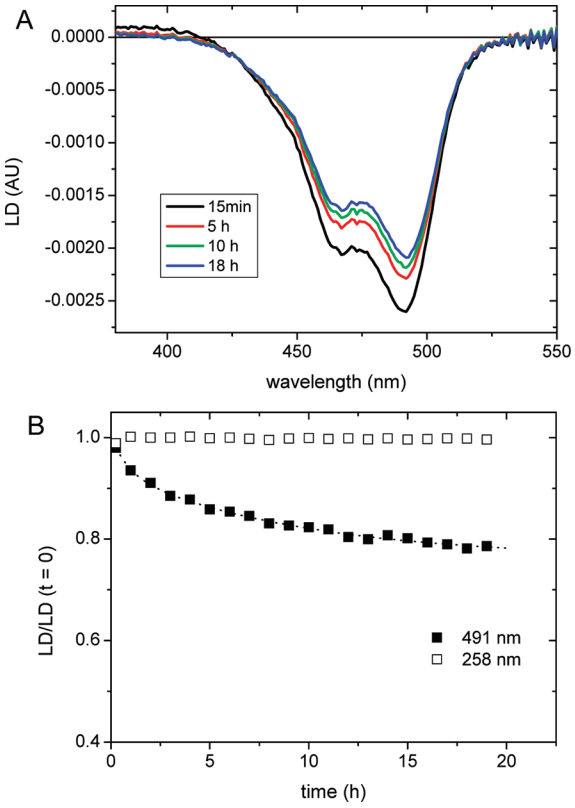

Figure 8. Long-term stability of DNA/YOYO complex in PVA. (A) LD spectra of YOYO−DNA in PVA at the indicated times after the film was humidified and stretched to R_s = 1.5. (B) Normalized LD^n = LD(t)/LD(t = 0) at 258 and 491 nm as a function of time after the film was stretched. Curve is best fit of LD^n at 491 nm to a biexponential decay $LD^n = 0.76\ ^+/_-\ 0.01 + (0.07\ ^+/_-\ 0.01)e^{-t/t_1} + (0.17\ ^+/_-\ 0.01)e^{-t/t_2}$ with t_1 = 0.9 $^+/_-$ 0.3 h and t_2 = 9.2 $^+/_-$ 1.9 h.

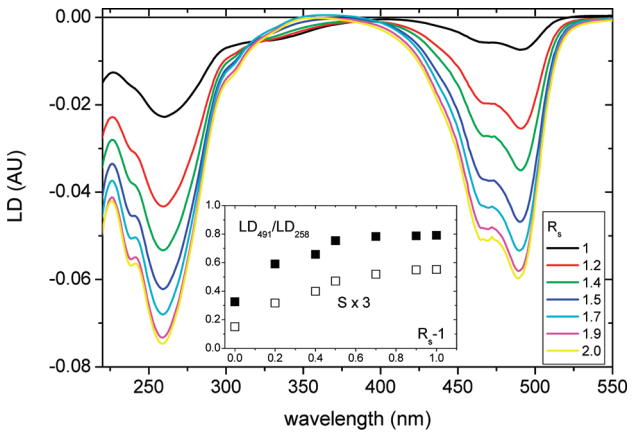

Figure 9. LD spectra of YOYO/DNA in a humid PVA film at indicated stretching ratios R_s, recorded after an equilibration time of 20 min before each spectrum. Inset: the ratio LD(491)/LD(258) (solid) and the helix alignment S (open) calculated from LD(258) as described in the text.

increases with increasing R_s thus indicates that the amount of DNA-bound YOYO increases as the film is stretched. The data in Figure 9 took 2 h to collect, so it is worth noting that any YOYO dissociation that may occur in that time span (Figure 8) would affect the LD ratio in the opposite direction.

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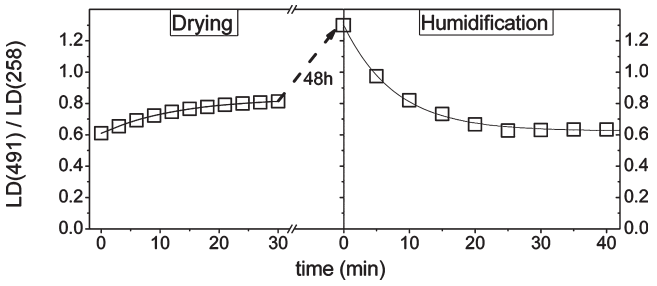

Figure 10. Ratio of dye LD (491 nm) and DNA LD (258 nm) during drying and rehumidification calculated from the data in Figures SZ A and B (Supporting Information). The drying process was monitored for 30 min, and then the film was dried for another 48 h before the humidification step. Curves are fits to monoexponential process with time constants 12.7 $^{+}/_{-}$ 0.3 min for drying and 7.8 $^{+}/_{-}$ 0.3 min for humidification.

Drying and Rehydration of PVA—DNA/YOYO films. In further experiments PVA films containing DNA/YOYO complexes were dried for 2 days and then humidified once again. During drying (Figure S4 in Supporting Information) the amplitude of both the DNA and dye LD bands decreased, but they stayed centered at 258 and 491 nm, respectively. During the rehumidification both bands grew in amplitude again, similar to the increase in the DNA amplitude seen during humidification of the DNA—PVA films (Figure 3), and the peak wavelengths again remained at 258 and 491 nm.

Figure 10 shows the time dependence of the ratio of the LD signals at 491 and 258 nm during the drying and the subsequent rehumidification. The ratio tends to reach a plateau after about 30 min of drying, but it is also seen that the ratio increases further upon prolongeddesiccation for 48 h. During the rehumidification the LD ratio decreases gradually and reaches the value in the original humid film (0.61) after about 30 min.

DISCUSSION

In aqueous solution the three YO-based dyes studied here (Figure 1) bind to double-stranded DNA by intercalation,[29,38] with binding constants of 10^6 M^{-1} or higher (Table 1). Here we investigate how these dyes bind DNA if the aqueous environment is replaced by a humid PVA film. The film samples were prepared by mixing PVA solutions with preformed DNA—dye complexes, and the high binding constants means that more than 99% of the dye molecules were bound to the DNA at the outset of the PVA experiments (see Supporting Information). We first discuss the mode of DNA binding in the PVA matrix and how stable the dye/DNA complexes are compared to aqueous solution. These findings are then discussed in terms of how dielectric and steric effects of the PVA matrix may affect the DNA and its interaction with the YO type of dyes. Finally we consider the properties of the PVA—DNA films as a composite biomaterial, looking at both humidified and dried states of the films.

DNA Binding of the YO Type of Dyes in PVA Films. *YO.* When YO binds to DNA in aqueous solution its absorption spectrum is red-shifted by 10 nm compared to the nonbound dye (Figure S1 of Supporting Information; Table 1), but when the same YO/DNA complex is inside a humid PVA film (Figure 2) the absorption maximum is at the same wavelength (481 nm) that YO exhibits itself in a film without DNA (Figure 3 and Table 1). This observation indicates that YO is not bound to DNA in the film since a red-shift is commonly observed when intercalators such as ethidium bromide[39] and YO-PRO[34] bind DNA. The LD spectrum of the YO/DNA sample in a stretched PVA film (Figure 5) supports this conclusion by the positive sign and the spectral shape being the same as for YO in a PVA film that lacks DNA (Figure 5), as expected if the dye is fully dissociated.

YO-PRO. The maximum absorption of YO-PRO is at the same wavelength (488 nm) whether the PVA films contain DNA or not (Table 1), indicating that YO-PRO is not DNA bound in PVA after 4 h as was concluded for YO. However, the LD spectra in Figure 6 show that the dissociation of YO-PRO from DNA takes at least one hour, much slower than YO, which is fully dissociated already after 15 min (Figure 5). Importantly, the YO-PRO dissociation is not an effect of the film stretching per se because the same spectral signs of YO-PRO dissociation are observed if the LD is measured on one and the same stretched film over time (Figure 7) or on different films that were equilibrated for different times before being stretched (Figure 6). Below we discuss how the PVA film environment may destabilize the YO-PRO/DNA complex compared to an aqueous solution.

The derived LD spectrum of bound YO-PRO in PVA (Figure 7 dashed) indicates that this dye binds DNA by intercalation. The negative LD sign excludes other modes such as groove binding and is consistent with intercalation. Second, the 7 nm red shift compared to free YO-PRO in a PVA film (Figure 7, dotted) is similar to the 9 nm red shift observed when YO-PRO intercalates DNA in aqueous solution.[29] Finally the bound spectrum has a more marked shoulder than the free form, in agreement with how DNA binding affects the shape of the absorption spectra of YO (Figure S1 of Supporting Information) and YO-PRO.[29]

YOYO. Our results show that YOYO forms a stable DNA complex in humid PVA films by intercalation. The absorption maximum of YOYO is red-shifted by 5 nm when the PVA film contains DNA (Table 1), in contrast to the lack of DNA-induced absorption shift we obtain with YO and YO-PRO, which indicates that a majority of the YOYO molecules remain bound to DNA in the PVA matrix after 4 h. Even more convincing is that the negative LD in the dye absorption band around 491 nm gives a dye LD^r level, which is more negative than the LD^r of the DNA bases around 260 nm (Figure.8), in agreement with observations on the intercalated YOYO/DNA complex in aqueous solution.[29] An intercalative mode of binding also explains why the amount of bound YOYO increases in parallel with the degree of DNA alignment (inset of Figure 10) because the affinity of intercalators is known to increase when the DNA-molecules are stretched mechanically.[40]

Dye Affinity and Dissociation from DNA in PVA Film. *Mechanism of Dye Dissociation.* The results in Figure 6 show that the fraction of free YO-PRO dye increase in a two-step fashion, since a monoexponential decay of bound dye failed to describe the data whereas a biexponential fit worked satisfactorily. We propose that the two steps correspond to that YO-PRO initially dissociates from the DNA molecule and binds/interacts with the PVA polymers in its vicinity (where the dye becomes aligned with a positive LD), and that the second step corresponds to further transport of the dissociated YO-PRO into the rest of the PVA film which would make available interaction "sites" in the part of the PVA matrix that is close to the DNA molecule. That the first step is too fast to be monitored by our present approach is reasonable since it take seconds or less for YO-PRO to dissociate from DNA in aqueous solution.[33] The diffusion into to the rest of the PVA film can be expected to be retarded by the steric or attractive YO-PRO/PVA interactions that are evident from the alignment of dissociated YO-PRO, so a time scale of 30 min is not

unreasonable but needs to be confirmed by studies of how fast YO-PRO itself moves in humid PVA films.

Dye Affinity for DNA and PVA. The dissociation of YOYO is considerably slower than with YO-PRO since the longer time constant is 9 h for YOYO (Figure 8) compared to 30 min for YO-PRO (Figure 6). On the other hand YO dissociates in less than 15 min (Figure 4) and thus faster than YO-PRO. The same trend of decreasing dissociation rates in the order YO, YO-PRO, and YOYO has been observed in agarose hydrogels and aqueous solution.[33]

A more direct measurement is the relative amount of dye that is bound to DNA when the dissociation process has ended in a binding equilibrium. That the LD spectrum of YO (Figure 4) does not change with time shows that the dye is 100% dissociated already after 15 min. With YO-PRO the LD spectra (Figure 6) indicate that about 75% of the dye has dissociated at long times, in agreement with the fact that the LD spectrum of YO-PRO/DNA deviate from that of free YO-PRO even after 24 h (Figure 5). In the case of YOYO we were not able to deconvolute the spectra into contributions from bound and free dye due to the slow dissociation, but the final level in the fit in Figure 8 suggests than a majority of the YOYO molecules (roughly 75%) remain bound in the limit of long times.

Taken together the dissociation rates and final LD spectra indicate that the affinity to DNA in PVA increases in the same order as in aqueous solution: YO < YO-PRO < YOYO (Table 1). However, in the polymeric environment a competitive binding of the dye to the PVA affects noncovalent interactions between the dyes and double-stranded DNA. The estimated equilibrium fraction of PVA-bound dyes (100, 75, and 25%, respectively) indicates that dye affinity to PVA is increasing in the order YOYO < YO-PRO < YO and is inversely proportional to that of DNA. The binding constant 10^6 M^{-1} for YO means that more than 99% would be bound in aqueous solution at the DNA and dye concentrations we use (see Supporting Information), in contrast to the 100% DNA dissociated and instead bound to PVA in polymer environment. A similar destabilization of the DNA complexes with YO-PRO and YOYO in PVA is evident from the fact that both dyes dissociate and start interacting with polymer to a substantial degree in the films (75 and 25%, respectively) in spite of their higher binding affinity to DNA in aqueous solution (Table 1).

Dielectric and Steric Environment of Humid PVA Films. Dissociation of the YO-type dyes from DNA in humid PVA films may be due to a reduction of the forces that drive the dyes to bind DNA in the first place and/or to dye−PVA interactions that compete with the DNA-binding.

All three dyes become aligned when by themselves in stretched films (dotted curves in Figures 4, 5, and 7), which shows that they interact with the aligned PVA matrix. One possible cause is steric (repulsive) confinement of the dye molecules between the PVA chains as the film is stretched, it is known that viscous solvents increase the quantum yield of free YOYO[34] in a way similar to what we observe with free YOYO in humid PVA films (Figure S5 of Supporting Information). The over-riding type of dye−PVA interaction seems to be attractive however: Figure 5 shows that YO-PRO molecules become aligned even if they enter the PVA matrix after it has been stretched and in Boltzmann terms it is unlikely that the dye becomes oriented by entering a repulsive cage. Attractive PVA−dye interactions can also explain the enhanced quantum yield of free YOYO in PVA (Figure S5 of Supporting Information) because binding to PVA is expected to restrict the intradye rotation that is known to quench the YO fluorescence in aqueous solution.[34] If present, such attractive dye−PVA interactions would contribute to the destabilization of the DNA−dye complexes we observe.

The PVA environment may also reduce the attractive interactions that drive the YO-type of dyes to bind DNA. That YO and YO-PRO both are red-shifted in their absorption compared to the same free dyes in water (Table 1) indicates that the PVA matrix is a less polar environment since a similar red-shift is observed when the two dyes enter the hydrophobic interior of the DNA helix from water (Table 1). The behavior of free YOYO supports this picture; the shape of its absorption spectrum is in fact a sensitive probe of the dielectric environment. In aqueous solution YOYO exhibits a reversed spectral shape compared to YO and YO-PRO (the shoulder of YOYO is on the blue side of its maximum), which has been ascribed to stacking of the two YO chromophores of YOYO in water.[34] Figure 2 shows that in humid PVA films the YOYO spectrum instead has the shoulder on the red side of the maximum, similar to the shape in less polar solvents such as ethanol.[34] Interaction with the hydroxyl groups of the PVA matrix is a possibility since glycerol has a similar effect on the YOYO spectrum[34] as the PVA films but seems unlikely since YOYO exhibits the same reversed spectral shape in an aqueous solution of PVA (Figure 2) as in pure water.[34] Taken together these observations indicate that the humid PVA film presents an environment which is less polar to the YO-type of dyes than an aqueous solution.

The reduced polarity of PVA films is likely due to a reduced water activity compared to a pure aqueous solution, perhaps because water molecules can hydrogen bond to the PVA chains. At any rate one result is a lower effective dielectric constant in the PVA environment, which would in fact strengthen the electrostatic attraction that is known[32] to be important to drive the cationic YO dyes to bind DNA at the low ionic strength we use here. However, a reduced water activity will also weaken the hydrophobic and stacking interactions that are essential for intercalation, and such effects, in combination with competitive dye interactions with the PVA matrix, are the most likely sources behind the increased dye dissociation from DNA in the PVA films. A lower water activity would also strengthen any hydrogen bonds between dye and DNA, but those are of little importance for intercalators compared to groove binders.[31]

Material Properties of PVA−DNA Biocomposite Films. *DNA Secondary Structure and Alignment.* The spectral LD shifts in Figure 3 confirm[35] that DNA is in B form in fully humidified PVA films. This is an important observation for the present study because it means that when YO type of dyes binds the DNA molecules they have the same secondary structure in PVA as in aqueous solution where the properties of the complexes are well characterized. Figure 3 also shows that the B-form is reached faster when the salt concentration in the film is raised from 2 to 17 mM NaCl. Since the A to B transition requires a certain level of water activity,[15] the faster B conversion indicates that PVA films are more rapidly humidified when they contain more salt (perhaps because they are more hygroscopic) and that equilibrium degree of film humidity may take as long as 40 min to reach at low salt.

The slow alignment of DNA at 2 mM NaCl (Figure 3A) occurs in parallel to a similarly slow increase in the PVA alignment (Figure 3B) and since the change in DNA secondary structure is observed in the same time range all three effects are most likely due to an ongoing humidification after the film was stretched. That the PVA and DNA change in parallel is supported by the observation in Figure 3C (high salt): if the PVA alignment is fast

so is the DNA alignment. That the final degree of DNA alignment is lower but attained faster in films that contains more salt (Figure 3) can be understood from the fact that the persistence length of double-stranded DNA decreases from 85 nm at 2 mM NaCl to 55 nm at 17 mM NaCl[41] since a more flexible DNA polymer is expected to exhibit a lower degree of helix alignment but also to adapt faster to the stretching of the PVA matrix.

Long-Term Stability. The PVA films are sturdy as an environment for DNA molecules and their dye complexes. The DNA alignment is stable for many hours also in humid PVA films (Figure 8B), which is surprising since in aqueous solution DNA molecules of the lengths used here would relax in milliseconds and in seconds in polymeric hydrogels.[42] The results of Figure 10 show that PVA films with DNA−YOYO complexes can be stored in dry state for at least 2 days and then be rehydrated in a reversibly fashion in about 30 min. The increasing and then decreasing ratio LD(491)/LD(258) suggests that the amount of bound YOYO increases during drying and decreases upon rehumidification, a reversibility that supports that a majority of the YOYO remains bound to DNA at equilibrium also in humid PVA. The DNA maximum remains at 258 nm during both the drying and rehydration step, and there are no signs of the shift to 261 nm seen with DNA itself (Figure 3). This observation suggests that YOYO stabilizes the B-form of DNA when the PVA-films are dried.

The weak DNA alignment we observe also without stretching the films (Figure 9) probably arises due to a DNA combing effect when the PVA solutions are allowed to dry, as has been observed at the receding meniscus of a drying droplet of aqueous DNA solution.[43] The dry films exhibit a weak DNA LD, which is reinforced as the film is humidified (data not shown), similar to the increase in DNA LD seen when films only containing DNA are humidified (Figure 3A).

CONCLUSION

Dyes from the cyanine group YO, YO-PRO-1, and YOYO were investigated here in terms of binding properties in PVA humid gels to DNA. PVA in the film state strongly destabilizes noncovalent interactions between intercalating dyes and double-stranded DNA. Polymeric film environment effect both dye as well as DNA properties. Low water activity in highly dense polymeric environment weaken the stacking interactions in the DNA double helix, which further affects dye binding. That promotes attractive interactions between dye molecules and PVA, which leads to steric confinement of the dye in a polymer matrix. All molecules are bound to PVA in the case of YO dye. Divalent YO-PRO remains partially intercalated (25%) within the double helix, whereas bisintercalation of YOYO is predominant (75%) in films. We can conclude that binding, structural properties, and dynamics of dyes and drugs with higher affinity to B DNA than 10^6 M^{-1} can be investigated in humid elastic gels. The DNA−drug complexes can be stored even in dry state of PVA and reformed by rehumidifying. Easiness of controlling the hydration levels and long-term stability of the films (orientation stability as long as 48 h and intercalation of homodimer at 24 h) allow restraining DNA for weeks without losing its unique molecular properties. This research is a first step to better understand the behavior of DNA in a strongly hydrophobic environment. Anisotropic PVA can be a good host medium for studying DNA secondary structure when it is doped with intercalating dyes.

ASSOCIATED CONTENT

Supporting Information. Figures depicting estimates of the binding constants of YO and YOYO to mixed-sequence DNA, absorption spectrum of YO in aqueous solution, LD spectra of PVA film with and without DNA, amount of bound dye estimated from the binding constants, analysis of the dissociation YOPRO from DNA in PVA film, LD spectra during drying and rehumidification of DNA/YOYO-PVA film, and YOYO and YOYO−DNA fluorescence intensity in humid PVA. This material is available free of charge via the Internet at http://pubs.acs.org.

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REFERENCES

(1) Steckl, A. J. *Nat. Photon.* **2007**, *1*, 3–5.
(2) Prasad, P. N. *Introduction to Biophotonics*; John Wiley and Sons: New York, 2003; pp 545−557.
(3) Roy, I.; Ohulchanskyy, T. Y.; Bharali, D. J.; Pudavar, H. E.; Mistretta, R. A.; Kaur, N.; Prasad, P. N. *Proc. Natl. Acad. Sci.* **2005**, *102*, 279–284.
(4) Hannestad, J. K.; Sandin, P.; Albinsson, B. *J. Am. Chem. Soc.* **2008**, *130*, 15889–15895.
(5) Samoc, A.; Miniewicz, A.; Samoc, M.; Grote, J. G. *J. Appl. Polym. Sci.* **2007**, *105*, 236–245.
(6) Zhang, S. *Nat. Biotechnol.* **2003**, *21*, 1171–1178.
(7) Choi, J. W.; Nam, Y. S.; Fujihira, M. *Biotech. Bioproc. Eng.* **2004**, *9*, 76–85.
(8) Levine, L. A.; Williams, M. E. *Curr. Op. Chem. Biol.* **2009**, *13*, 669–677.
(9) Petka, W. A.; Harden, J. L.; McGrath, K. P.; Wirtz, D.; Tirrell, D. A. *Science* **1998**, *281*, 389–392.
(10) Teles, F. R.; Fonseca, L. P. *Talanta* **2008**, *77*, 606–623.
(11) Kopeček, J. *Biomaterials* **2007**, *28*, 5185–5192.
(12) Johnston, A. P. R.; Zelikin, A. N.; Caruso, F. *Adv. Mater.* **2007**, *19*, 3727–3730.
(13) Lucarelli, F.; Palchetti, I.; Marrazza, G.; Mascini, M. *Talanta* **2002**, *56*, 949–957.
(14) Ohmichi, T.; Kawamoto, Y.; Wu, P.; Miyoshi, D.; Karimata, H.; Sugimoto, N. *Biochemistry* **2005**, *44*, 7125–7130.
(15) Bloomfield, V.; Crothers, D.; Tinoco, I. *Nucleic acids: structures, properties, and functions* ; University Science Books: Sausalito, CA, 2000.
(16) Murakami, Y.; Maeda, M. *Biomacromolecules* **2005**, *6*, 2927–2929.
(17) Gras, S. L.; Mahmud, T.; Rosengarten, G.; Mitchell, A.; Kalantar-Zadeh, K. *ChemPhysChem* **2007**, *8*, 2036–2050.
(18) Norden, B.; Seth, S. *Biopolymers* **1979**, *18*, 2323–2339.
(19) Shin, M. K.; Kim, S. H.; Jung, S.; Kim, S. I.; Kim, S. J.; Kim, B. J.; So, I. *App. Phys. Lett.* **2008**, *93*, 171903.
(20) Papancea, A.; Valente, A J. M.; Patachia, S.; Miguel, M. G.; Lindman, B. *Langmuir* **2008**, *24*, 273–279.
(21) Aoi, K.; Takasu, A.; Okada, M. *Polymer* **2000**, *41*, 2847–2853.
(22) Rodger, A.; Norden, B. *Circular Dichroism and Linear Dichroism*; Oxford University Press: Oxford, 1997.
(23) Matsuoka, Y.; Norden, B. *Biopolymers* **1982**, *21*, 2433–2452.
(24) Kimura, T.; Nam, K.; Mutsuo, S.; Yoshiwaza, H.; Okada, M.; Furuzono, T.; Fuijsato, T.; Kishida, A. *Mol. Theor.* **2006**, *13*, 75.

(25) Kimura, T.; Okuno, A.; Miyazaki, K.; Furuzono, T.; Ohya, Y.; Ouchi, T.; Mutsuo, S.; Yoshizawa, H.; Kitamura, Y.; Fujisato, T.; Kishida, A. *Mater. Sci. Eng. C* **2004**, *24*, 797–801.
(26) Straws, G.; Broyde, S. B.; Kurucsev, T. *J. Phys. Chem.* **1971**, *76*, 2727–2733.
(27) Balan, N.; Hari, M.; Nampoori, V. P.N. *Appl. Opt.* **2009**, *48*, 3521–3525.
(28) Kelly, G. L.; Kurucsev, T. *Biopolymers* **1976**, *15*, 1481–1490.
(29) Larsson, A.; Carlsson, C.; Jonsson, M.; Albinsson, B. *J. Am. Chem. Soc.* **1994**, *116*, 8459–8465.
(30) Glazar, A. N.; Peck, K.; Mathies, R. A. *Proc. Natl. Acad. Sci.* **1990**, *87*, 3851–3855.
(31) Dervan, P. B.; Becker, M. M. *J. Am. Chem. Soc.* **1978**, *100*, 1968–1970.
(32) Petty, J.; Bordelon, J. A.; Robertson, M. E. *J. Phys. Chem. B* **2000**, *104*, 7221–7227.
(33) Eriksson, M.; Karlsson, H. J.; Westman, G.; Akerman, B. *Nuc. Ac. Res.* **2003**, *31*, 6235–6242.
(34) Carlsson, C.; Larsson, A.; Jonsson, M.; Albinsson, B.; Norden, B. *J. Phys. Chem.* **1994**, *98*, 10313–10321.
(35) Matsuoka, Y.; Norden, B. *Biopolymers* **1983**, *22*, 1731–1746.
(36) Norden, B. *App. Spec. Rev.* **1978**, *14*, 157–248.
(37) Norden, B.; Kubista, M.; Kurucsev, T. *Q. Rev. Biophys.* **1992**, *25*, 51–171.
(38) Spielmann, H. P.; Wemmer, D. E.; Jacobsen, J. P. *Biochemistry* **1995**, *34*, 8542–8553.
(39) Bloomfield, V.; Crothers, D.; Tinoco, I. *Nucleic acids: structures, properties, and functions* ; University Science Books: Sausalito, CA, 2000; Chapter 6.
(40) Vladescu, I. D.; McCauley, M. J.; Nunez, M. E.; Rouzina, I.; Williams, M. C. *Nat. Met.* **2007**, *4*, 517–522.
(41) Baumann, C. G.; Smith, S. B.; Bloomfield, V. A.; Bustamante, C. *Proc. Natl. Acad. Sci.* **1997**, *94*, 6185–6190.
(42) Magnusdottir, S.; Åkerman, B.; Jonsson, M. *J. Phys. Chem.* **1994**, *98*, 2624–2633.
(43) Bensimon, A.; Simon, A.; Chiffaudel, A.; Croquette, V.; Heslot, F.; Bensimon, D. *Science* **1994**, *265*, 2096–2098.