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Interaction of YOYO-3 with Different DNA Templates to Form H-Aggregates

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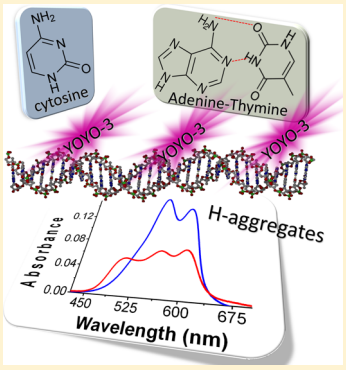
Supporting Information

ABSTRACT: Homodimeric cyanine dyes are DNA intercalators that display a large enhancement of fluorescence emission when bound to double-stranded DNA. However, other different interaction modes are possible, such as H-type molecular aggregates of the dye, templated by the nucleic acid. In this paper, we study in depth the formation of nonfluorescent H-aggregates of the cyanine homodimer YOYO-3 with two different DNA templates using absorption and both steady-state and time-resolved fluorescence spectroscopy. First, a nonfluorescent YOYO-3 H-aggregate complex was found to form in single-stranded polycytidine chains, resulting in the appearance of a new absorption band at approximately 500 nm. The specific interaction of cytosine bases suggests the involvement of the C-rich i-motif in facilitating the formation of the H-aggregate complex. Second, the interaction of YOYO-3 with double-stranded poly(A·T) tracts also led to the appearance of a new absorption band at approximately 500 nm, and hence of a different type of H-aggregate. We found that the aggregate is formed mainly in double-stranded regions with consecutive adenine bases in the same strand (and thymine bases in the complementary strand). These poly(A·T) tracts provide narrow minor grooves and enhanced electrostatic negative potential to promote the aggregation of the negatively charged cyanine. As the YOYO-3 H-aggregates are nonfluorescent, our results provide an important basis to quantitatively understand the fluorescence emission of this cyanine dye in the presence of DNA strands.



INTRODUCTION

DNA intercalators are a class of fluorescent dyes that associate with double-stranded DNA (dsDNA). Some of these intercalator dyes exhibit a remarkable increase in fluorescence quantum yield when they intercalate into dsDNA. This fluorescence can be used as a highly advantageous analytical signal in nucleic acid biosensor design.[1,2] Dimeric cyanine dyes are a family of fluorophores used in nucleic acid staining because of their intercalating properties. Among the more popular cyanine dyes are the oxazole yellow (YO), and its symmetric homodimers YOYO-1 and YOYO-3 (see Figure 1 for the structure). These dyes have large extinction coefficients (>84000 M^{-1} cm^{-1}), and show a fluorescence enhancement factor of approximately 3200 when bound to dsDNA.[3,4] Importantly, YOYO-1 and YOYO-3 have a greater affinity for DNA than ethidium bromide and other cyanine dyes.[5] These features make the YOYO dyes very useful for the detection[3,6,7] and fluorescence microscopy imaging of DNA.[8−10] However, the binding of cyanine dyes to DNA is far from simple. Previous studies of the interactions of these dimeric cyanine dyes, as well as others such as BOBO-3, with double stranded DNA (dsDNA) have demonstrated the presence of different binding modes,[11−14] displaying different spectral characteristics. The predominant mode of binding is bis-intercalation, with the long axis of the YO chromophore oriented parallel to the long axis of the base pair pocket. A secondary DNA binding mode, where the dipole of the dye molecule was aligned with the

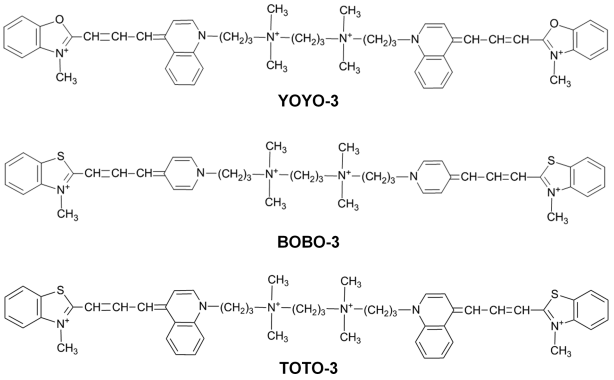


Figure 1. Chemical structures of the homodimeric cyanine dyes YOYO-3, BOBO-3, and TOTO-3.

DNA grooves, was also established for high dye/base pair ratios.[11,13] This binding mode coexisted and competed with the typical intercalation mechanism when the dye interacted with dsDNA. Furthermore, differences in the environment of the dyes, and hence in their spectral properties, also arise from the different base composition of the DNA sequence. For instance, Netzel and colleagues showed that the emission enhancement,

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Table 1. Sequences of the Tested Single-Stranded DNA Oligonucleotides

sequence	name
5′-AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA-3′	$ssPolyA_{30}$
5′-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-3′	$ssPolyT_{30}$
5′-CCCCCCCCCCCCCCCCCCCCCCCCCCCCCC-3′	$ssPolyC_{30}$
5′-CGGGCGGGCGGGCGGGCGGGC-3′	$ssPolyG_{21}$
5′-CCCCATGCGTATCAATAGTAA GCCCC-3′	$(Olig4C)_{26}$
5′-CCCCCATGCGTATCAATAGTAAGCCCCC-3′	$(Olig5C)_{28}$
5′-CCCCCCATGCGTATCAATAGTAAGCCCCCC-3′	$(Olig6C)_{30}$
5′-AATGCGTATCCCCCCAATAGTAAG-3′	$(Olig6Ccenter)_{24}$
5′-CCCCCCCCCCCATGCGTATCAATAGTAAGCCCCCCCCCC-3′	$(Olig10C)_{38}$

the quantum yield, and the fluorescence lifetime of 10 monomeric and bichromophoric cyanine dyes were drastically affected by the base composition of the duplex.[15] For instance, the differences in fluorescence lifetime of thiazole yellow homodimer have been suggested to be useful to develop a platform for GC-content estimation with single molecule resolution.[16] Likewise, in a previous work, we found that the cyanine homodimer BOBO-3 (Figure 1) displays a high affinity for AT pairs and occupies four sites in its primary binding mode, whereas in CG double strands, the dye covers six sites, has a lower affinity, and its fluorescence lifetime is 1 ns longer than in AT sites.[17]

The picture of the interaction of cyanine dyes with DNA is not yet complete. The extensive π-conjugate system over the dye moiety makes it prone to self-aggregate. This dye aggregation commonly occurs in aqueous solution because of strong van der Waals attractive forces. The presence of nonfluorescent molecular aggregates in solution, which are disrupted upon binding to DNA, has been reported to significantly contribute to the low quantum yield of homodimeric cyanines in solution.[18] Cyanine dyes have the capability to self-assemble into different types of aggregates with modified electronic configuration: a face-to-face stacking (H-aggregates), or a head-to-tail arrangement (J-aggregates).[19] H-aggregates show extensive π-packing that causes a hypsochromic shift of the absorption band and a reduced fluorescence emission because of strong excitonic coupling,[15,20,21] whereas J-aggregates present a staggered self-assembly, causing a bathochromic shift of the absorption band, and they are highly fluorescent.[19] However, these aggregates not only form in solution; in other cases, the self-assembly needs some type of templating that typically is facilitated by materials with a large negatively charged surface[21−23] because the cyanine dyes are usually positively charged. Among these templates, DNA molecules also have the potential to promote cyanine dye self-aggregation,[24−26] due to the extensively charged phosphate backbone.

Our recent interest lies in the nature of the cyanine dye H-aggregates templated by DNA molecules, as these interactions may play a critical role in interpreting quantitative fluorescence data of DNA staining. In a previous study, we found an unprecedented H-aggregate of BOBO-3 templated by single-stranded DNA (ssDNA), in particular, only promoted by consecutive cytidine bases.[27] Several striking features made this finding very unique: it was the first time that a cyanine H-aggregate promoted by ssDNA was detected; the interaction was specifically promoted only by consecutive cytosine bases; and, most importantly, the H-aggregate complex was so stable that its formation even prevented the single DNA strand to hybridize with its complementary sequence.[27] More recently, in our desire to find cyanine intercalators as DNA markers at the single molecule regime, i.e., in very diluted solutions, we chose the homodimeric cyanine dye YOYO-3 as a suitable dye for that use. Our studies on the photophysics and binding properties of the homodimeric cyanine YOYO-3 with double-stranded DNA (dsDNA) also revealed the formation of a YOYO-3 complex with the spectral features of an H-aggregate at high [YOYO-3]/[base-pair] ratios.[14] Within this background, we further explore in this paper the nature of DNA-templated H-aggregates of YOYO-3. On one hand, we check whether the formation of an H-aggregate promoted by poly cytidine ssDNA may be a general characteristic of dimeric homocyanine dyes. On the other hand, we explore the H-aggregate of YOYO-3 templated by dsDNA, and the involvement of different base composition and the nature of the grooves in the formation of the aggregate. Our interest here lies in exploring the features, stoichiometry and stability of these H-aggregates of YOYO-3 using absorption and steady-state and time-resolved fluorescence spectroscopy.

EXPERIMENTAL METHODS

Reagents. All experiments were performed using chemicals of analytical reagent grade and Milli-Q water. The stock solutions of all chemicals were protected from sunlight and kept at approximately 4 °C in a refrigerator. The pH of the buffer solutions was adjusted using diluted spectroscopic-grade NaOH and HCl (Sigma-Aldrich, Spain) dissolved in Milli-Q water. YOYO-3 iodide (Quinolinium, 1,1′-[1,3-propanediylbis-[(dimethyliminio)-3,1-propanediyl]]bis[4-[3-(3-methyl-2(3H)-benzoxazolylidene)-1-propenyl]]-, tetraiodide) stock solution (LifeTechnologies, Carlsbad, CA, USA) was freshly diluted in a buffer solution at a pH of 7.35 that contained 10 mM Tris, 1 mM EDTA, and 100 mM NaCl (TEN buffer). The buffer salt of Tris, sodium chloride, and EDTA were obtained from Sigma-Aldrich (Spain). In the staining experiments, YOYO-3 was added to the corresponding DNA solution and incubated for 10 min in the dark at 25 °C. Unless otherwise specified, all the experiments were performed in TEN buffer at pH 7.35.

Oligonucleotides. The chemically synthesized homonucleotides and other sequences of oligonucleotides were obtained from IBA Technologies (Germany). Polyadenosine ($ssPolyA_{30}$), polythymidine ($ssPolyT_{30}$), and polycytidine ($ssPolyC_{30}$) oligonucleotides were synthesized with 30 nucleotides. Synthesis and purification of polyguanosine oligonucleotides is not possible because a guanine higher-order structure, guanine quadruplex, is formed during the synthesis.[28,29] The interactions of deoxyguanosine and the pair CG with YOYO-3 were characterized using a mixed oligonucleotide. This mixed oligonucleotide was a $C(GGGC)_5$ strand (named $ssPoliG_{21}$), together with its complementary

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strand (see Table 1). The synthesis of long polyinosine oligonucleotides is also complicated. The number of inosine residues left after purification cannot be specified and ranged between 30 and 50. All synthesized single-stranded oligonucleotides were purified by double HPLC and dissolved in TEN buffer. The stock ssDNA concentration was verified by absorption measurements at 260 nm. All subsequent dilutions for the working solutions were prepared in the same buffer. The sequences of all DNA employed in this work are shown in Table 1. To perform the hybridization, the ssDNA sequences were annealed with their corresponding complementary sequences by heating at 75 °C for 5 min followed by slow cooling to room temperature, obtaining the respective dsDNA. The oligonucleotide solutions used in this work ranged between 1 and 100 nM dsDNA or ssDNA.

Instruments. Absorption spectra were recorded at 25 °C using a PerkinElmer Lambda 650 UV/vis spectrophotometer equipped with a Peltier temperature-controlled cell holder. Circular dichroism (CD) spectra were measured on a Jasco J-715 spectropolarimeter (Jasco, USA), equipped with a Peltier (PTC-348W1) for temperature control. The measurements were performed at 25 °C using a 4 × 10 mm quartz cuvette (the optical path used was 10 mm). Steady-state fluorescence emission spectra were collected at 25 °C on a JASCO FP-6500 spectrofluorometer using a 450-W xenon lamp as the excitation source and an ETC-273T temperature controller. Fluorescence decay traces were recorded in time-correlated single photon counting (TCSPC) mode, using a FluoTime 200 fluorometer furnished with a TimeHarp 200 collection card and equipped with a fiber-coupled 531 nm LDH-P-FA-530 pulsed laser (PicoQuant GmbH, Germany), working at a 20 MHz repetition rate. The fluorescence was collected at a 90° geometry after crossing a polarizer set at the magic angle (54.7°) and using a 2 nm bandwidth monochromator. The instrument response function (IRF), measured using a Ludox scatterer suspension, has a full width at half-maximum of 330 ps, and the sample decay traces were recorded until reaching 2 × 10^4 counts in the peak channel. The fluorescence decay traces of aqueous solutions of YOYO-3 in solution and in the presence of the single-stranded polyhomonucleotides ([YOYO-3]/[base] ratio range: 0.003−0.03) were recorded at 531 nm for excitation and 600, 610, and 620 nm for emission. This range was selected to avoid energy transfer processes between YOYO-3 molecules in close proximity, that has been reported to occur at [YOYO-3]/[DNA bp] ratios higher than 0.1.[14] The three decay traces of each sample were globally analyzed by a least-squares minimization deconvolution method in terms of the multiexponential functions using FluoFit software (PicoQuant GmbH). The decay times were treated as shared parameters, and the pre-exponential factors were treated as local adjustable parameters. The shortest lifetime was kept fixed to the lifetime of YOYO-3 in solution, 0.30 ns because this value is close to the instrumental response. The reduced chi-squared value, χ^2, the weighted residuals, and the autocorrelation functions were indicators of the goodness of fit. All fluorescence experiments were performed using 5 × 10 mm quartz cuvettes.

RESULTS AND DISCUSSION

YOYO-3 H-Aggregate Templated by ssDNA. In a previous work, we demonstrated that the dimeric cyanine BOBO-3 forms an H-aggregate templated by, at least, six consecutive cytidine nucleotides in a single DNA strand. Herein, we have checked whether this may be a general property of dimeric cyanine dyes by testing the interactions of YOYO-3 with single-stranded poly homonucleotides by using UV−vis absorption spectrometry, and steady-state and time-resolved fluorimetry. In the visible region, free YOYO-3 displays two absorption bands with maxima at 560 and 605 nm. The 560 nm absorption band is caused by the formation of internal dimers.[12,15] The addition of the single-stranded poly homonucleotides ssPolyA$_{30}$, ssPolyT$_{30}$ or ssPolyG$_{21}$ (see Table 1) practically did not alter the shape of the absorption spectra of YOYO-3 (Figure 2a and Table 2), although the absorbance

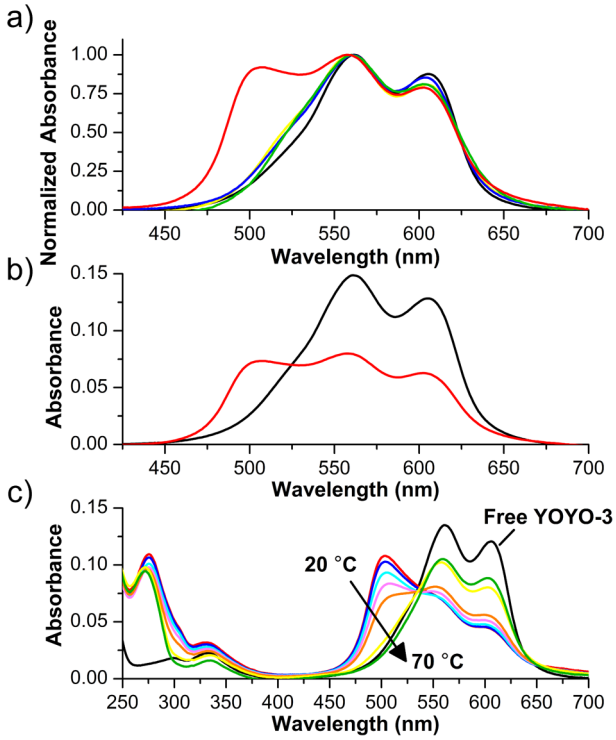

Figure 2. (a) Normalized absorption spectra of YOYO-3 (1.5 × 10^{-6} M) free in TEN buffer solution (pH 7.35) (black) and upon addition of single-stranded polyhomonucleotides: ssPolyT$_{30}$ (yellow), ssPolyG$_{21}$ (blue), ssPolyA$_{30}$ (green), and ssPolyC$_{30}$ (red). (b) Absorption spectra of YOYO-3 (1.5 × 10^{-6} M) free in TEN buffer solution (pH 7.35) (black) and upon addition of ssPolyC$_{30}$ (red). [YOYO-3]/[DNA base] ratio = 0.5. (c) Absorption spectra of YOYO-3 (1.5 × 10^{-6} M) (black) with ssPolyC$_{30}$ (3 × 10^{-7} M) at different temperatures: 20 °C (red); 30 °C (blue); 40 °C (cyan); 50 °C (pink); 55 °C (orange); 60 °C (yellow); and 70 °C (green).

decreased when larger amounts of these polyhomonucleotides were added (see Figure SI.1 in the Supporting Information). In contrast, the addition of ssPolyC$_{30}$ clearly gave rise to the appearance of a new absorption band at approximately 508 nm (Figure 2 and Table 2). This band is similar to that previously observed for BOBO-3 in the presence of similar polycytidine ssDNA.[27]

Regarding the fluorescence emission, free YOYO-3 in aqueous solution is poorly fluorescent, showing a fluorescence maximum centered at 620 nm. The addition of any of the four studied single-stranded polyhomonucleotide did not significantly modify either the fluorescence intensity or the spectrum shape (Figure SI. 2 and Table 2). These results are consistent with the inability of the YOYO-3 dye to intercalate into single-stranded polyhomonucleotides, and the only possible binding

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Table 2. Summary of Some Spectral Characteristics of YOYO-3 in Solution and Interacting with Single- and Double-Stranded Polyhomonucleotides

	$absorption_{max}$ (nm)	$emission_{max}$ (nm)	lifetime (ns)
YOYO-3 in solution	560/605	620	0.30 ± 0.04
YOYO-3/ssPolyA_{30}	560/605	620	1.07 ± 0.02 0.30[a]
YOYO-3/ssPolyT_{30}	560/605	620	0.99 ± 0.03 0.30[a]
YOYO-3/ssPolyC_{30}	508/560/603	620	1.66 ± 0.23 0.30[a]
YOYO-3/ssPolyG_{21}	560/605	620	3.38 ± 0.11 1.51 ± 0.23 0.30[a]
YOYO-3/ dsPoly$(AT)_{30}$	503/563/605	625	3.26 ± 0.21 1.26 ± 0.12 0.30[a]
YOYO-3/ dsPoly$(CG)_{21}$	563/605	630	3.37 ± 0.14 1.58 ± 0.17 0.30[a]

[a]Because the value of the shortest lifetime is close to the instrumental response, during the fitting, this value remained fixed to the one experimentally obtained for the free YOYO-3 in aqueous solution (in the absence of DNA).

mode is driven by electrostatic interactions with the phosphate groups. These interactions do not enhance the fluorescence emission of the cyanine homodimer dyes.[11,13] Interestingly, the additional absorption band found in the presence of ssPolyC_{30} (centered at 508 nm) was confirmed to be nonfluorescent, as evidenced by the excitation spectra (λ_{em} = 630 nm), which displayed no excitation band of YOYO-3 around 500 nm. Likewise, the samples did not show any appreciable emission when directly excited at 508 nm (data not shown). Time-resolved fluorescence also provided some insights into the interaction of YOYO-3 with the ssDNA polynucleotides. The fluorescence decay profile of free YOYO-3 in aqueous solution was found to follow a monoexponential function with a short lifetime of 0.30 ± 0.04 ns, which agrees with the low quantum yield of free YOYO-3.[15] The fluorescence decay curves of YOYO-3 in the presence of ssPolyC_{30}, ssPolyA_{30}, and ssPolyT_{30} displayed two lifetimes, corresponding to a major contribution of free dye, and a small population of the dye bonded to the single strand, with a lifetime between 1 and 1.7 ns (Table 2). This value is in good agreement with the lifetime recorded for other cyanine dyes interacting with ssDNA by electrostatic interactions,[13,17] and the absence of bis-intercalation, because self-hybridization is not possible for these homonucleotide strands. However, when ssPolyG_{21} was present, YOYO-3 showed triexponential decay traces, with a third longer lifetime component of 3.38 ns (Table 2), similar to the decay traces found in dsDNA.[14] The ssPolyG_{21} sequence is not only homogeneously composed of guanidine nucleotides, but it also contains some cytidine nucleotides (Table 1), due to the impossibility of synthesizing pure polyG oligonucleotides. Hence, in our ssPolyG_{21} sequence, a G-quadruplex structure,[30] partial self-hybridization between adjacent strands, and several hairpins are potentially possible. These structures can even be stabilized by the intercalating dye,[31] allowing the formation of transient contacts and the binding of YOYO-3 in an intercalation mode, which is responsible for the longest lifetime component.

Hence, we have found that only the poly cytidine oligonucleotide is capable of promoting the appearance of a different absorber structure of YOYO-3. The hypsochromic effect of the new absorption band is characteristic of H-aggregates. These type of aggregates are well described for cyanine dyes interacting with negatively charged templates such as polyelectrolytes[21,32] silica films,[33] nanoparticles,[22] calix[4]-arene,[23] and also double-stranded DNA molecules,[24−26] such as the ones formed by thiazole orange and Cyan 13 dyes.[34] These H-aggregates usually present hypsochromic shifts in the absorption bands and aggregate in a parallel way, plane-to-plane stacking, to form a sandwich-type arrangement.[19] In contrast, other dyes may aggregate in a head-to-tail arrangement (end-to-end stacking) and present a staggered J-aggregate configuration,[19] which leads to red-shifted absorption bands, such as the aggregate formed by the dye Cyan βiPr[35] or by TO-PRO-3.[36] Most of the previous reported models of H-aggregates templated by DNA required hybridized, double DNA strands. The only previous report of an H-aggregate templated by ssDNA involved another bischromophoric, cyanine dye, BOBO-3, and polycytidine ssDNA.[27] Herein, we have found that the formation of this type of H-aggregate may be a general property of dimeric cyanine dyes, and that only cytosine, but not the other bases, allows the formation of the complex, which suggests that the structure of this base has to be crucially involved in the formation of the aggregate.

One of the most striking features of the BOBO-3 H-aggregate with polycytidine oligonucleotides is that its formation is so stable that it even prevents the hybridization of the ssDNA with its complementary strand.[27] We have also tested whether this is the case with the YOYO-3 dye. We used single-stranded polyinosine oligonucleotide (ssPolyI_{30-50}) as the complementary sequence of the polycytidine ssPolyC_{30}. We started with the YOYO-3 dye interacting with ssPolyC_{30}, forming the H-aggregate complex. When the complementary strand (ssPolyI_{30-50}) was added, the 508 nm absorption band disappeared completely (Figure SI.3a), suggesting that ssPolyC_{30} shows a higher affinity to hybridize with ssPolyI_{30-50} than to remain forming the H-aggregate complex with YOYO-3. When the double strand is formed, YOYO-3 subsequently intercalates into the duplex DNA, as demonstrated by the increased emission intensity (Figure SI.3b), characteristic of bis-intercalation of the cyanine dye. These findings suggest that the stability of the H-aggregate of YOYO-3 is lower than that of the equivalent complex of BOBO-3. The main reason for this different behavior lies on the higher affinity toward intercalation of the YOYO dyes compared to the BOBO family.[11−13,17] Indeed, the largest binding constants for the intercalative mode of the homodimeric cyanine dyes are reported for the YOYO and TOTO dyes.[4,11,37] Both dyes contain two quinolinium groups, whereas the BOBO dyes contain two pyridine moieties at the same positions (see Figure 1 for structures). The presence of the quinolinium groups in the YOYO-3 structure might favor the π-stacking required for the bis-intercalation into the double helix, making this binding mode more favored than the H-aggregate with the single-stranded ssPolyC_{30}.

We also studied the thermal stability of the H-aggregate complex between YOYO-3 and ssPolyC_{30} using UV−vis absorption measurements. The absorption spectra of the solutions at different temperatures, between 20 and 70 °C, showed the gradual decrease of the absorption band of the H-aggregate (at 508 nm) with increasing temperatures. The H-aggregate band was practically undetectable at temperatures

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above 60 °C. These results confirm that the H-aggregate formed by YOYO-3 using cytidines as the template is less stable than the analogous H-aggregate formed by BOBO-3, because for the latter, an increase in temperature up to 70 °C does not eliminate the complex.[27]

To gain more insights into the stoichiometry and 3D arrangement of the H-aggregates of YOYO-3 with single-stranded polycytidine nucleotides, we investigated the minimal sequence of consecutive bases required to effectively form the complex. First, YOYO-3 was titrated with single-stranded oligonucleotides with different number of consecutive cytidines (from 4 to 10) at each end (see Table 1 for sequences). The absorption spectra of YOYO-3 during the titration with $(Olig4C)_{26}$ and $(Olig5C)_{28}$ (four and five consecutive cytosines at each end) did not appreciably displayed the characteristic shoulder of the H-aggregate complex but did show reduced absorbance with increasing ssDNA concentrations (Figures SI.4), which is a result of the binding mode of YOYO-3 through electrostatic interactions.[13] This result suggests that when the number of consecutive cytosines in the sequence is low, the three-dimensional conformation of the bases is inappropriate for the formation of the H-aggregate complex. However, when the titration was performed with ssDNA containing six or more consecutive cytosine bases ($(Olig6C)_{30}$ and $(Olig10C)_{38}$), the absorption band at approximately 500 nm was manifested, even though the intensity of the band was less pronounced than with polycytidine oligonucleotides (Figure 3a and Figure SI.5a). We also investigated whether the position of the cytidines in the oligonucleotide strand was a determining factor. We performed a similar titration with an ssDNA sequence containing six consecutive cytosines, but in the middle of the chain

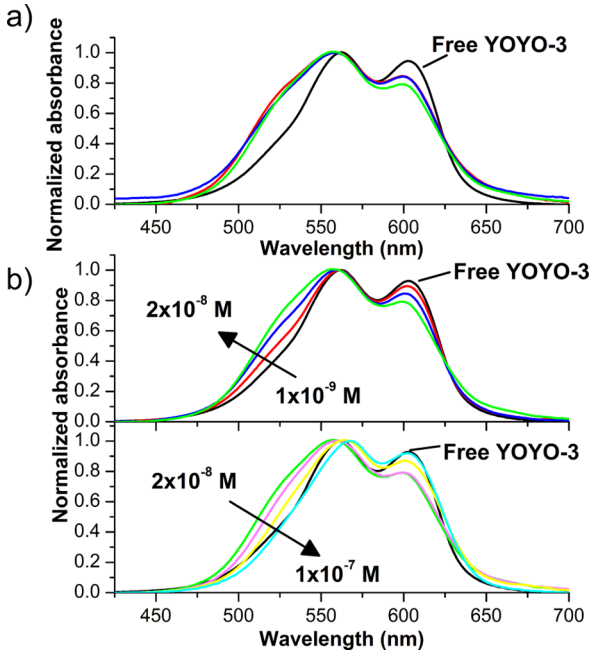

Figure 3. (a) Absorption spectra of free YOYO-3 (1.5×10^{-6} M) (black) and YOYO-3 with single-stranded oligonucleotides (2×10^{-8} M) containing a different number of consecutive cytidines: $(Olig6C)_{30}$ (red); $(Olig6Ccenter)_{24}$ (blue); and $(Olig10C)_{38}$ (green). (b) Absorption spectra of titrations of YOYO-3 (1.5×10^{-6} M) (black) with the oligonucleotide $(Olig10C)_{38}$. Concentration range: 1×10^{-9} M (red); 1×10^{-8} M (blue); 2×10^{-8} M (green); 3.5×10^{-8} M (pink); 7×10^{-8} M (yellow); and 1×10^{-7} M (cyan).

$(Olig6Ccenter)_{24}$ (see Table 1 for sequence). With this oligonucleotide, the absorption band of the YOYO-3 H-aggregate is evident (Figure SI.5b), confirming that the H-aggregate forms stacked on consecutive cytidines, regardless of the position of the cytidine along the ssDNA chain. An interesting feature of all these titrations was that, above a certain concentration of ssDNA, the intensity of the H-aggregate absorption band diminishes until it disappears (Figure 3b, and Figure SI.5). The decrease in the H-aggregate absorption band occurred along with a concomitant increase in the fluorescence emission of the YOYO-3 (see Figure SI.6). The fluorescence emission indicates that the YOYO-3 moiety is protected in an intercalative binding mode. Indeed, the sequences $(Olig6C)_{30}$, $(Olig6Ccenter)_{24}$, and $(Olig10C)_{38}$ allow up to four consecutive matches for intermolecular self-dimerization, that may occur at high ssDNA concentration and are stabilized by the presence of the intercalating dye.[31] This behavior is consistent with the lower stability of the YOYO-3 H-aggregate compared to that of BOBO-3.

Our results suggest that the formation of an H-aggregate complex of YOYO-3 with cytosines requires at least six consecutive cytidines in a row, possibly related to its 3D configuration. This stoichiometry is in good agreement with other stoichiometries found in the literature for templated H-aggregates of cyanine dyes. For instance, the dyes pseudoisocyanine and pinacyanol using polystyrene-sulfonate as a negatively charged template formed an H-aggregate with a sulfonate group/dye molar ratio of approximately 3:1.[21] In a different study, the dye 3,3′-diethylthiadicarbocyanine aggregated in the minor grooves of double stranded poly(AT), with a stoichiometry of 2.5 base pairs per dye.[24] Because YOYO-3 is a homodimer dye with two cyanine units, the requirement of six cytosine bases to form the H-aggregate complex with one YOYO-3 molecule is very similar to the analogous 6 sulfonate/2 cyanine units,[21] or 5 base pair/2 cyanine units[24] from the aforementioned previous works. Furthermore, the stoichiometry of the YOYO-3 aggregate is the same as that of the analogous BOBO-3 polycytidine-templated aggregate.[27] Nevertheless, the H-aggregate of BOBO-3 was promoted not only by consecutive cytidines but also by adenosine nucleotides, suggesting the involvement in the formation of the complex of hydrogen bonding through the primary amine groups of the bases. In contrast, YOYO-3 only aggregates with cytosines as template. This fact, along with the lower stability of the YOYO-3 aggregate, implies a different nature of the interactions. One possible candidate for such specificity is the ability of C-rich repeats to fold into an i-motif structure. In the i-motif, two cytidine stretches form a parallel-stranded duplex with hemiprotonated C·CH+ pairs, with two of such duplexes associate head-to-tail by base pair intercalation into a quadruplex.[38,39] The formation of this structure is favored at acidic pH, or pH close to neutrality at low temperature (4 °C),[40] but it also depends on the total concentration, and the specific sequence.[41,42] To investigate whether the formation of i-motif could be related to the H-aggregate complex with YOYO-3, we recorded the absorption spectra of solutions containing the same amount of YOYO-3 and $ssPolyC_{30}$ in TEN buffer but at different pH values (Figure SI.7). The absorption band of the H-aggregate complex was clearly observed from acidic pH (pH 4.2) to pH slightly basic (pH 7.5), but at more basic pH (pH > 8) it started to decrease. These results suggest that the i-motif structure is likely involved in the formation of the YOYO-3 H-aggregate with the cytosine chains. Even

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Table 3. Sequences of the Tested Double-Stranded DNA Oligonucleotides

sequence	name
5′-AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA-3′	dsPoly(AT)
5′-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-3′	
5′-CCCCCCCCCCCCCCCCCCCCCCCCCCCCCC-3′	dsPoly(CI)
5′-IIIIIIIIIIIIIIIIIIIIIIIIIIIIII$(I)_{1-20}$-3′	
5′-CGGGCGGGCGGGCGGGCGGGC-3′	dsPoly(CG)
5′-GCCCGCCCGCCCGCCCGCCCG-3′	
5′-GCGTATCAAAAAAAAAAAAAAAAAAAACAATAGG-3′	ds(Olig20AT)
5′-CCTATTGTTTTTTTTTTTTTTTTTTTGATACGC-3′	
5′-ATATATATATATATATATATATATATATAT-3′	ds(OligATAT)
self-hybridized	

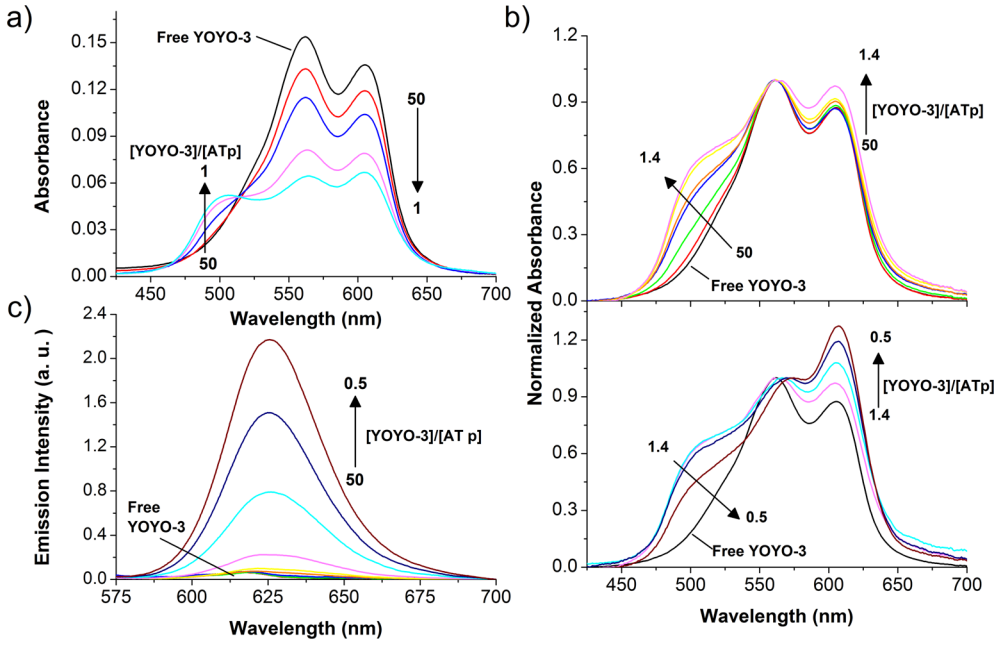

Figure 4. (a) Absorption spectra from titrations of YOYO-3 in solution (black) and YOYO-3 with dsPoly(AT). (b) Normalized absorption, and (c) emission spectra from titrations of free YOYO-3 (black) with dsPoly(AT). [YOYO-3] = 1.5 × 10^{-6} M (TEN buffer pH 7.35). (λ_{ex} = 560 nm). [YOYO-3]/[AT pair] ratio: 50 (red), 5.0 (green), 3.3 (blue), 2.5 (orange), 2.0 (yellow), 1.4 (pink), 1.0 (cyan), 0.7 (navy), and 0.5 (wine).

thought at pH > 7 the C·CH+ pairing needed for the formation of the i-motif structure is unlikely, the presence of YOYO-3 might favor its formation at room temperature and stabilize the complex.

YOYO-3 H-Aggregate Templated by dsDNA. In a previous work, we found that YOYO-3 in the presence of dsDNA can also form a characteristic aggregated with features resembling an H-aggregate, at high [YOYO-3]/[base-pair] ratios.[14] This previously reported H-aggregate must be different to that described above, because it was detected with dsDNA. Herein, we have also focused in the formation of this H-aggregate of YOYO-3 promoted by dsDNA, specially giving attention to DNA sequence specificity. Hence, we measured the UV−vis absorption spectra of YOYO-3 in the presence of double-stranded poly homonucleotides (see Table 3). When dsPoly(CG) (21 base pairs) was added to YOYO-3, the absorbance spectrum of the dye displayed an increase of the band at 605 nm, and a concomitant decrease of the 560 nm band (Figure SI.8a). These changes in the absorption spectrum were accompanied by a prominent enhancement of the emission intensity (Figure SI.8b), and a shift in the emission maximum from 620 to 630 nm. Fluorescence decay traces of YOYO-3 interacting with dsPoly(CG) required three exponential decay times: the short lifetime corresponding to YOYO-3 in solution (0.3 ns), and two long decay times of 1.58 ± 0.17 and 3.37 ± 0.14 ns. These features are in agreement with a conventional bis-intercalation model of the YOYO-3 within the double helix, where the dye is more protected from the solvent, which increases its quantum yield and long fluorescence decay time, along with some contribution of external binding in the minor grooves giving rise to the intermediate decay time.[13] This behavior does not differ from YOYO-3 intercalating into dsDNA containing all four bases in random sequence.[14]

Nonetheless, the addition of dsPoly(AT) (30 base pairs, see Table 3) to free YOYO-3 showed different absorption features. The interaction with dsPoly(AT) led to the appearance of a new band centered at 503 nm and a decrease in the absorption intensity of the other two bands (Figure 4a). This blue-shifted absorption band suggests the formation of a new H-aggregate of YOYO-3 templated by the minor grooves of dsDNA AT-rich regions, similar to previously reported cases of other cyanine dyes. There are several models of H-aggregates formed in DNA, such as dimers of 3,3′-diethylthiadicarbocyanine that grow cooperatively by adding additional dimers to adjacent sites,[24,25,43] the half-intercalation model proposed for TO and

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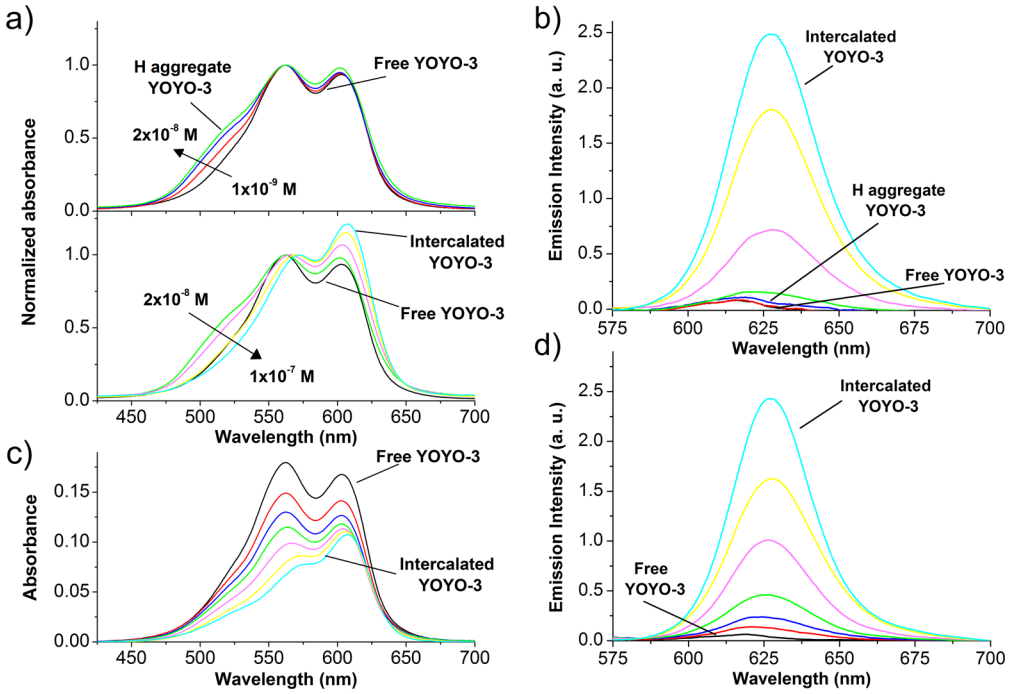

Figure 5. (a) Normalized absorption, and (b) emission spectra from titrations of free YOYO-3 (black) with ds(Olig20AT). (c) Absorption, and (d) emission spectra from titrations of YOYO-3 with ds(OligATAT). [YOYO-3] = 1.5×10^{-6} M (TEN buffer pH 7.35). λ_{ex} = 560 nm. Concentration range: 1×10^{-9} M (red); 1×10^{-8} M (blue); 2×10^{-8} M (green); 3.5×10^{-8} M (pink); 7×10^{-8} M (yellow); and 1×10^{-7} M (cyan).

Cyan 13,[19] or the high order arrangements described for unsymmetrical cyanine dyes.[36]

To investigate the optimal conditions for the formation of the H-aggregate, YOYO-3 was titrated with increasing amounts of dsPoly(AT) in a concentration range between 1×10^{-9} and 1×10^{-7} M (corresponding to a [YOYO-3]/[AT pair] ratio between 50 and 0.5). At high [YOYO-3]/[AT pair] ratios (>1), an isosbestic point in the absorption spectra was clearly observed (Figure 4a), indicating two main absorbent species in equilibrium: YOYO-3 free in solution and YOYO-3 stacked forming an H-aggregate. When the amount of dsPoly(AT) was increased up to a ratio [YOYO-3]/[AT pair] of 0.5, the shape of the absorption spectra changed. To show the changes more clearly, Figure 4b shows the normalized absorption spectra of YOYO-3 during the complete titration. The absorption band assigned to the formation of the H-aggregate complex reaches a maximum when the [YOYO-3]/[AT pair] ratio is 1.4, but at higher concentrations of dsPoly(AT) (lower ratios), this band begins to decrease and finally disappears (Figure 4b). Simultaneously, from the same [YOYO-3]/[AT pair] ratio, the intensity of the band at 605 nm starts to be enhanced, which indicates that the intercalative mode of binding appears and increases.[11,13] Regarding the fluorescence emission (Figure 4c), the fluorescence spectra and intensity of free YOYO-3 remained practically unchanged when the [YOYO-3]/[AT pair] ratio was higher than 1.4. However, when the concentration of dsPoly(AT) increased, the fluorescence maxima shifted to 625 nm, and the emission intensity was drastically enhanced. The fluorescence decay traces of YOYO-3 in the presence of dsPoly(AT), at low [YOYO-3]/[AT pair] ratios (from 0.003 to 0.03, to avoid homoenergy transfer processes),[14] confirmed the presence of free YOYO-3 (with a decay time of 0.30 ns), the secondary external binding (with a decay time of 1.26 ± 0.12 ns), and the intercalated mode (with a decay time of 3.26 ± 0.21 ns). Unlike other homodimer cyanine dyes such as TOTO-1[16] and BOBO-3,[17] the long lifetime of YOYO-3 does not show significant differences when bonded to AT or CG sites (Table 2). Temperature melting experiments of the YOYO-3 H-aggregate in dsPoly(AT) showed that the characteristic absorption band of the H-aggregate diminished at 40 °C and completely disappeared when the temperature was higher than 60 °C (Figure SI.9). Interestingly, after heating above the duplex melting temperature and slow cooling, the characteristic absorption band at 503 nm did not reappear, and the YOYO-3 presented enhanced fluorescence emission. This means that when the duplex DNA is formed in the presence of the dimeric cyanine, a more stable situation is reached with the dye intercalated into the dsDNA, and the H-aggregates are not formed.

Our results seem to entail that at high [YOYO-3]/[AT pair] ratios, the dye is kinetically trapped preferentially in the nonfluorescent, H-aggregate form. The YOYO-3 is saturating the minor grooves of the dsPoly(AT), preventing the solvent-protected intercalation within the double strand. Conversely, at [YOYO-3]/[AT pair] ratios lower than 1.4, there are more sites available for bis-intercalation, and this binding mode is facilitated, disrupting the H-type parallel stacking. To confirm this behavior, we performed CD measurements of YOYO-3 interacting with dsPoly(AT). The phenomenon of induced CD makes achiral molecules, such as YOYO-3, to be optically active when bound to chiral molecules, such as dsDNA.[44] The recorded CD spectra (Figure SI.10) showed that at high [YOYO-3]/[AT pair] ratios (>1) the YOYO-3 was exclusively active in the 500 nm region, whereas no optical activity was detected in the 550−650 nm range. This means that the YOYO-3 molecules bound to dsDNA are exclusively absorbing in this spectral region corresponding to the H-aggregate. In contrast, when the [YOYO-3]/[AT pair] ratio was decrease to 0.1, the typical ∓ pattern in the 550−650 nm range was detected due to the bis-intercalated YOYO-3.

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We further explored the preferential binding of this YOYO-3 H-aggregate to AT sites, and its relation with the minor groove dimensions. First, YOYO-3 was titrated with a double stranded oligonucleotide containing 20 AT consecutive base pairs ($ds(Olig20AT)_{34}$), in which all adenine bases were in the same strand and all thymine bases were in the complementary strand (Table 3). At a low concentration of the ds(Olig20AT) ($<2 \times 10^{-8}$ M, high [YOYO-3]/[DNA bp] ratios), the characteristic absorption band at approximately 500 nm is clearly observed (Figure 5a), whereas the emission intensity is negligible (Figure 5b). Both facts indicate that the H-aggregate of YOYO-3 is preferentially formed with this oligonucleotide, even in the presence of the other bases, and no intercalated dye is detected. At higher concentrations of oligonucleotide, the formation of the H-aggregate competes with the intercalation mode, and the band begins to decrease and finally disappears, with the concomitant increase in the intensity of the absorption band at 605 nm (Figure 5a) and the emission intensity (Figure 5b). Indeed, the fluorescence spectra become significant when the concentration of oligonucleotide is 3.5×10^{-8} M or higher, which corresponds with a [YOYO-3]/[DNA bp] ratio of approximately 1.4. Both spectral features are typical of the intercalated binding mode and confirm the change of the interaction mode. We also studied a dsDNA oligomer that contains an AT-rich region, but with alternating bases (-TATA-, see Table 3). In this case, YOYO-3 does not form the H-aggregate, and the distinctive absorption band at 500 nm is not detected (Figure 5c). From the beginning of the titration, the absorption spectra show the features of the dye intercalated into the duplex, i.e., the increase in the absorption band at 605 nm coupled with the concomitant decrease in of the band centered at 560 nm (Figure 5c). Simultaneously, the shift of the emission maximum is observed from the addition of the first aliquot, and the emission intensity rises gradually (Figure 5d). These observations clearly indicate that the only interaction mode of YOYO-3 with this oligonucleotide is by intercalation and are in good agreement with the behavior of YOYO-3 intercalated into the double helix of CG pairs (see Figure SI.8), in which the H-aggregate is not formed. These experiments clearly demonstrated that the formation of the H-aggregate requires polyA·polyT tracts in dsDNA. The main distinctive feature of polyA·polyT tracts is that they provide stiffness to the double strand and these regions display the narrowest minor grooves.[45] Indeed, AAAA·TTTT minor grooves have a 3.9 Å width, whereas TATA·ATAT regions display minor grooves of 5.7 Å width.[46] Moreover, the negative electrostatic potential is enhanced in narrow minor grooves, providing an attractive charged environment to promote self-assembly of the cationic dyes.[24,35] Moreover, AT-rich regions are usually preferred opposite to the CG grooves because of a lower steric hindrance caused by the absence of amine groups in adenine bases.[24,25,35,36,43] Hence, this second type of H-aggregate formed by YOYO-3 in polyA·polyT tracts is likely to be mainly caused by a narrow fit, enhanced negative electrostatic potential in the minor groove, and less steric hindrance.

CONCLUSIONS

We have explored the formation of two different types of H-aggregate of YOYO-3 in either single- and double-stranded DNA. In ssDNA, a nonfluorescent H-aggregate of YOYO-3, characterized by the appearance of an absorption band at 500 nm, is formed in polycytidine single strands. This H-aggregate promoted by polycytidine single strands is very similar to that previously reported for BOBO-3,[27] suggesting that it may be a general property of dimeric cyanine dyes. The formation of this H-aggregate is promoted when at least six consecutive cytidines are present. The pH dependency of the formation of this H-aggregate supports the involvement of the C-rich i-motif structure in the formation of the aggregate. Interestingly, this H-aggregate is less stable than that of BOBO-3. The BOBO-3 H-aggregate strikingly prevented the aggregation of the single strands with their complementary. In contrast, the YOYO-3 H-aggregate is easily disrupted in the presence of the complementary single-strand. The presence of the quinolinium groups in the YOYO-3 structure may be responsible of the more stable and preferred intercalation mode.

The second type of YOYO-3 H-aggregate was found in polyA·polyT tracts in double-stranded DNA when the [DNA]/[YOYO-3] ratio is low enough. The formation of the H-aggregate is accomplished only when either consecutive adenines or thymines are in the same strand. Our results suggest that the narrower minor grooves of polyA·polyT tracts provide a kinetic tract for the formation of the H-aggregate, that is disrupted at higher [dsDNA]/[YOYO-3] ratios at which the YOYO-3 prefers the bis-intercalation mode.

ASSOCIATED CONTENT

Supporting Information

Supporting Figures S-1 to S-10. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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