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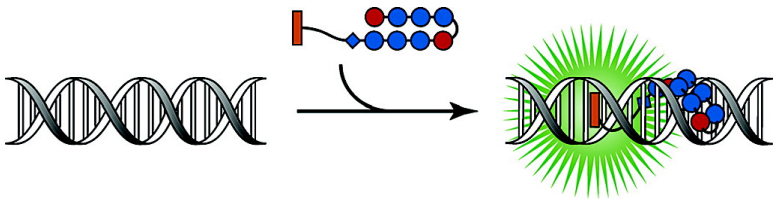

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Sequence-Specific Fluorescence Detection of DNA by Polyamide−Thiazole Orange Conjugates

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Abstract: Fluorescent methods to detect specific double-stranded DNA sequences without the need for denaturation may be useful in the field of genetics. Three hairpin pyrrole-imidazole polyamides **2**−**4** that target their respective sequences 5′-WGGGWW-3′, 5′-WGGCCW-3′, and 5′-WGWWCW-3′ (W = A or T) were conjugated to thiazole orange dye at the C-termini to examine their fluorescence properties in the presence and absence of match duplex DNA. The conjugates fluoresce weakly in the absence of DNA but showed significant enhancement (>1000-fold) upon the addition of 1 equiv of match DNA and only slight enhancement with the addition of mismatch DNA. The polyamide−dye conjugates bound specific DNA sequences with high affinity ($K_a > 10^8$ M^{-1}) and unwound the DNA duplex through intercalation (unwinding angle, ϕ, ∼8°). This new class of polyamides provides a method to specifically detect DNA sequences without denaturation.

Introduction

Interest in the detection of specific nucleic acid sequences in homogeneous solution has increased due to major developments in human genetics. Single nucleotide polymorphisms (SNP's) are the most common form of variation in the human genome and can be diagnostic of particular genetic predispositions toward disease.[1] Most methods of DNA detection involve hybridization by an oligonucleotide probe to its complementary single strand nucleic acid target to generate a fluorescent signal.[2−4] Examples include the molecular beacons, in which a fluorophore and quencher are conjugated to a DNA hairpin at the 5′ and 3′ ends, respectively.[2] Fluorescence emission is detected upon probe hybridization to its target single-stranded DNA site by separating the fluorophore from the quencher. Another class, the "light-up probes", utilize a peptide nucleic acid (PNA) conjugated to a thiazole orange (TO) cyanine dye.[4] The PNA directs the conjugate to a specific hybridization site, where emission of the relatively nonfluorescent dye is enhanced by approximately 50-fold. In both cases signal enhancement is only reported upon hybridization to single-stranded DNA, thus requiring harsh denaturation conditions for the detection of a DNA sequence. A series of peptide−thiazole orange conjugates have fluorescence enhancement properties upon binding to calf thymus (CT) DNA but were not shown to discriminate different DNA sequences.[5] Fluorescent methods to detect specific double-stranded DNA sequences require the integration of sequence specific double strand DNA binding molecules with enhanced fluorescent properties of a dye upon DNA complexation.

Pyrrole-imidazole polyamides are a class of minor groove-binding ligands that can be programmed to recognize specific DNA sequences with affinities and specificities comparable to DNA binding proteins.[6] Polyamides with tetramethyl rhodamine (TMR) tethered to an internal pyrrole ring have been found to display modest sequence-specific fluorescence enhancement (≥10-fold) upon binding to match duplex DNA.[7] Although these conjugates bound DNA with slightly reduced affinity relative to their parent compounds (those not containing TMR), the detection method proved useful in probing the interactions between polyamides and non-Watson−Crick DNA base pairs.[7] We recently demonstrated that acridine covalently linked to a hairpin polyamide permits both intercalation and minor groove binding, while the polyamide's capacity for sequence discrimination is retained.[8] These polyamide−intercalator conjugates were utilized to inhibit the binding of major-groove-binding transcription factors, presumably by an allosteric mechanism (helix distortion). Utilizing the programmable nature of a minor-groove-binding polyamide combined with a DNA-binding

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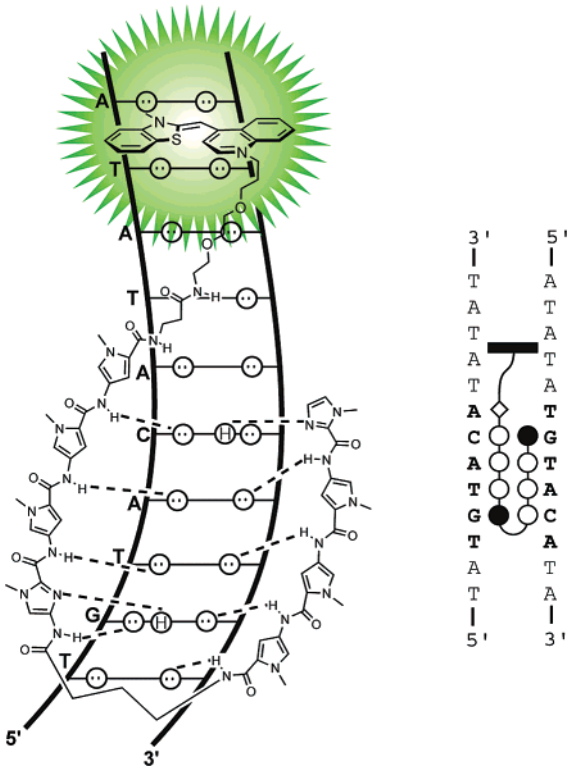

Figure 1. DNA binding model for the eight-ring hairpin polyamide conjugate ImPyPyPy-γ-PyPyPyIm-β−PEG_2−TO bound to the minor groove of 5′-TGTACAT-3′. (Left) Circles with dots represent lone pairs of N3 of purines and O2 of pyrimidines. Circles containing an H represent the N2 hydrogens of guanine. Putative hydrogen bonds are illustrated by dotted lines. (Right) Solid circles represent imidazoles (Im), open circles represent pyrroles (Py), and diamonds denote β-alanine (β). The shaded bar depicts the thiazole orange intercalator. Fluorescence emission from the conjugate−DNA complex is represented in green. According to the pairing rules, Im/Py codes for G-C, Py/Py for A-T or T-A, Py/Im for C-G, and β-alanine for A,T.

fluorophore such as TO (**1**) that enhances upon DNA binding (intercalation) may provide a highly specific and sensitive method for the detection of specific sequences of double helical DNA without the need for denaturation (Figure 1).

Three eight-ring hairpin pyrrole-imidazole polyamides that target their respective sequences 5′-WGGGWW-3′, 5′-WGGCCW-3′, and 5′-WGWWCW-3′ were chosen for this case study.[6a] The parent hairpins were previously well characterized by quantitative footprinting titration with regard to affinity and sequence specificity (i.e. sensitivity to single base pair mismatches).[6b−d] From prior studies, we anticipate that polyamides **2** and **3** with the larger number of Im/Py pairs (which distinguish G-C from C-G, A-T, and T-A) versus Py/Py pairs (which do not distinguish A-T from T-A) would be the more specific in the series. Our usual experience is that attachment of dyes to polyamides[7] decreases the overall affinity by a factor of 10. It was of interest to examine whether the thiazole orange dye, a putative intercalator, will restore that energetic penalty.

We report here the synthesis and fluorescence properties of three hairpin polyamide−TO conjugates **2**−**4** and their abilities to detect specific sequences of double helical DNA in homogeneous solution (Figure 2). The polyamide provides a sequence-specific scaffold to deliver a TO intercalator to a predetermined DNA site in the minor groove. The TO fluorophore was chosen for its fluorescence enhancement properties upon intercalation.[9] In addition, the dye's structure can be modified to tune its

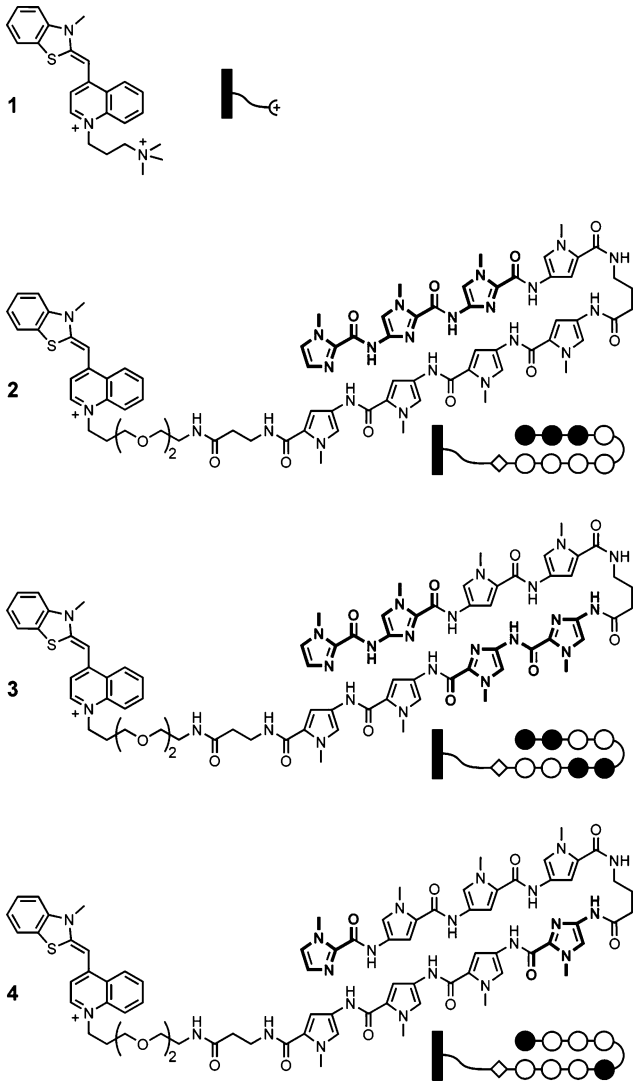

Figure 2. Structures of TO−PRO-1 **1** and hairpin polyamide−TO conjugates **2**−**4**.

absorption and emission profiles.[10] On the basis of previous polyamide-intercalator designs,[8] the polyamide C-terminus was linked using poly(ethylene glycol) (PEG). Solution-structure data of a thiazole orange dimer (TOTO)[11] directed the linker attachment to the TO at the minor-groove-residing quinoline nitrogen. We find that the conjugates **2**−**4** bind specifically to target match site double helical DNA without any compromise with regard to affinity and specificity. In addition, these hybrid DNA binding molecules unwind closed circular DNA (ccDNA), supporting the intercalation model of thiazole orange within this construct. As anticipated, the probes are relatively nonfluorescent in the absence of DNA and display a substantial increase in

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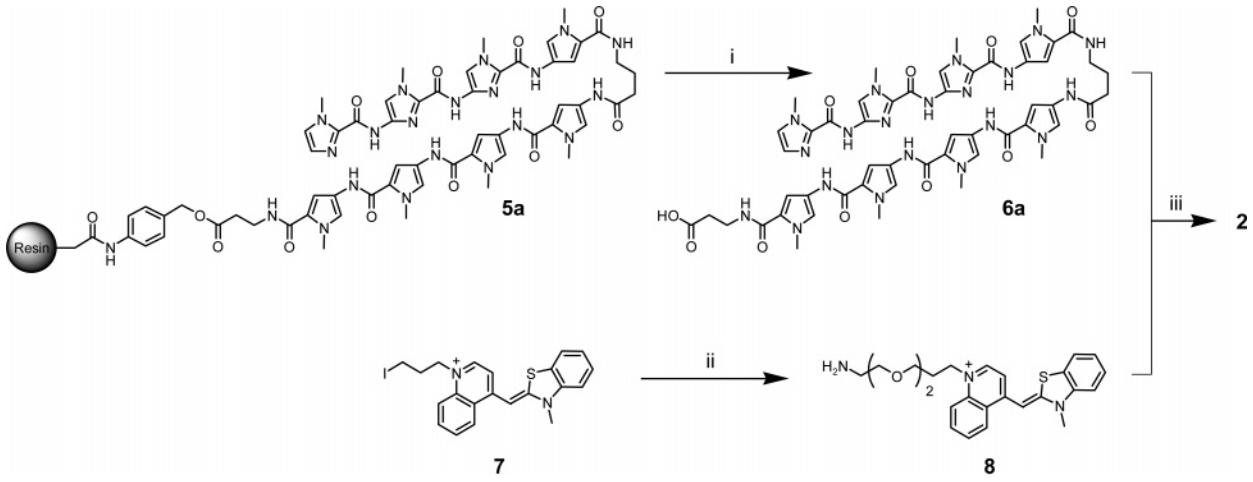

Figure 3. Synthesis of polyamide−TO conjugates. (i) 1 M LiOH, MeOH, THF, 37 °C, 2 h. (ii) 2-(2-Boc-aminoethoxy)ethanol, DTBMP, AgO_3SCF_3, CCl_4, 12 h. (iii) DPPA, TEA, DMSO, 2 h.

fluorescence (>1000-fold) upon addition of duplex DNA containing a match binding site.

Results and Discussion

Each polyamide−TO conjugate **2**−**4** was synthesized by linking a sequence-specific DNA binding domain of a hairpin polyamide to a nonspecific thiazole orange dye that fluoresces upon intercalation. DNA binding affinities and sequence specificities were characterized by quantitative DNase footprinting.[12] Evidence for DNA unwinding caused by sequence-specific intercalation was examined using the Crothers−Zeeman helical unwinding assay.[13] The three conjugates were then investigated for fluorescence properties in the presence of match and mismatch hairpin oligomers to determine specific enhancement effects.

Synthesis of Polyamide−Thiazole Orange Conjugates. Resin-bound eight-ring hairpin polyamides **5a**−**5c** were synthesized in a stepwise manner on Boc-*β*-alanine phenylacetamidomethyl (PAM) resin according to established Boc-chemistry protocols (Figure 3).[14] The corresponding carboxylate-containing polyamides **6a**−**6c** were cleaved from PAM resin through overnight treatment with LiOH in methanol. The iodopropyl-linked thiazole orange **7** was lengthened using 2-(2-Boc-aminoethoxy)ethanol to generate **8**. Following purification, the polyamide carboxylate was coupled to the free amine of TO−PEG **8** with diphenylphosphoryl azide (DPPA) and TEA in DMSO for 2 h. Crude conjugates **2**−**4** were each purified by preparatory reverse phase HPLC and stored as dry aliquots at −80 °C.

Binding Energetics and Sequence Specificity. DNA-binding properties of **2**−**4** were investigated by quantitative DNase I footprinting titration assays (Figure 4).[12] The 5′-^{32}P-labeled PCR-amplified fragment of pEF15 (Figure 4a) contains one match site for each of the three polyamide−TO conjugates. The equilibrium association constants (K_a) at their target match sites are 3×10^8 M^{-1}, 4×10^9 M^{-1}, and 1×10^{10} M^{-1}, for **2**, **3**, and **4**, respectively. By comparison, the parent polyamides of **2**−**4** (those containing *N*,*N*-dimethylaminopropylamine in lieu of **8** at the C-terminus) bound DNA with approximately the same affinities (4×10^8 M^{-1}, 4×10^9 M^{-1}, and 2×10^{10} M^{-1}, respectively).[15] Because polyamides conjugated to nonintercalating moieties (such as fluorescent dyes and peptides) generally display decreased binding affinities relative to their parent polyamides,[16] it appears that the conjugated TO moiety *contributes* to the DNA binding affinity. Additionally, tethering TO to these polyamides does not diminish their sequence specificities, as conjugates **2**−**4** display strong binding to their targeted match sites and only weak binding to mismatch sites at the highest concentrations measured (Figure 4b). Given the similar binding properties of conjugates **2**−**4** to the parent hairpin polyamides, we would anticipate that conjugates **2**−**4** have comparable specificities for single base pair mismatch sites. The apparent increased binding site size is likely a combined result of DNase I enzyme cleaving characteristics and additional protection from the thiazole orange.

Helical Unwinding Angle Determination. The DNA-unwinding properties of the commercially available TO−PRO-1 **1** and polyamide−TO conjugate **3** were determined from a helical unwinding assay, developed by Crothers and Zeeman, capable of providing an unwinding angle (ϕ) from sequence-specific interactions.[13] A series of relaxation reactions were carried out using topoisomerase I (Topo I) on closed-circular pUC19 DNA preequilibrated with varying concentrations of polyamides. Plasmids were extracted from the polyamide conjugates with phenol-chloroform and separated by two-dimensional (2D) agarose gel electrophoresis to differentiate the resulting distribution of topoisomers. Increased DNA unwinding shifts the final topoisomer distribution toward a more negatively supercoiled population. Each reaction containing dye **1** or conjugate **3** resulted in a negative distribution of topoisomers, whereas control experiments lacking added intercalators resulted in a primarily positive distribution of topoisomers (Figure 5a). Mathematical analysis of the topoisomer distributions showed decreasing apparent unwinding angles (ϕ_{ap}) for simultaneously decreasing conjugate and plasmid concentrations (Figure 5b). The actual average unwinding angles (ϕ) for binding

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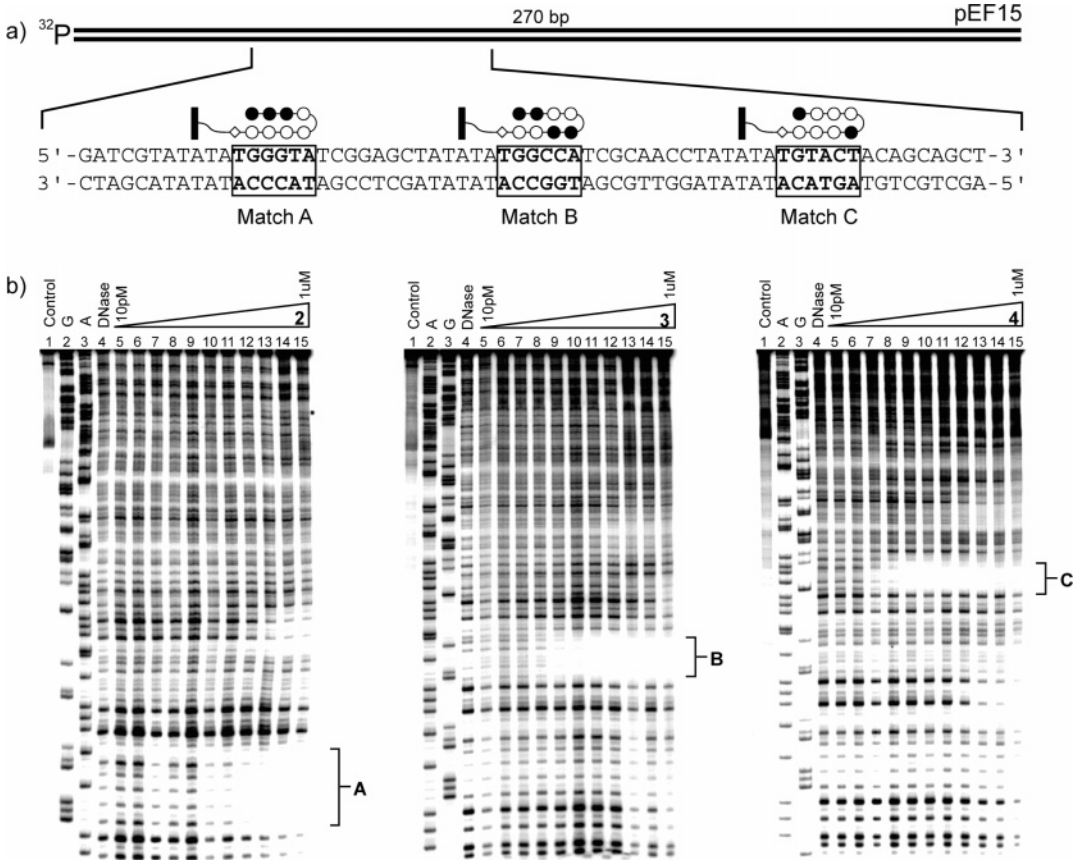

Figure 4. (a) Sequence of the synthesized insert from the pEF15 plasmid containing the boxed 7-bp target sites match A, match B, and match C for conjugates **2**, **3**, and **4**, respectively. (b) Quantitative DNase I footprint titration experiments[21] with conjugates **2** (left), **3** (middle), and **4** (right) on the PCR-amplified 5′-^{32}P-labeled fragment from pEF15. Lane 1, intact DNA; lanes 2 and 3, sequencing reactions; lane 4, DNase I standard; lanes 5−15, DNase I digestion products in the presence of 10, 30, 100, and 300 pM, 1, 3, 10, 30, 100, and 300 nM, and 1 μM polyamide, respectively.

to pUC19 were determined from the ordinate intercepts. TO−PRO-1 **1** unwinds DNA ∼17°, whereas polyamide−TO conjugate **3** unwinds pUC19 an average of ∼8° per binding event. The decreased propensity of unwinding by **3** is not surprising, as slight linker modifications have been shown to significantly alter the binding properties of TO derivatives.[17] It is also noteworthy that the negative inverses of the slopes from this assay are proportional to the average binding affinities for each dye to pUC19. In accord with the higher affinity of conjugate **3** found by DNase I footprinting, the slope shown for **3** is less negative than the slope of **1**. The evidence of intercalation and the high binding affinity of **3** suggest that polyamide−TO conjugates are likely good candidates for fluorescence enhancement upon DNA binding.

Detection of Specific Double-Stranded DNA Sequences. The fluorescence properties of polyamide−TO conjugates **2**−**4** were examined in the presence and absence of three different hairpin-forming DNA oligomers (Figures6 and 7). Each synthetic 37-mer was designed with a match site for one of three polyamide−TO conjugates (**2**, **3**, and **4** are programmed to bind oligo A, oligo B, and oligo C, respectively; Figure 7a). Figure 6a shows the absorption spectra of TO−PRO-1 (**1**), unconjugated polyamide (**5b**), and polyamide conjugated to thiazole orange (**3**) free in water at room temperature. The polyamide−TO absorption pattern overlays both the unconjugated polyamide absorbance (∼310 nm) and the fluorophore alone (∼510 nm). The fluorescence of 100 nM solutions of polyamide−TO conjugates in aqueous solution (1× TKMC) without DNA is almost negligible, indicative of free rotation between the benzothiazole and quinoline rings (causing nonradiative energy loss),[11] as well as minimal back-bonding of the fluorophore to the polyamide core.[18] Addition of match DNA to the conjugates increases their fluorescence intensities (Figure 6b). Fluorescence enhancements of all three conjugates in the presence of their match oligomers are ∼1000-fold at 1:1 DNA−polyamide stoichiometry.

The ability of each conjugate **2**−**4** to distinguish its match site from nonmatch DNA was examined by direct laser excitation scanning through the bottom of polystyrene plates. The emission data for polyamide−TO conjugates in the presence of each hairpin oligomer at 1:1 DNA:polyamide stoichiometry and 100 nM are presented in Figure 7. The increased fluorescence enhancement of each conjugate when bound to its corresponding match DNA oligomer relative to mismatch and control mixtures can be seen visually in Figure 7b. The normalized fluorescence data collected by direct excitation at 532 nm for conjugates **2**−**4** bound to each oligomer are shown in Figure 7c. Conjugates **2** and **3**, containing three and four Im/Py pairs, respectively, showed the highest selectivity for fluorescence emission with little enhancement in the presence of mismatch oligos. Conjugate **4**, containing only two Im/Py

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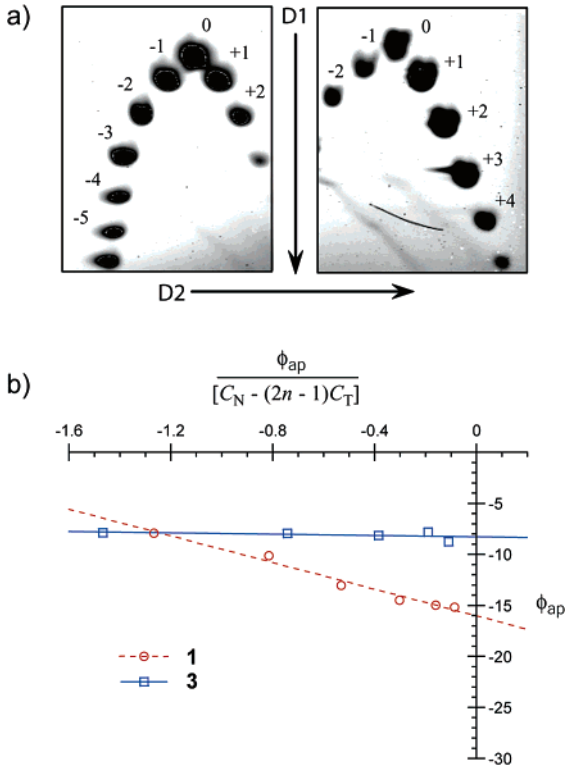

Figure 5. (a) Images of topoisomer bands from reactions containing and lacking polyamide−TO conjugate **3** (left and right, respectively). The directions of electrophoresis and ΔLk values are shown. (b) Unwinding plots for TO−Pro-1 **1** (○) and polyamide−TO conjugate **3** (□) on pUC19. Each data point was calculated from one set of topoisomer distributions from reactions containing polyamide compared to a control distribution lacking polyamide. Interception of the ordinate yields the unwinding angle (ϕ) per polyamide−TO conjugate.

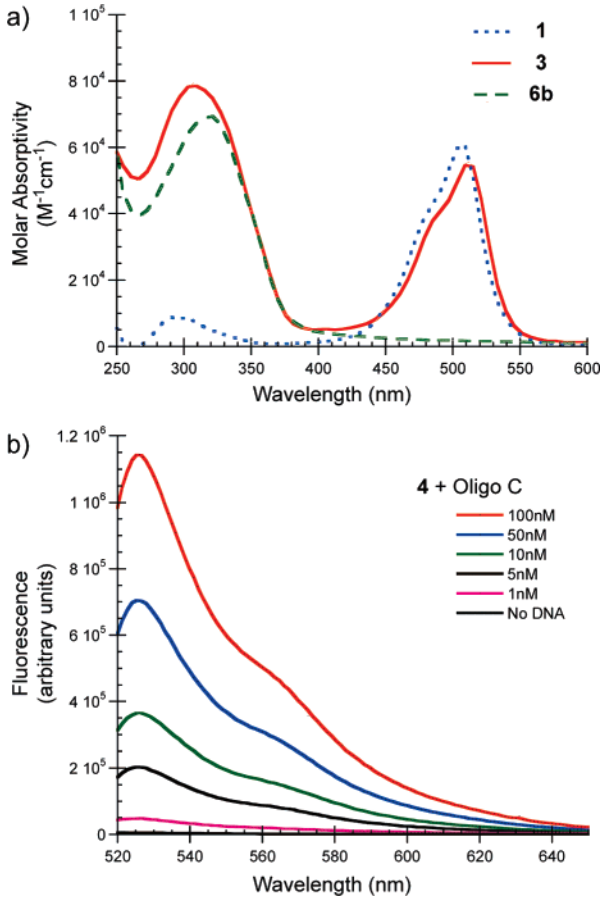

Figure 6. (a) Absorption spectra of free polyamide−TO conjugate **3** (—), free unconjugated polyamide **6b** (− −), and free TO−Pro-1 **1** (····). (b) Fluorescence emission profile for polyamide−TO conjugate **4** at 100 nM concentration in the presence of increasing concentrations of oligo C (1−100 nM).

pairs, showed slightly decreased specificity by the fluorescence assay. Polyamides typically have higher specificity with increased imidazole content,[15] which may account for the different specificities of **2**−**4** in the presence of the nanomolar concentrations of DNA required for these experiments. Nonetheless, with each oligomer, the fluorescence enhancement is relatively small for conjugates not programmed to bind the sequence and significantly increased for the conjugate containing the match pyrrole-imidazole polyamide.

These data support a model where the polyamide moiety directs the nonfluorescent conjugate to a specific site of the DNA duplex and delivers the tethered thiazole orange fluorophore to an adjacent site. Upon polyamide binding, the thiazole orange likely intercalates the DNA and the conformation of the normally free rotating fluorophore is restricted, resulting in substantial fluorescence emission following excitation.

Polyamide−TO conjugates represent a new class of DNA-binding molecules that detect specific sequences of double-stranded DNA. The conjugates bind specifically and with high affinity to target match site double-helical DNA and unwind DNA, presumably by intercalation. The generally nonfluorescent conjugates also show significant fluorescent enhancement only upon excitation in the presence of DNA containing the match site tested. These conjugates show promise as probes for biologically important sequences, such as triplet repeats and transposable elements, and as chromosome paints for telomeric and centromeric repeats.

Experimental Section

Materials. TO−PRO-1 (**1**) was purchased from Molecular Probes. Lepidine (4-methylquinoline) and 3-methyl-2-(methylthio)benzothizolium *p*-toluenesulfonate were purchased from Aldrich. Restriction endonucleases were purchased from New England Biolabs and used as noted in the manufacturer's protocol. [γ-^{32}P]-Adenosine-5′-triphosphate (≥6000 Ci/mmol) was obtained from ICN. Purified pUC19 DNA for unwinding angle determination was isolated from transformed JM109 *Escherichia coli* using the Qiagen protocol. EDTA, dithiothreitol (DTT), ultrapure agarose, and calf thymus Topo I were purchased from Gibco/BRL. Micron 50 microconcentrators were purchased from Amicon. Microcolumns were purchased from Amersham Pharmacia Biotech, Inc. Water (18 MΩ) was obtained from a Millipore MilliQ water purification system, and all buffers were 0.2 μm filtered. Polystyrene plates (#62409−068) were from VWR Scientific. Reagent-grade chemicals were used as received, unless otherwise stated.

UV spectra were measured in water on a Beckman model DU 7400 diode array spectrophotometer. Fluorescence spectrophotometer measurements were obtained at room temperature on an ISS K2 spectrophotometer employing 5 nm emission and excitation slits in conjunction with a Hg lamp. TO fluorescence was measured in polystyrene plates on a Molecular Dynamics Typhoon, employing a 532-nm excitation laser and a 526-nm short-pass emission filter. HPLC analysis was performed on a Beckman Gold system using a RAININ C_{18}, Microsorb MV, 5 μm, 300 × 4.6 mm reversed-phased column in 0.1% (v/v) TFA with acetonitrile as eluent and a flow rate of 1.0 mL/min. Preparatory reversed-phase HPLC was performed on a Beckman HPLC using a Waters DeltaPak 25 × 100 mm, 100 μm C18 column equipped with a

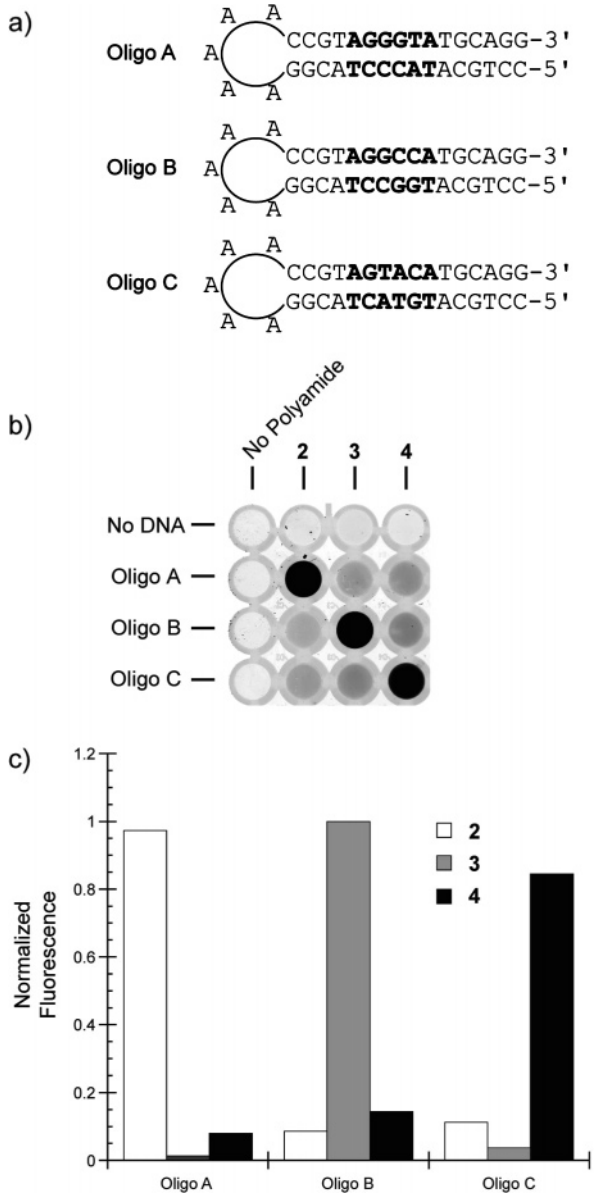

Figure 7. (a) Design of hairpin-forming duplex oligonucleotides oligo A, oligo B, and oligo C containing match sites (shown in bold) for polymides **2**, **3**, and **4**, respectively. (b) Plate experiment conducted at 100 nM polyamide−TO in the presence of 100 nM of three different oligonucleotides. Each conjugate preferentially binds and shows fluorescence enhancement at the designed DNA match site. The fluorescence intensity correlates with the darkness of the well. (c) Normalized data for the plate experiment using the buffer well as the background fluorescence. The fluorescence intensities of the polyamides without DNA are >1000-fold lower than the maximum intensities and are not shown on the bar graph.

guard, 0.1% (wt/v) TFA, 0.25% acetonitrile/min. Matrix-assisted laser desorption/ionization time-of-flight mass spectrophotometry (MALDI-TOF) was performed using an Applied Biosystems Voyager DE-Pro.

ImImImPy-γ-PyPyPyPy-β-PEG$_2$−TO (2). Hairpin polyamide **6a** (1 μmol aliquot) was dissolved in 500 μL of DMSO and treated with **8** (3.5 μmol), diphenylphosphoryl azide (DPPA, 5 μL), and TEA (20 μL), and the mixture stirred at room temperature for 2 h. The mixture was diluted with 0.1% (w/v) TFA (8 mL) and DMF (2 mL), and the resulting solution was purified by reversed-phase HPLC. Lyophilization provided ImImImPy-γ-PyPyPyPy-β-PEG2-TO (**2**) as a red powder (0.65 mg, 42% recovery). MALDI-TOF-MS (monoisotopic): calcd for $C_{77}H_{86}N_{23}O_{12}S$ (M + H) 1557.7, found 1557.7.

ImImPyPy-γ-ImImPyPy-β-PEG$_2$−TO (3) was synthesized as described for **2** starting from 1 μmol of **6b** (0.75 mg, 48% recovery). MALDI-TOF-MS (monoisotopic): calcd for $C_{76}H_{85}N_{24}O_{12}S$ (M + H) 1558.7, found 1558.9.

ImPyPyPy-γ-ImPyPyPy-β-PEG$_2$-TO (4) was synthesized as described for **2** starting from 1 μmol of **6c** (0.69 mg, 44% recovery). MALDI-TOF-MS (monoisotopic): calcd for $C_{78}H_{87}N_{22}O_{12}S$ (M + H) 1556.7, found 1557.0.

ImImImPy-γ-PyPyPyPy-β-OH (6a). A sample of ImImImPy-γ-PyPyPyPy-β-resin (200 mg, 0.38 mmol/g) **5a** was suspended in 6 mL of THF, 1.5 mL of methanol, and 1.5 mL of LiOH (1M) and heated at 37 °C for 12 h. The reaction mixture was filtered to remove resin, concentrated in vacuo, and diluted with 0.1% (w/v) TFA (8 mL) and DMF (2 mL). The resulting solution was purified by reversed-phase HPLC. Lyophilization provided ImImImPy-γ-PyPyPyPy-β-OH (**6a**) as a white powder (5.6 mg, 7% recovery). MALDI-TOF-MS (monoisotopic): calcd for $C_{52}H_{58}N_{20}O_{11}$ (M + H) 1139.5, found 1139.4.

ImImPyPy-γ-ImImPyPy-β-OH (6b) was synthesized as described for **6a** using ImImPyPy-γ-ImImPyPy-β-resin **5b** (6.8 mg, 8% recovery). MALDI-TOF-MS (monoisotopic): calcd for $C_{51}H_{57}N_{21}O_{11}$ (M + H) 1140.5, found 1140.6.

ImPyPyPy-γ-ImPyPyPy-β-OH (6c) was synthesized as described for **6a** using ImPyPyPy-γ-ImPyPyPy-β-resin **5c** (9.7 mg, 12% recovery). MALDI-TOF-MS (monoisotopic): calcd for $C_{53}H_{59}N_{19}O_{11}$ (M + Na) 1160.5, found 1160.8.

1-{3-[2-Aminoethoxy)ethoxy]propyl}-4-(3-methyl-3*H*-benzothiazol-2-ylidenemethyl)quinolinium (8). 1′-(3′-Iodopropyl)-3-methyl-oxa-4′-cyanine iodide **7**[9d,19] (200 mg, 0.340 mmol) and 2-(2-Boc-aminoethoxy)ethanol[20] (697 mg, 3.4 mmol) were dissolved in 50 mL of chloroform. To this were added 2,6-di-*tert*-butyl-4-methylpyridine (DTBMP, 140 mg, 0.68 mmol) and AgO_3SCF_3 (175 mg, 0.68 mmol), and the reaction was stirred overnight at room temperature under Ar. The solvent was filtered and removed in vacuo, and the remaining residue was dissolved in 200 mL of 15% TFA/DCM and allowed to stir for 4 h. The mixture was concentrated in vacuo and 50 mg of crude residue was dissolved in 0.1% (wt/v) TFA (8 mL) and DMF (2 mL) and purified by reversed-phase HPLC. Lyophilization provided **8** as a red powder (3.7 mg, 10% overall recovery). ^{1}H NMR (DMSO-d_6): δ8.80 (d, 1H), 8.56 (d, 1H), 8.05 (m, 2H), 7.78 (m, 2H), 7.62 (m, 1H), 7.40 (m, 2H), 6.93 (s, 1H), 6.51 (s, 1H), 4.63 (t, 2H), 4.02 (s, 3H), 3.50 (m, 6H), 3.26 (m, 4H), 2.95 (m, 2H), 2.08 (m, 2H). MALDI-TOF-MS (monoisotopic): calcd for $C_{25}H_{30}N_3O_2S$ 436.2, found 436.0.

Construction of Plasmid DNA. The plasmid pEF15 was constructed by insertion of the following hybridized inserts into the *Bam* HI/*Hind*III polycloning sites in pUC19: 5′-GATCG TATAT ATGGG TATCG GAGCT ATATA TGGCC ATCGC AACCT ATATA TGTAC TACAG C-3′ and 5′-AGCTG CTGTA GTACA TATAT AGGTT GCGAT GGCCA TATAT AGCTC CGATA CCCAT ATATA C-3′. The insert was obtained by annealing complementary *Hind*III restriction fragments of pUC19 using T4 DNA ligase. The ligated plasmid was then used to transform JM109 subcompetent cells (Promega). Colonies were selected for α-complementation on 25-mL Luria-Bertani agar plates containing 50 mg/mL ampicillin. Cells were harvested after overnight growth at 37 °C. Large-scale plasmid purification was performed using WizardPlus Midi Preps from Promega. The presence of the desired insert was determined by dideoxy sequencing.

Preparation of 5′-End-Labeled Fragments. Two 21-base-pair primer oligonucleotides, 5′-GAATT CGAGC TCGGT ACCCG G-3′ (forward) and 5′-TGGCA CGACA GGTTT CCCGA C-3′ (reverse), were constructed for PCR amplification. The forward primer was radiolabeled using [γ-^{32}P]-dATP and polynucleotide kinase, followed by purification using MicroSpin G-50 columns. The desired DNA segment was amplified as previously described.[12] The labeled fragment

(19) Brooker, L. G. S.; Keyes, G. H.; Williams, W. W. *J. Am. Chem. Soc.* **1942**, *64*, 199−210.
(20) Kim, Y.-S.; Kim, K. M.; Song, R.; Jun, M. J.; Sohn, Y. S. *J. Inorg. Biochem.* **2001**, *87*, 157−163.

was loaded onto a 7% nondenaturing preparatory polyacrylamide gel (5% cross-link) and the desired 270-base-pair band was visualized by autoradiography and isolated. Chemical sequencing reactions were performed according to published protocols.[21]

Quantitative DNase I Footprint Titrations. All reactions were carried out in a volume of 400 μL according to published protocols.[12] Quantitation by storage phosphor autoradiography and determination of equilibrium association constants were as previously described.[12]

Unwinding Angle Determination. Relaxation reactions and numeric analyses were carried out as described.[8a,13]

Optical Characterization. All measurements were performed in TKMC buffer [10 mM Tris-HCl (pH 7.0), 10 mM KCl, 10 mM $MgCl_2$, and 5 mM $CaCl_2$]. The concentration of polyamide−TO conjugate was 100 nM and the volume of solution used was 500 μL. In a fluorimeter, polyamides **2**−**4** were excited at 490 nm and measured over the interval from 520 to 650 nm. A 100 nM solution of each conjugate was irradiated in the presence of an increasing concentration of the appropriate match DNA (up to 100 nM) to generate the fluorescence enhancements reported. Absorption spectra were recorded under the same conditions as emission spectra at higher concentrations.

(21) (a) Maxam, A. M.; Gilbert, W. S. *Methods Enzymol.* **1980**, *65*, 499−560. (b) Iverson, B. L.; Dervan, P. B. *Methods Enzymol.* **1996**, *15*, 7823−7830.

Plate Characterization. In the dark, 100 μL solutions were made by titrating together a fixed concentration of 100 nM conjugate against 100 nM of each DNA oligo A−C. The solutions were gently swirled and then allowed to sit for 6 h before measurements were made. No changes in fluorescence intensity were observed for longer equilibration times. Plates containing **2**−**4** were excited at 532 nm and data were collected with a 526-short pass filter. ImageQuant software (Molecular Dynamics) was used to analyze the fluorescence intensity of each experiment. The normalized data were calculated by using the relation $(F_{obs} - F_{min})/(F_{max} - F_{min})$, where F_{obs} is the sample fluorescence and F_{max} and F_{min} are the maximum and minimum fluorescence intensity, respectively.

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Supporting Information Available: Equlibrium binding isotherms for **2**−**4** and unwinding experimental data for compounds **1** and **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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