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Sequence Specific Fluorescence Detection of Double Strand DNA

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Abstract: Methods for the fluorescent detection of specific sequences of double strand DNA in homogeneous solution may be useful in the field of human genetics. A series of hairpin polyamides with tetramethyl rhodamine (TMR) attached to an internal pyrrole ring were synthesized, and the fluorescence properties of the polyamide−fluorophore conjugates in the presence and absence of duplex DNA were examined. We observe weak TMR fluorescence in the absence of DNA. Addition of ≥1:1 match DNA affords a significant fluorescence increase over equimolar mismatch DNA for each polyamide−TMR conjugate. Polyamide−fluorophore conjugates offer a new class of sensors for the detection of specific DNA sequences without the need for denaturation. The polyamide-dye fluorescence-based method can be used to screen in parallel the interactions between aromatic ring pairs and the minor groove of DNA even when the binding site contains a non-Watson−Crick DNA base pair. A ranking of the specificity of three polyamide ring pairs—Py/Py, Im/Py, and Im/Im—was established for all 16 possible base pairs of A, T, G, and C in the minor groove. We find that Im/Im is an energetically favorable ring pair for minor groove recognition of the T·G base pair.

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 (SNPs) 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 leading to signal generation.[2−7] One example is the "molecular beacon" which consists of a hairpin DNA labeled in the stem with a fluorophore and a quencher.[4] Binding to a complementary strand results in opening of the hairpin and separation of the quencher and the fluorophore. However, detection by hybridization requires DNA *denaturation* conditions, and it remains a challenge to develop sequence specific fluorescent probes for DNA in the double strand form.[8−10]

Hairpin polyamides are a class of synthetic ligands that can be programmed to recognize specific DNA sequences with affinities and specificities comparable to DNA binding proteins.[11] The large number of DNA sequences which can be targeted with hairpin polyamides suggests that polyamide−fluorophore conjugates might be useful in fluorescence-based detection of specific nucleic acid sequences. Within the context of targeting sites in gigabase size DNA, Laemmli demonstrated that polyamides with Texas Red or fluorescein at the C-terminus specifically stain 5′-GAGAA-3′ repeats in *Drosophila* satellites, as well as teleomeric repeats in insect and human chromosomes

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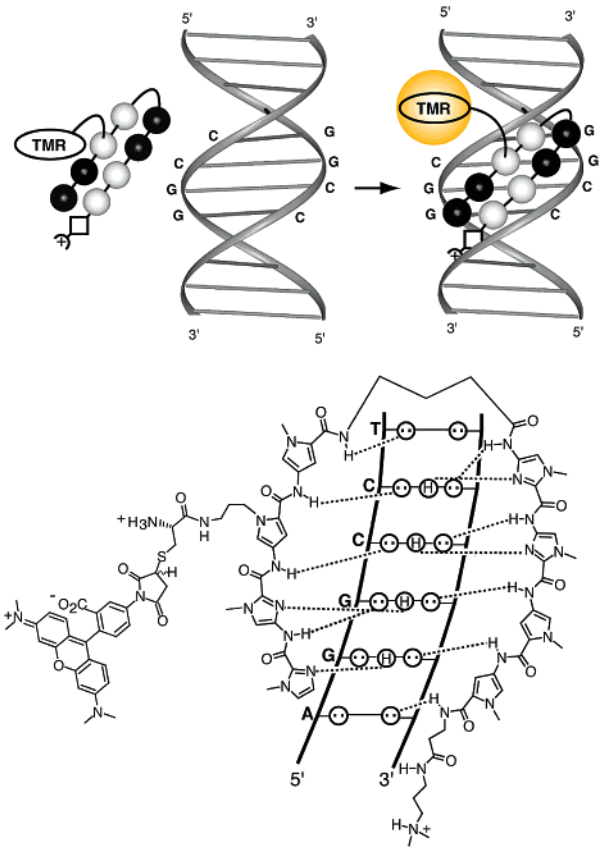

Figure 1. (Top) Hairpin polyamide−fluorophore conjugate is weakly fluorescent in the absence of DNA, but affords fluorescence enhancement in the presence of match duplex DNA. (Bottom) Model of polyamide−TMR conjugate **1** bound to match site.

(5′-TTAGG-3′ and 5′-TTAGGG-3′, respectively).[12] Similarly, Trask has demonstrated the use of polyamide−dye conjugates to fluorescently label specific repetitive regions on human chromosomes 9, Y and 1 (TTCCA repeats) for discrimination in cytogenetic preparations and flow cytometry.[13]

We report here that hairpin polyamide−fluorophore conjugates are capable of detecting specific sequences within short segments of double helical DNA in homogeneous solution (Figure 1). The fluorescence of our polyamide-tetramethyl rhodamine (TMR) conjugates is largely quenched in the absence of DNA. Addition of duplex DNA containing a match site to a polyamide-TMR conjugate affords a ≥10-fold increase in fluorescence. Attenuated signal results when mismatch DNA is added at the same concentration. This fluorescence detection method allows us to analyze the specificity of hairpin polyamides that target non-Watson−Crick base pairs.

Results and Discussion

Detection of Specific DNA Sequences in Homogeneous Solution. As a model study, six hairpin pyrrole-imidazole (Py-Im) polyamides with TMR[14] attached at an internal pyrrole ring were synthesized (Figure 2). Each eight-ring polyamide **1**−**6** was programmed according to the pairing rules[11] for a different 6-base pair DNA match sequence (Figure 3). Tetramethyl rhodamine-5-maleimide was coupled as a thioether to an internal pyrrole ring by a cysteine linker. This point of attachment was chosen to direct the fluorophore into aqueous solution (away from the DNA helix) to minimize possible unfavorable steric interactions when the hairpin is bound in the minor groove (Figure 1). The synthetic details for conjugates **1**−**6** (as well as an unusual conjugate **7** containing an Im/Im pair for a subsequent mismatch study) are described in the Supplemental Section. The purity and identity of conjugates **1**−**7** were verified by reversed phase analytical HPLC and MALDI/TOF mass spectrometry (Figure 2).

The parent hairpins typically bind with equilibrium association constants $K_a \geq 10^9$ M^{-1}.[11] To determine the energetic consequences for covalent attachment of fluorophores to the N1 position of at internal pyrrole ring in the 8-ring hairpin, the equilibrum association constants for conjugates **1**−**6** at six sites were determined by quantitative footprinting titration. The affinities of conjugates **1**−**6** at DNA match sites are reduced by a factor of 10−50 from the parents (Table 1A).[11] Despite the energetic penalty, the expected sequence specificities are maintained K_a (match) > K_a (single mismatch) > K_a (multiple mismatch) (Table 1B).

The fluorescence properties of the polyamide-TMR conjugates **1**−**6** in the presence and absence of short segments of duplex DNA (17-mer) were examined (Figure 4). The absorption maxima of conjugates **1**−**6** (∼560 nm) are red-shifted and are attenuated relative to the absorption maximum (550 nm) of the TMR fluorophore **8** alone (Figure 4a), presumably due to electronic interactions between the fluorophore and the polyamide in the ground state.[16] In the absence of DNA, the fluorescence intensities of the polyamide-TMR conjugates are significantly diminished relative to the TMR control **8** (pH 7.0, TKMC buffer). The addition of increasing amounts of match DNA to the conjugate affords an increase of fluorescence until a 1:1 DNA:conjugate stoichiometry is reached (Figure 4bc). Using polyamide **3** as an example, fluorescence data from the addition of an increasing amount of a 17 base pair (17-mer) DNA duplex (0.05−1.0 μM concentration) containing the match sequence 5′-tAGTACTt-3′ to an aqueous solution of 1 μM concentration of match conjugate **3** results in an increase in fluorescence intensity characteristic of all conjugates **1**−**6**. Fluorescent enhancement is greatest when the DNA duplex contains the polyamide's match sequence (Table 2). Addition of 17-bp DNA duplex containing a match sequence beyond the 1:1 DNA:polyamide stoichiometry does not lead to further significant increase of emission intensity which indicates the occurrence of a single binding event between the conjugate and each DNA duplex (Figure 4c).

For the most effective discrimination, the stabilities of the different polyamide-DNA complexes should be match > single base mismatch > multiple base mismatch as determined from our footprinting study (Table 1). The ability of each conjugate **1**−**6** to distinguish one match from an ensemble of six different DNA sequences by fluorescence was examined by direct laser excitation scanning through the bottom of polystyrene plates.

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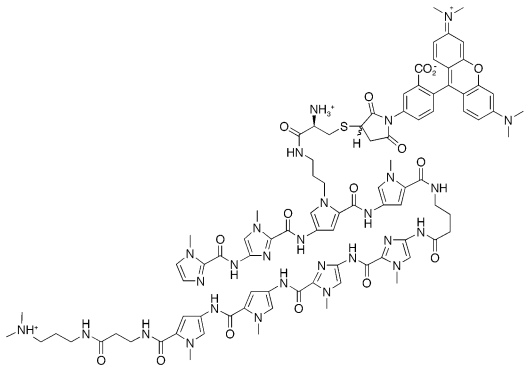

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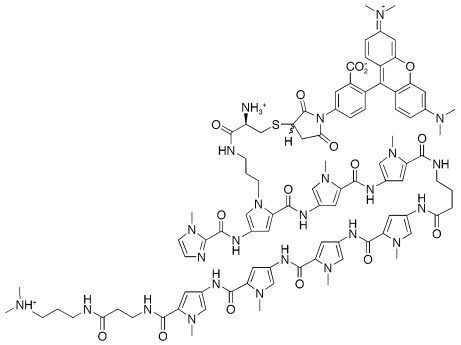

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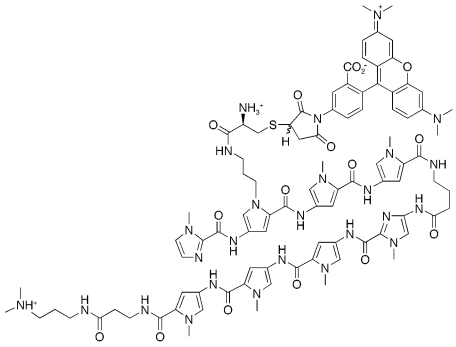

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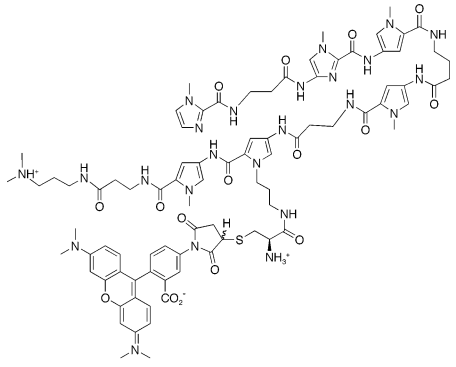

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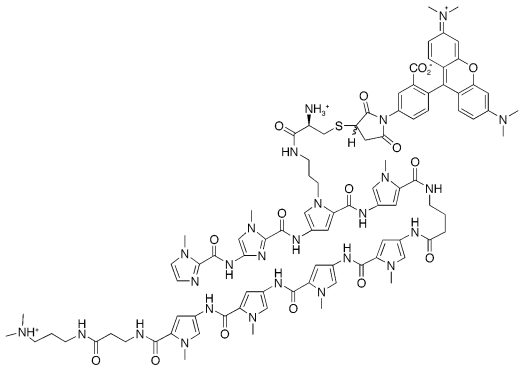

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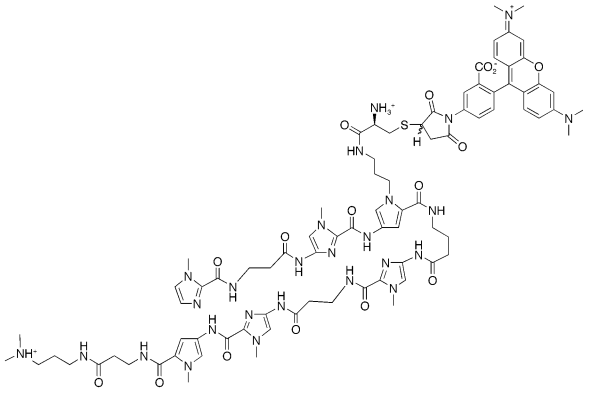

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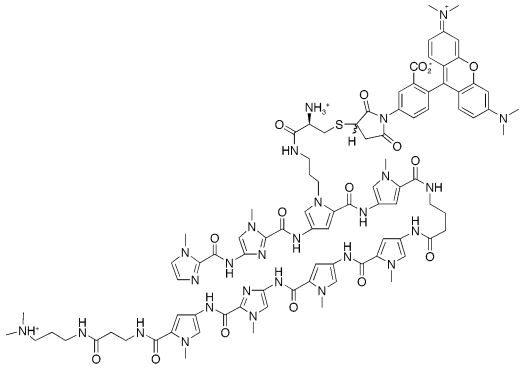

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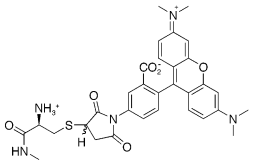

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Figure 2. Structures of conjugates **1**−**7** and control TMR fluorophore **8**.

Table 1. (A) Equilibrium Association Constants (M^{-1}) for Conjugates **1**−**6** at Sequences Shown on Left (B) Normalized Values of Relative Affinities of Match vs Mismatch Sites from Table 1A[a]

A	1	2	3	4	5	6
taGGCCtt	$\mathbf{2.1 \times 10^9}$	$<10^7$	$<10^7$	2.0×10^7	$\leq 2.8 \times 10^7$	$<10^7$
taGTATtt	$<10^7$	$\mathbf{2.5 \times 10^8}$	5.2×10^7	$\leq 5.2 \times 10^8$	$<10^7$	$\leq 5.4 \times 10^7$
taGTACtt	$<10^7$	5.0×10^7	$\mathbf{1.7 \times 10^9}$	$\leq 4.2 \times 10^8$	3.2×10^8	$\leq 1.4 \times 10^8$
taGTGTtt	$<10^7$	1.0×10^8	$<10^7$	$\mathbf{1.4 \times 10^9}$	$\leq 4.5 \times 10^7$	$\leq 2.0 \times 10^8$
taGGTAtt	$<10^7$	1.0×10^8	$<10^7$	1.10×10^8	$\leq \mathbf{9.7 \times 10^8}$	$<10^7$
taGCGCtt	$<10^7$	$<10^7$	$<10^7$	3.0×10^7	$\leq 8.7 \times 10^7$	$\leq \mathbf{4.9 \times 10^8}$

B	1	2	3	4	5	6
taGGCCtt	1	<0.05	<0.01	0.01	≤0.03	≤0.02
taGTATtt	<0.01	1	0.03	≤0.37	<0.01	≤0.11
taGTACtt	<0.01	0.20	1	≤0.30	0.33	≤0.29
taGTGTtt	<0.01	0.40	<0.01	1	≤0.05	≤0.41
taGGTAtt	<0.01	0.40	<0.01	0.08	1	<0.02
taGCGCtt	<0.01	<0.05	<0.01	0.02	≤0.09	1

[a] The assays were carried out at 22 °C at pH 7.0 in the presence of 10 mM Tris-HCl, 10 mM KCl, 10 mM $MgCl_2$, and 5 mM $CaCl_2$.

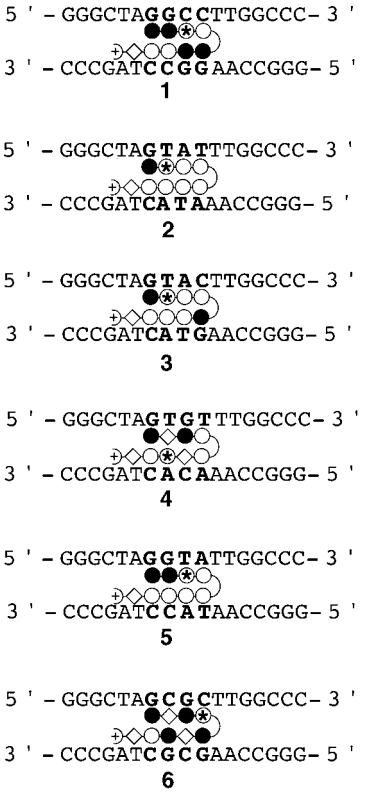

Figure 3. Ball and stick model of six different polyamide−TMR conjugates **1**−**6** which code for six different match sites within a 17-mer DNA duplex. Im and Py residues are shown as black and white circles, respectively; β-alanine is diamond. TMR is covalently attached at the pyrrole containing the asterisk.

The data were acquired on a Molecular Dynamics Typhoon optical scanner employing a 532 nm 10−20 mW excitation laser and a 580 nm emission filter (±15 nm fwhm) suitable for TMR fluorescence detection. The emission data for hairpin polyamide conjugates **1**−**6** interacting with six different double stranded 17-mers is presented in Figure 5a. Polyamide−TMR conjugates are mostly quenched, except in the presence of increasing concentrations of a match DNA sequence. The data were normalized and plotted against the concentration of DNA titrant (0.02 μM to 1 μM) added to a 1 μM solution of conjugate (Figure 5b). The 1:1 stoichiometry end point provides a measure of the relative sequence specificity of each polyamide−TMR conjugate **1**−**6**. We note the agreement between the normalized specificities determined by DNase I footprinting titration (Table 1B) and those we observe from this new optical assay (Table 2).

The observation that the fluorescence of the TMR fluorophore is significantly diminished when attached by a short tether to an internal pyrrole ring position in a series of hairpin polyamide−TMR conjugates was not expected. We have found that other fluorophores, such as fluorescein, when conjugated to an internal pyrrole of hairpin polyamides behave similarly. Presumably, the short linker holds the fluorophore in very close proximity or in direct contact with the hairpin polyamide. After excitation, the polyamide−fluorophore decays from the excited state via a nonradiative pathway. Incorporation of the aliphatic β-alanine (β) residues leads to a decrease in fluorescence quenching. The more flexible aliphatic β may allow increased conformational freedom to the conjugates **4** and **6**, possibly disrupting the association of dye and polyamide. The perturbed ground-state absorption spectrum of the fluorophore suggests a molecular association between polyamide and fluorophore that allows quenching to occur.[17]

The simplest model is that, upon binding DNA, the hairpin is sequestered in the minor groove moving the TMR fluorophore away from the polyamide and into the surrounding aqueous environment. One can imagine two conformations for the polyamide−dye conjugate: polyamide and TMR are in close proximity when free in solution (fluorescence quenched), and polyamide and TMR are separated in an extended conformation with the TMR projecting away from the polyamide when bound to DNA (fluorescence unquenched).

Both Laemmli and Trask have demonstrated the utility of polyamide-dye conjugates in the context of *gigabase size* DNA as sequence specific chromosome "paints".[12,13] This report extends the properties of polyamide-dye molecules for specific DNA sequence detection in solution *within short segments* of DNA. The short segments of DNA are not immobilized on a chip nor is there a need for end labeling. It may be that applications might be related to "SNP typing" in which the identity of the SNP containing sequence is known. In a formal

(17) Addition of a four-ring polyamide to TMR (intermolecular association) affords a 10 nm bathochromic absorption maximum shift of TMR, similar to that observed for conjugates **1**−**6** (vs control TMR **7**). Fluorescence emission data in the presence of linearly increasing polyamide concentration affords a linear Stern−Volmer plot.[16]

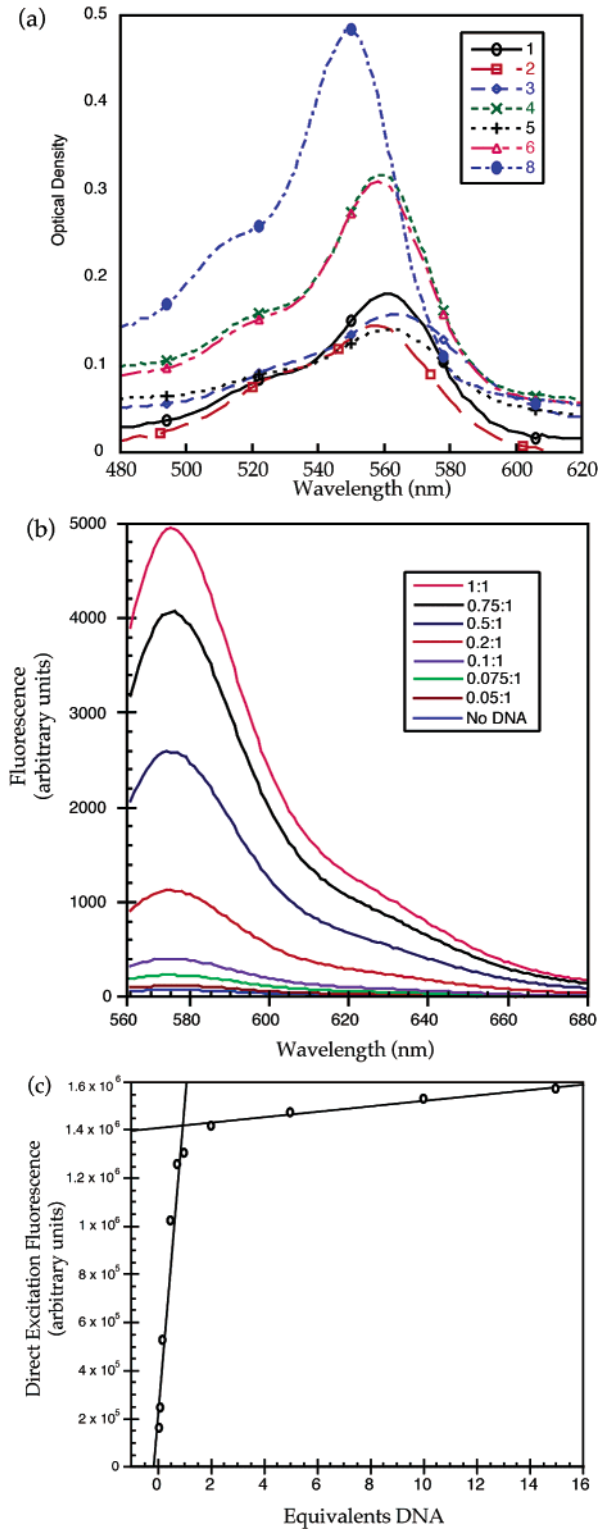

Figure 4. (A) Absorption spectra of **1**−**6**, **8**. All conjugates **1**−**6** exhibit a ~10 nm bathochromic shift and absorption attenuation relative to control compound **8**. (B) Fluorescence emission profiles for conjugate **3** at 1 μM concentration in the presence of increasing concentrations of DNA (0.05−1 μM) containing the polyamide's match site 5′-taGTACtt-3′ (10 mM Tris-HCl (pH 7.0), 10 mM KCl, 10 mM $MgCl_2$, and 5 mM $CaCl_2$). Solutions were allowed to equilibrate for 4 h at 22 °C. Excitation wavelength is 545 nm. (C) Direct excitation data of **3** with various equivalent of match DNA was collected on a Typhoon optical scanner as described in the Experimental Section. Addition of more than one equivalent of match dsDNA generates no appreciable increase in fluorescence for conjugates as shown for **3**. The two lines intersect at 0.96:1 polyamide:DNA yielding the stoichiometry of a single binding event.

Table 2. Normalized Fluorescence Intensity for Polyamide−TMR Conjugates **1**−**6** at 1 μM Concentration Interacting with Six Different DNA Duplexes at 1 μM Concentration

	1	2	3	4	5	6
taGGCCtt	1	0.04	0.03	0.04	0.05	0.08
taGTATtt	0.04	1	0.09	0.30	0.20	0.10
taGTACtt	0.02	0.22	1	0.12	0.01	0.27
taGTGTtt	0.04	0.30	0.01	1	0.08	0.18
taGGTAtt	0.05	0.13	0.03	0.09	1	0.07
taGCGCtt	0.07	0.02	0.05	0.07	0.04	1

[a] The assays were carried out at 22 °C at pH 7.0 in the presence of 10 mM Tris-HCl, 10 mM KCl, 10 mM $MgCl_2$, and 5 mM $CaCl_2$.

sense, recognition and detection in these bifunctional polyamide-dye molecules can be tuned separately; the polyamide module is programmed for a desired DNA sequence by the pairing rules and the dye is chosen for optimal absorption/emission properties.[18]

Polyamides as Molecular Calipers for Non-Watson−Crick Base Pairs in the DNA Minor Groove. Our fluorescence DNA detection method allows us to screen, in parallel, how a hairpin polyamide will behave when it is forced to bind over a non-Watson−Crick base pair. To date, the energetics of polyamide binding over non-Watson−Crick base pairs has not been examined, mainly due to the labor intensive effort required for the preparation of special end-radiolabeled DNA fragments containing discrete non-Watson−Crick base pairs for use in quantitative footprinting titration analysis. Three hairpin polyamide−fluorophore conjugates **3**, **5**, and **7** were used to screen Py/Py, Im/Py, and Im/Im ring pairs, respectively, against all possible 16 pairing permutations of the nucleic acid bases A, T, G, and C (Figure 6). Polyamide **7** contains an unusual Im/Im ring pairing, previously shown to be energetically unfavorable for recognition of any of the four Watson−Crick base pairings.[19] However, Wang and co-workers have reported an NMR structure for the polyamide dimer $(ImImImDp)_2$ that indicates binding with a minor groove site containing G·T and T·G base pairs under the Im/Im pair.[20] Im/Im recognition of a G·T and T·G wobble mismatch is the first example of ring pairs for specific recognition of non-Watson−Crick base pairs.[21]

Hairpin-forming oligonucleotides (37-mer) were synthesized and annealed (pH 7.0, tris-EDTA buffer). Each duplex was designed to allow for base pair variance at a single unique X/Y position within the context of a 6-base pair hairpin match site. Assays were conducted in 30 μL solutions with the concentration of polyamide conjugate fixed at 1 μM, and the concentration of oligonucleotide was varied from 0.1 μM to 1 μM. After 4 h incubation (22 °C), fluorescence intensities at the 16 variable positions were acquired in parallel on the optical scanner (16 *X*/*Y* position × 5 concentrations).

Figure 6 shows the data collected by direct excitation fluorescence and the normalized data represented in bar graph format. Polyamide **3** and **5** reveal strong fluorescence intensity, and hence affinity, for a sequence containing a match site as dictated by the pairing rules, i.e., Py/Py prefers A·T and T·A

(18) Although polyamide-Hoechst hybrids[10] have the property of enhanced fluorescence when bound to match DNA, the minor groove binding Hoechst dye requires an A/T tract adjacent to the DNA target site and hence is not as versatile.
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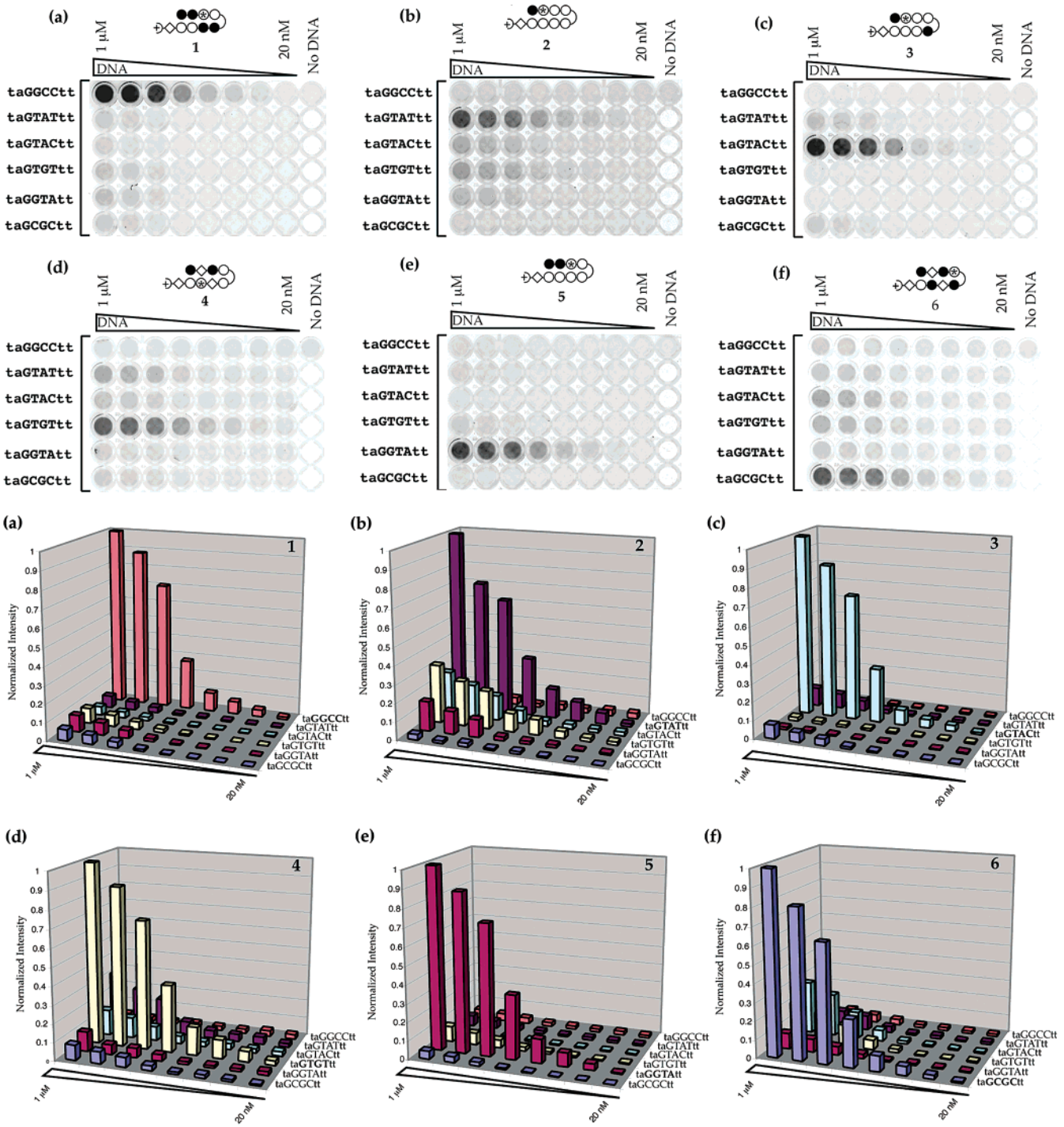

Figure 5. (A) Plate experiments conducted at 1 μM of polyamide-TMR in the presence of 0.02 μM to 1 μM concentration gradient of six different 17-mer DNA duplexes. Each polyamide-TMR conjugate **1**−**6** reveals a preference for one of the six sequences of DNA. The "minus" DNA well is shown for measurement of background fluorescence. Fluorescence intensity correlates with the dark color of a well. (5B) Normalized data for the 54-well experiment using the background as the lowest observable fluorescence intensity.

over the 14 other base pairs; Im/Py prefers G·C over the 15 other base pairs. Polyamide **8** with the Im/Im pair shows a slight preference for G/C over A/T, but does not discriminate G·C from C·G as expected for a symmetrical ring pair. Remarkably, the Im/Im pair binds T·G with the highest affinity validating the Lee−Sugiyama−Wang model.[20] However, in our study Im/Im distinguishes T·G from G·T, a result not anticipated in the NMR structure, which may be due to different sequence contexts used in the two experiments. It is noteworthy that a minor groove binding polyamide can target *a single non-Watson−Crick base pair* out of 12 unique possibilities.

In summary, polyamide-fluorophore conjugates have been used to target sequence repeats in chromosomes for interrogation of sequence location and estimation of repeat length.[12,13] In this study, we extend the use of polyamide-fluorophore conjugates to distinguish match and mismatch sequences within short segments of DNA in solution. The correlation of sequence identity with fluorescence enhancement may be useful in other

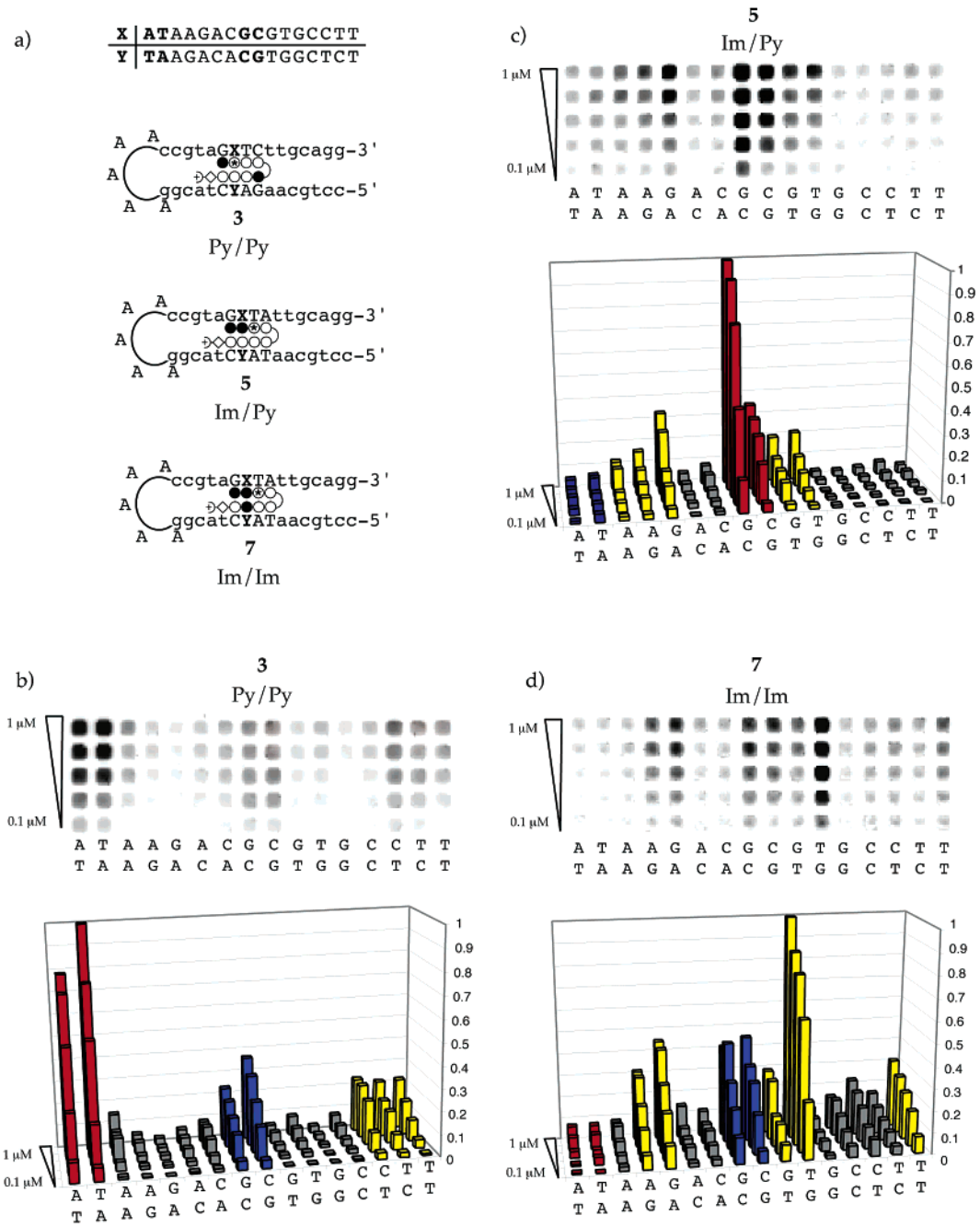

Figure 6. (A) Design of hairpin forming duplex oligonucleotides with polyamide match sites for conjugates **3**, **5** and **7** which vary at a single internal X/Y position all possible 16 base pairs of A, T, G, and C. (B−D) Fluorescence intensities acquired on an optical scanner for the interaction of **3**, **5**, and **7** (1 μM concentration) with 0.1−1 μM of each of 16 different oligonucleotides listed above. Plotted below is normalized graphical representation of polyamides pairing preference as reported by the optical assay.

applications such as screening for sequence variation in specific segments of genomic DNA.

Experimental Section

Materials. UV spectra were measured in water on a Beckman model DU 7400 spectrophotometer. MALDI-TOF mass spectrometry data was collected by the Protein and Peptide Microanalytical Facility at The California Institute of Technology. HPLC analysis was performed using a Beckman Gold Nouveau system using a Rainin C_{18}, Micosorb MV, 5 μm, 300 $\times$ 4.6 mm reversed phase column using 0.1% (wt/v) TFA with acetonitrile as eluent and a flow rate of 1.0 mL/min, gradient elution 1.25% acetonitrile/min. Preparatory reversed phase HPLC was performed on a Beckman HPLC with a Waters DeltaPak 25 $\times$ 100 mm, 100 μm C_{18} column equipped with a guard, 0.1% (wt/v) TFA/0.25% acetonitrile/min. Water was from either a Millipore MilliQ water purification system or RNase free water from a USB. All buffers were 0.2 μm filtered prior to storage. Oligonucleotides were synthesized and purified by The Caltech Biopolymer Synthesis and Analysis Resource Center and Genbase Inc., San Diego. Enzymes for molecular biology were purchased from either New England Biolabs or Boeringer−Mannheim. [α-^{32}P] adenosine triphosphate and [α-^{32}P] thymidine triphosphate were purchased from New

England Nuclear. L-Boc(Trt)−Cys−OH was purchased from Bachem. Tetramethyl rhodamine-5-maleimide was from Molecular Probes. Polystyrene plates were from VWR Scientific. Fluorescence spectrophotometer measurements were made at room temperature on an ISS K2 spectrophotometer employing 5 nm emission and excitation slits in conjunction with a Hg lamp. Polystyrene plate measurements were made on a Molecular Dynamics Typhoon employing a 532 nm 10−20 mW excitation laser. The emission filter employed is 580 ± 15 nm filter for observation of tetramethyl rhodamine fluorescence.

Synthesis of Polyamide-Fluorophore Conjugates. Polyamides **1**−**7** were synthesized by solid-phase methods. A new monomer, 4-[(*t*-butoxycarbonyl)amino]-1-(phthalimidopropyl)-pyrrole-2-carboxylic acid (**15**) was incorporated in the usual manner by DCC/HOBt activation (DMF, 4 h., 37 °C). (See Supplemental Figures S1 and S2.) Polyamide **3** was simultaneously deprotected and cleaved from the resin by aminolysis with 3-(dimethylamino)-propylamine (60 °C, 10 h.). Purification by reversed phase HPLC afforded a diamine which was allowed to react with one equiv of L-Boc(Trt)−Cys−OH activated with DCC/HOBt. The polyamide-cysteine conjugate **10** was characterized by analytical HPLC and MALDI-TOF mass spectrometry. The L-cysteine modified polyamides in DMF were allowed to react with one equivalent of TMR-5-maleimide in a minimal volume of DMF. This reaction was monitored by analytical HPLC and was usually complete in less than 15 min. The products were purified by reversed phase preparatory HPLC. All products were analyzed by MALDI/TOF MS. For **1** (monoisotopic) [M + H] 1853.6 (1853.8 calcd for $C_{89}H_{105}N_{28}O_{16}S$); **2** (monoisotopic) [M + H] 1850.54 (1850.81 calcd for $C_{92}H_{108}N_{25}O_{16}S$); **3** (monoisotopic) [M + H] 1851.74 (1851.81 calcd for $C_{91}H_{107}N_{26}O_{16}S$); **4** (monoisotopic) [M + H] 1749.9 (1749.79 calcd for $C_{85}H_{105}N_{24}O_{16}S$); **5** (monoisotopic) [M + H] 1851.8 (1851.81 calcd for $C_{91}H_{107}N_{26}O_{16}S$); **6** (monoisotopic) [M + H] 1751.6 (1751.78 calcd for $C_{83}H_{103}N_{26}O_{16}S$); **7** (monoisotopic) [M + H] 1852.7 (1852.63 calcd for $C_{90}H_{106}N_{27}O_{16}S$); **8** (monoisotopic) [M + H] 617.4 (617.2 calcd for $C_{32}H_{34}N_5O_6S$). For **1**−**3**, **5**, **7** UV (H_2O) $\lambda_{max}(\epsilon)$ 312 (68 800), **4** and **6**, UV (H_2O) $\lambda_{max}(\epsilon)$ 300 (51 600), **1**−**6**, Vis (H_2O) $\lambda_{max}(\epsilon)$ 563 (65 700), **8** vis (H_2O) $\lambda_{max}(\epsilon)$ 550 (95 000).

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−TMR conjugate was 1 μM and the volume of solution used for each measurement was 150 μL. In the fluorimeter, polyamides **1**−**6** were excited at 545 nm and measured over the interval of 570 nm to 680 nm. Emission maxima are 575 nm for **1**−**8**. 1 μM solutions of each conjugate were irradiated in the presence and absence of 1 μM of the appropriate match DNA to generate the fluorescence enhancements reported in Supplemental Figure S4. Absorption spectra for **1**−**6** and **8** were recorded under the same conditions as emission with **1**−**6** at higher concentration.

96-Well Plate Characterization. In the dark, 150 μL solutions were made by titrating together a fixed concentration of 1 μM conjugate against 20 nM to 1 μM of each DNA 17-mer. The solutions were gently swirled for a few minutes and then allowed to sit for 4 h before measurements were made. No changes in fluorescence intensity were observed for longer equilibration times. Plates containing polyamides **1**−**7** were excited at 532 nm, and data were collected at 580 (±15 nm fwhm) nm. ImageQuant software (Molecular Dynamics) was used to analyze the fluorescence intensity of each titration experiment. From these data, histograms were constructed to assist in determining DNA sequence preference for each conjugate. The normalized data used in each histogram is polyamide specific assuming the background to be the lowest observable fluorescence intensity, which is subtracted in the denominator from the highest observed fluorescence intensity, that of the match sequence at 1:1 conjugate:duplex as given by the relation $(F_{obs} - F_{min})/(F_{max} - F_{min})$.

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