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Detection of CAG repeat DNA sequences by pyrene-functionalized pyrrole-imidazole polyamides

Toshikazu Bando,* Jun Fujimoto, Masafumi Minoshima, Ken-ichi Shinohara, Shunta Sasaki, Gengo Kashiwazaki, Masatoshi Mizumura and Hiroshi Sugiyama*

Department of Chemistry, Graduate School of Science, Kyoto University, Sakyo, Kyoto 606-8502, Japan

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Abstract—Five *N*-methylpyrrole-*N*-methylimidazole (Py–Im) polyamides possessing a fluorescent pyrene were synthesized by Fmoc solid-phase synthesis using Py/Im monomers and pyrenylbutyl-pyrrole monomer compound **9**. The steady state fluorescence of conjugates **1**–**5** was examined in the presence and absence of $(CAG)_{12}$-containing oligodeoxynucleotides (ODNs) 1 and 2. Of the conjugates, conjugate **1** showed no background emission around 470 nm in the absence of ODNs, and a clear increase of emission at 475 nm was observed upon addition of ODNs 1 and 2. The emission of conjugate **1** at 475 nm increased linearly with the concentration of ODN and the number of CAG repeats. The results indicate that conjugate **1** efficiently forms a pyrene excimer upon binding in the minor groove of DNA.
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1. Introduction

A number of chemical and biological studies have demonstrated that *N*-methylpyrrole-*N*-methylimidazole (Py–Im) polyamides precisely recognize each of the four Watson–Crick base pairs.[1,2] Sequence-specific DNA recognition by Py–Im polyamides bound in the minor groove depends on the sequence of side-by-side aromatic amino acid pairings oriented in the amino-carboxyl (N–C) direction with respect to the 5′–3′ direction of the DNA helix. An antiparallel pairing of *N*-methylimidazole (Im) opposite *N*-methylpyrrole (Py) (Im/Py) recognizes a G·C base pair, whereas a Py/Py pair recognizes T·A and A·T base pairs. A β-alanine/β-alanine (β/β) pairing reads T·A and A·T pairs in the same way as do Py/Py pairs. Thus, Py–Im polyamides can sequence-specifically recruit various functional groups in DNA.[3,4] We have developed various types of sequence-specific alkylating Py–Im polyamides and have investigated their chemical and biological properties.[5–7] In addition to such basic applications, the sequence-specific binding properties of Py–Im polyamides have been explored in the detection of specific sequences of duplex DNA for potential diagnostic applications.[8–11]

Fluorescent biosensors are powerful tools for probing the sequence and structures of biomolecules. A typical example of such probe systems is the molecular beacons, which have proven to be of significant utility in the detection of DNA sequences. With respect to fluorescent biosensors, we are especially interested in pyrene, which has been used for detecting DNA sequences.[12] Pyrene-based fluorescence exhibits large extinction coefficients, excellent quantum yields, and good stability in aqueous solution. Pyrene also forms an excited state dimer, termed an excimer, with readily detected emission that is red-shifted by about 100 nm relative to the monomer. In spite of these useful properties, pyrene excimer emission has only been used in applications for the detecting of single-strand DNA sequences.[13–18] Because formation of an excimer is strongly distance- and geometry-dependent, specific DNA binding of Py–Im polyamides could precisely control excimer-based fluorescence. To develop a fluorescence-based probe for the detection of repeated DNA sequences in duplex form, we have designed a novel Py–Im conjugate, which changes fluorescence when bound in the minor groove of duplex DNA. Here we report the molecular design of a pyrene excimer-based Py–Im conjugate **1**, synthesized by Fmoc

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* Corresponding authors. Tel.: +81 75 753 4002; fax: +81 75 753 3670; e-mail: hs@kuchem.kyoto-u.ac.jp

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solid-phase methods, which has potential in the field of chemical biosensors for double-stranded DNA.

2. Results and discussion

2.1. Molecular design and synthesis

It has been clearly demonstrated that an expansion of triplet repeat DNA sequences is associated with various diseases. As a model, we examined techniques to detect the CAG repeat sequence, which is known to be expanded in Huntington disease.[19,20] Py–Im polyamides possessing more than five contiguous Py–Im rings show poor binding to the minor groove of B-DNA because of over-curvature compared with the minor groove of DNA. By introducing β-alanine (β) as an aliphatic substitute for a Py ring in the hairpin polyamides, we take advantage of the better flexibility of the β residue to allow the crescent-shaped polyamide to match the curvature of B-DNA.[21] Accordingly, we have designed and synthesized Py–Im polyamides targeting a seven-base pair sequence, 5′-AGCAGCA-3′ (Fig. 1).

We prepared 4-(9-fluorenylmethoxy carbonyl) amino-1-pyrenylbutylpyrrole-2-carboxylic acid (compound **9**) as a monomeric unit to introduce a pyrene functionalized Py residue to Py–Im polyamides. Compound **9** was synthesized according to the five-step procedure shown in Scheme 1. Methyl 4-nitro-1-(4-pyrenylbutyl)pyrrole-2-carboxylate (compound **7**) was prepared from the corresponding starting material **6** in a mixture of 1-(4-bromobutyl)pyrene and potassium carbonate in the presence of a small amount of tetrabutylammonium iodide. The resultant compound **7** was converted to Boc-protected pyrrole carboxylic acid (compound **8**) by subsequent reduction and protection using palladium-carbon and $(Boc)_2O$ under hydrogen gas pressure, then hydrolysis by LiOH. Under these conditions, a relatively high yield of compound **8** was obtained without isolation of an O_2-sensitive amino pyrrole intermediate. The removal of the *t*-Boc protecting group, followed by Fmoc protection of compound **8**, produced compound **9**.

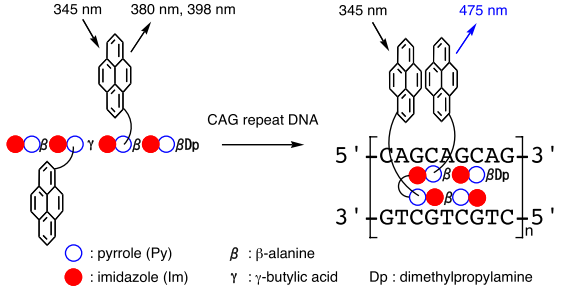
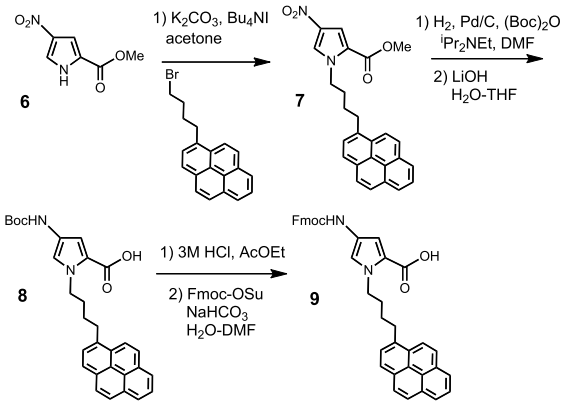


Figure 1. Molecular interaction model of Py–Im polyamides with double-stranded DNA. The excimer-based probe for the detection of the CAG repeat sequences is composed of two *N*-(1-pyrenyl) butyl groups conjugated to a Py–Im polyamide. The pyrene moieties are segregated in the absence of target (left). Upon target binding, excimer formation occurs with consequent longer wavelength excimer emission (475 nm) (right).



Scheme 1. Preparation of Fmoc-protected pyrenylbutyl-pyrrole monomer for solid-phase synthesis.

The polyamides **1**–**5** were synthesized by Fmoc solid-phase synthesis, starting with Fmoc-β-Ala-CLEAR-acid resin and using Fmoc protected Py/Im monomers and compound **9**.[22–24] After completion of the solid-phase synthesis, the polyamides were cleaved from the resin by aminolysis with 3-(dimethylamino)propylamine (Dp), to produce the desired polyamides **1**–**5**, in which 4-pyrenylbutyl groups were incorporated at the corresponding N1-position of Py in the hairpin Py–Im polyamide. After purification by HPLC, Py–Im polyamides **1**–**5** were obtained as a yellow powder. The purity of polyamides was >95% as judged by HPLC analysis. The bis-pyrenyl conjugates **1**–**5** adopt a hairpin conformation in the DNA minor groove when complexed with their target DNA duplex (Fig. 2).

2.2. Emission of conjugates 1–5 in the presence of CAG repeats

To evaluate the ability of conjugates to detect CAG repeat sequences, the fluorescence of conjugates **1**–**5** was examined in the presence and absence of $(CAG)_{12}$-containing ODNs 1 and 2. In the presence of ODNs 1 and 2, conjugate **1** showed an increasing emission centered at 475 nm with increasing concentrations of ODNs 1 and 2 (Fig. 3). The dramatic increase of emission can be explained by the formation of a pyrene excimer on

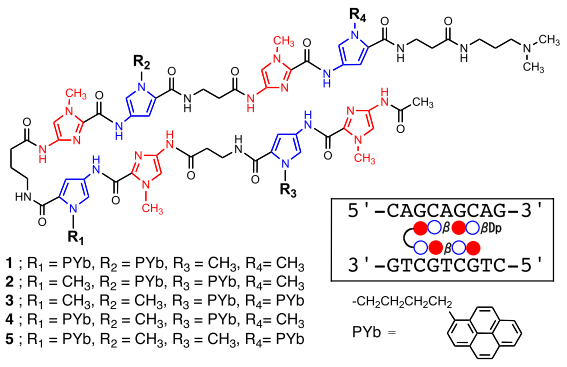


Figure 2. The molecular design of the DNA minor groove binder for the CAG repeat sequences. Chemical structures of bis-pyrenyl Py–Im polyamides **1**–**5**.

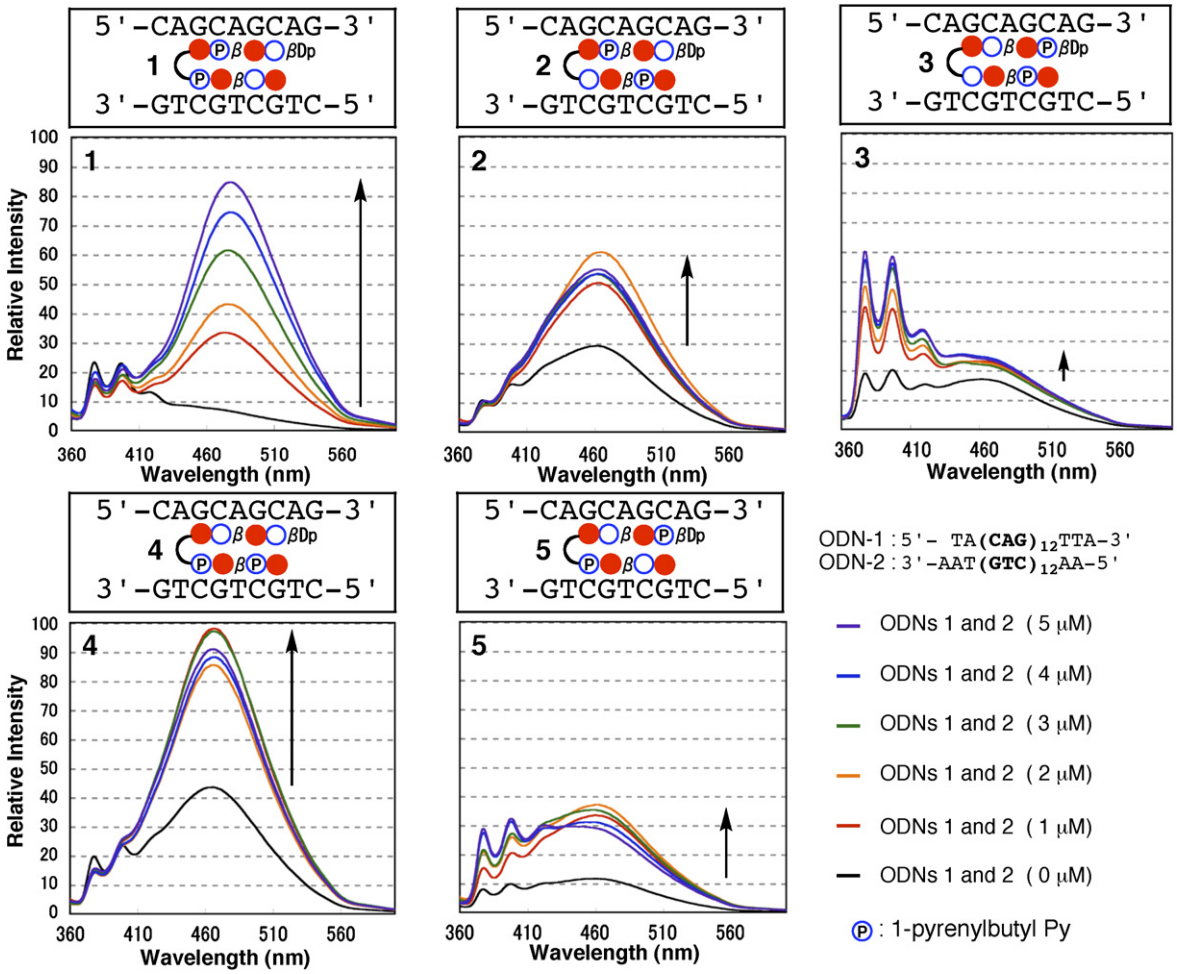


Figure 3. Fluorescence titration spectra of the conjugates **1**–**5** (10 μM) in a 5 mM Na phosphate buffer (pH 7 containing 7% v/v DMF) in the presence of a target repeated ODN-1: 5′-TA(CAG)$_{12}$TTA-3′/ODN-2: 5′-AA(CTG)$_{12}$TAA-3′ (0–5 μM); λ_{ex} = 345 nm.

binding of conjugate **1** to the minor groove of DNA. Importantly, the pyrene excimer of conjugate **1** did not form in the absence of DNA. In contrast, conjugates **2** and **4** showed excimer emission centered at 462 nm in the absence of ODNs 1 and 2, and the intensity of excimer fluorescence increased in a nonlinear fashion with the amount of target ODNs. In the case of conjugates **3** and **5**, fluorescence of the monomeric pyrene was observed even in the presence of ODNs 1 and 2. The results imply that, on binding to DNA, conjugate **1** changes its conformation to one in which two pyrene units can form an excimer. Accordingly, conjugate **1** represents an 'excimer-based' bis-pyrene Py–Im polyamide probe with fluorescence at 380 and 398 nm that shifts to 475 nm after the conjugate binds to target oligonucleotides in the minor groove of double-stranded DNA.

Because conjugate **1**, containing two pyrene units, showed optimal excimer fluorescence on DNA binding, we examined whether this excimer emission could be used to quantify the number of CAG repeat sequences (Fig. 4). Strong excimer emission of conjugate **1** was observed with 4–12 CAG repeat sequences, as shown in Figure 4b. The emission of conjugate **1** increased linearly with the concentration of target ODNs. In contrast, excimer emission was weak using ODNs 7 and 8, without CAG repeat, and emission did not increase with the amount of ODNs 7 and 8 used (Fig. 4c).

3. Conclusions

We have demonstrated that the excimer emission of Py–Im polyamides is dramatically changed by the substitution of a pyrenylbutyl group in the hairpin. The emissions of conjugate **1** excimer increased linearly with the number of CAG repeats and the concentration of ODNs containing CAG repeats, indicating the possible application of conjugate **1** for the quantification of CAG repeat DNA sequences.

4. Experimental

4.1. General

Reagents and solvents were purchased from standard suppliers and used without further purification. Abbreviations of some reagents: HCTU, 1-[bis(dimethylamino)methylene]-5-chloro-1*H*-benzotriazolium 3-oxide

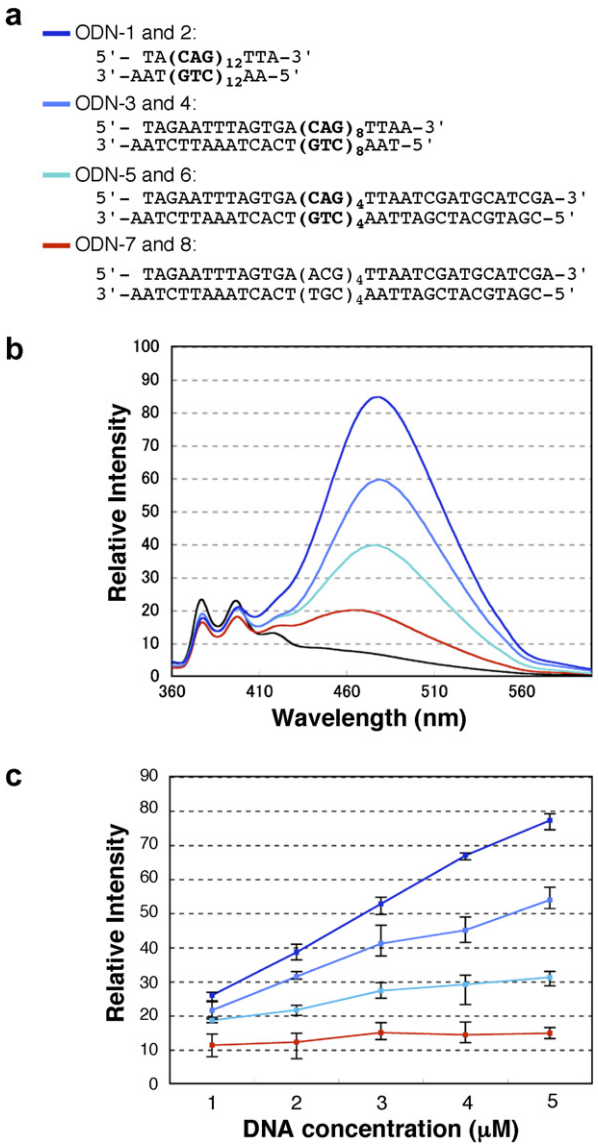


Figure 4. (a) Sequences of ODNs 1–8. (b) Fluorescence titration spectra of conjugate **1** (10 μM) in a 5 mM Na phosphate buffer (pH 7 containing 7% v/v DMF) in the presence of each ODNs (5 μM), respectively. Black line shows spectra in the absence of ODNs. (c) Fluorescence titration graph of conjugate**1** ([**1**], 10 μM; [Na phosphate], 5 mm, 7% v/v DMF, at pH 7.0, λ_{em} = 475 nm) in the presence of each ODN (0–5 μM). The relative intensity was obtained by subtracting the background level of emission of **1** in the absence of ODNs at 475 nm. Error bars represent standard deviation of the means of triplicate samples. λ_{ex} = 345 nm.

hexafluorophosphate; $(Boc)_2O$, di-*tert*-butyl dicarbonate; Fmoc-OSu, 9-fluorenylmethyl succinimidyl carbonate; DMF, *N*,*N*-dimethylformamide; DCM, dichloromethane; AcOEt, ethyl acetate; THF, tetrahydrofuran. NMR spectra were recorded with a JEOL JNM-FX 400 nuclear magnetic resonance spectrometer, and tetramethylsilane was used as the internal standard. Proton NMR spectra were recorded in parts per million (ppm) downfield relative to tetramethylsilane. The following abbreviations apply to spin multiplicity: s (singlet), d (doublet), t (triplet), m (multiplet), br (broad). Electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS) was produced on a BioTOF II (Bruker Daltonics) mass spectrometer. DNA oligonucleotides were purchased from Sigma–Aldrich Co. Fluorescence measurements were performed on Spectrofluorometer FP-6300 (JASCO). All machine-assisted polyamide syntheses were performed on Liberty Microwave Peptide Synthesizer (CEM Co.) with a computer-assisted operation system at a 0.10 mmol scale (200 mg of CLEAR resin, 0.52 meq/g) by using Fmoc solid-phase chemistry. HPLC purification was performed with a Chemcobond 5-ODS-H reversed phase column (10 × 150 mm) in 0.1% AcOH with acetonitrile as eluent at a flow rate of 6.0 mL/min, appropriate gradient elution conditions, and detection at 254 or 345 nm. Methyl 4-nitropyrrole-2-carboxylate **6** was prepared according to the published procedure.[25,26]

4.2. General preparation of the Py–Im polyamides 1–5 by Fmoc solid-phase synthesis

Typically, Fmoc-β-Ala-CLEAR-acid resin (200 mg, 0.10 mmol) was swollen in 3 mL of DMF in a 50-mL plastic centrifuge tube for 30 min and placed on the board of microwave synthesizer. DMF, DCM, 20% piperidine in DMF, 20% acetic anhydride in DMF, 0.45 M HCTU in DMF, 2 M $^{i}Pr_2NEt$ in DMF were placed in the external bottle, respectively. The amino acids were weighted out in 4 molar excess amounts and added DMF to get standard 0.2 M amino acid solution used on the instrument. Each monomer in DMF solution was placed on the loading position of microwave synthesizer, respectively. The synthesis was then started, controlled by the computer using the established program. The reactions were performed using deprotection steps of 180 s at 30 W (T_{max} = 75 °C) and coupling steps of 300 s at 25 W (T_{max} = 75 °C) and a final capping step of 120 s at 50 W (T_{max} = 75 °C). All couplings were carried out with single-couple cycles. After the completion of the synthesis on the peptide synthesizer, the resin was washed with four times DMF, methanol, and DCM, respectively, and dried in a desiccator at room temperature in vacuo.

4.2.1. AcImPy-β-ImPy*-γ-ImPy*-β-ImPy-β-Dp (1). The resin was then placed in a 10-mL glass scintillation vial, 3 mL of (dimethylamino)propylamine was added, and the solution stirred at 55 °C overnight. Resin was removed by filtration through a pad of Celite and washed thoroughly with DCM. The resultant filtrates were removed in vacuo. The residue was dissolved in approximately 0.5 mL of DMF. The polyamide solution was analyzed and purified by preparatory HPLC at 345 nm. Appropriate fractions were lyophilized to give the final desired polyamide **1** (3.5 mg, 4% for 12 steps) as light-yellow powder. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.30 (s, 2H, NH), 10.25 (s, 2H, NH), 9.88 (s, 4H, NH), 8.29–7.85 (m, 14H, NH+PyreneCH), 7.44 (s, 4H, CH), 7.30 (s, 2H, CH), 7.19 (s, 2H, CH), 6.92 (s, 2H, CH), 6.90 (s, 2H, CH), 4.36 (br s, 4H, CH_2), 3.93 (s, 18H, NCH_3), 3.92 (s, 6H, NCH_3), 3.40 (m, 12H, CH_2), 3.03 (br s, 2H, CH_2), 2.52 (br s, 2H, CH_2), 2.32 (m, 6H, CH_2), 2.14 (br s, 2H, CH_2), 2.05 (s, 6H, NCH_3), 2.00 (s, 3H, $COCH_3$), 1.85 (m, 4H, CH_2), 1.70

(m, 4H, CH_2), 1.48 (m, 4H, CH_2); ESI-TOF-MS *m/e* Calcd for $C_{102}H_{111}N_{26}O_{13}$: $[M+H]^+$ 1907.9. Found: 1908.0.

A synthetic procedure similar to that used for the synthesis of polyamide **1** was followed to prepare polyamides **2**–**5**, with yields of 2–4% for 12 steps as light-yellow powder.

4.2.2. AcImPy*-β-ImPy-γ-ImPy*-β-ImPy-β-Dp (2). ESI-TOF-MS *m/e* Calcd for $C_{102}H_{111}N_{26}O_{13}$: $[M+H]^+$ 1907.9. Found: 1908.1.

4.2.3. AcImPy*-β-ImPy-γ-ImPy-β-ImPy*-β-Dp (3). ESI-TOF-MS *m/e* Calcd for $C_{102}H_{111}N_{26}O_{13}$: $[M+H]^+$ 1907.9. Found: 1908.4.

4.2.4. AcImPy*-β-ImPy*-γ-ImPy-β-ImPy-β-Dp (4). ESI-TOF-MS *m/e* Calcd for $C_{102}H_{111}N_{26}O_{13}$: $[M+H]^+$ 1907.9. Found: 1908.4.

4.2.5. AcImPy-β-ImPy*-γ-ImPy-β-ImPy*-β-Dp (5). ESI-TOF-MS *m/e* Calcd for $C_{102}H_{111}N_{26}O_{13}$ $[M+H]^+$ 1907.9. Found: 1908.2.

4.2.6. Methyl 4-nitro-1-(4-pyrenylbutyl)pyrrole-2-carboxylate (7). To a solution of 1-(4-bromobutyl)pyrene (1.23 g, 3.64 mmol) in acetone (20 mL) was added K_2CO_3 (0.75 g, 5.46 mmol) and Bu_4NI (0.27 g, 0.72 mmol). After compound **6** (0.62 g, 3.64 mmol) was added, the reaction mixture was refluxed for 11 h. Evaporation of the solvent gave a brown residue, which was collected by filtration, washed with water, 5% aq HCl, then dried to produce **7** (1.46 g, 94% yield) as a brown powder. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.16–8.09 (m, 3H), 8.04 (d, J = 8.8 Hz, 2H), 7.96 (s, 2H), 7.93 (t, J = 7.6 Hz, 1H), 7.76 (d, J = 7.6 Hz, 1H), 7.49 (d, J = 2.0 Hz, 1H), 7.30 (d, J = 2.0 Hz, 1H), 4.30 (t, J = 7.2 Hz, 2H), 3.75 (s, 3H), 3.32 (t, J = 7.2 Hz, 2H), 1.89 (m, 2H), 1.84 (m, 2H); ESI-TOF-MS *m/e* Calcd for $C_{26}H_{23}N_2O_4$: $[M^+ + H]$ 427.17. Found: 427.19.

4.2.7. 4-[(*tert*-Butoxycarbonyl)amino]-1-(4-pyrenylbutyl)pyrrole-2-carboxylic acid (8). To a solution of compound **7** (1.46 g, 3.43 mmol) in DMF (25 mL) was slowly added 10% Pd–C (440 mg). After ^{i}Pr$_2$NEt (1.84 mL, 10.30 mmol) and $(Boc)_2O$ (1.18 mL, 5.14 mmol) were added dropwise, the reaction mixture was stirred for 5 h at room temperature under hydrogen gas pressure. The catalyst was removed by filtration through celite using AcOEt (200 mL). The organic phase was separated, washed with water (3× 20 mL), saturated NaCl (20 mL), dried over anhydrous Na_2SO_4. After the filtration, the solvent was evaporated in vacuo to produce crude amine, which was used in the next step without further purification. To a solution of crude amine in water–THF (1:2, 60 mL) was added LiOH monohydrate (2.9 g, 68.60 mmol). The reaction mixture was refluxed for 28 h and quenched by acetic acid (4.5 mL), then AcOEt (200 mL) was added. The organic phase was separated, washed with water (3× 20 mL), saturated NaCl (20 mL), dried over anhydrous Na_2SO_4. After the filtration, the solvent was evaporated in vacuo to produce compound **8** (1.46 g, 88% yield for 2 steps) as a yellow powder. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.13 (d, J = 8.8 Hz, 1H), 8.06 (d, J = 7.6 Hz, 2H), 8.01–7.98 (m, 3H), 7.92 (s, 2H), 7.88 (t, J = 7.6 Hz, 1H), 7.74 (d, J = 7.6 Hz, 1H), 7.16 (s, 1H), 6.63 (s, 1H), 6.17 (s, 1H), 4.22 (br t, 2H), 3.24 (t, J = 7.2 Hz, 2H), 1.88 (m, 2H), 1.77 (m, 2H), 1.42 (s, 9H); ESI-TOF-MS *m/e* Calcd for $C_{30}H_{31}N_2O_4$ $[M^+ + H]$ 483.23. Found: 483.27.

4.2.8. 4-[(9-Fluorenylmethoxycarbonyl)amino]-1-(4-pyrenylbutyl)pyrrole-2-carboxylic acid (9). The reaction mixture of compound **8** (1.46 g, 3.03 mmol) in 12 M HCl–AcOEt (1:3, 60 mL) was stirred for 5 h at room temperature. The precipitate was collected by filtration, washed with diethyl ether, and dried to produce crude amine (1.16 g) as a gray powder, which was used in the next step without further purification. To a solution of crude amine in water–DMF (1:1, 60 mL) was added $NaHCO_3$ (927 mg, 11.04 mmol) and Fmoc-OSu (1.02 g, 3.04 mmol). The reaction mixture was stirred for 5 h at rt under Ar gas. After the quenching by 5% aq HCl, the precipitate was removed by filtration. The solution was partitioned between AcOEt (200 mL) and water (20 mL). The organic phase was separated, washed with water (3× 20 mL), saturated NH_4Cl (20 mL), saturated NaCl (20 mL), dried over anhydrous Na_2SO_4. After the filtration, the solution was concentrated to a residue, which was subjected to column chromatography (silica gel, 25% AcOEt in hexane) to produce compound **9** (1.23 g, 65% yield for 2 steps) as a yellow powder. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.12 (d, J = 8.8 Hz, 1H), 8.04 (d, J = 7.6 Hz, 2H), 8.00–7.95 (m, 3H), 7.90 (s, 2H), 7.87 (t, J = 7.6 Hz, 1H), 7.71 (m, 3H), 7.53 (d, J = 7.2 Hz, 2H), 7.33 (br t, 2H), 7.25 (br t, 2H), 7.13 (s, 1H), 6.66 (s, 1H), 6.34 (s, 1H), 4.42 (d, J = 6.4 Hz, 2H), 4.23 (m, 3H), 3.24 (br t, 2H), 1.87 (m, 2H), 1.76 (m, 2H); ESI-TOF-MS *m/e* Calcd for $C_{40}H_{33}N_2O_4$: $[M^+ + H]$ 605.24. Found: 605.30.

4.3. Steady state fluorescence measurements of DNA fragments containing CAG/CTG repeats sequences

Fluorescence spectra were obtained on a Spectrofluorometer FP-6300 (JASCO) with an excitation wavelength of 345 nm in hybridization buffer, and oligonucleotides at corresponding concentrations of conjugates **1**–**5**. No special efforts were made to remove oxygen from the reaction solution at room temperature.

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