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Perylene-conjugated pyrrole polyamide as a sequence-specific fluorescent probe

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ABSTRACT

Perylene-conjugated pyrrole (Py)-polyamide **2** was designed and synthesized using the Fmoc solid-phase synthesis and a subsequent Sonogashira coupling reaction with 3-bromoperylene. Interestingly, conjugate **2** did not luminesce in water at 313 nm irradiation but was turned on in the presence of target double-stranded (ds) DNA, and showed strong emission with increasing DNA concentration, in particularly, by the binding to the target telomere sequences through heterodimer formation with partner **3**. Importantly, the excitation spectrum of **2** clearly indicates that the Py and Imidazole (Im) moieties in the polyamide effectively sensitize the perylene moiety to give rise to fluorescence emission. Energy transfer would occur from the Py moiety to the perylene. Thus, screening of perylene-conjugates will allow us to develop a novel "molecular light switch" with sequence-specificity.

1. Introduction

Fluorescence detection of specific dsDNA sequences has recently attracted intense interest because it provides a simple procedure that does not require manipulations, such as denaturation of double-stranded (ds) DNA and hybridization of the complementary strand.[1,2] Chemists have studied the possibility of switchable fluorescent molecules as useful tools for specific detection of dsDNA.[3–7] Our laboratory has explored the functional design and biological application of pyrrole (Py)–imidazole (Im) polyamides,[8] which bind in the minor groove of the dsDNA with the high affinity and specificity.[9,10] To develop sequence-specific dsDNA probes, we selected a Py–Im polyamide as a sequence-specific DNA binding scaffold and perylene as a fluorophore that emits light in visible region (ca. 460–490 nm).[11–13] Herein, we describe the new perylene-modified polyamide **2**, which emits strong fluorescence only in the presence of target DNA sequences.

2. Molecular design and synthesis

As our previous studies suggest the insertion of an alkynyl group between the pyrrole and the aromatic fluorophore improved DNA binding stability,[14] conjugate **2** was designed. The precursor polyamide **1** and binding partner polyamide **3** were synthesized by Fmoc solid-phase synthesis using HCTU as a coupling reagent, and subsequent treatment with *N*,*N*-dimethyl-1,3-propanediamine (Dp).[15–17] Conjugate **2** possessing a perylene moiety was produced by the coupling of **1** and 3-bromoperylene using Sonogashira conditions (Scheme 1).[18–20]

3. Absorption, excitation and fluorescence Spectra

The DNA binding processes of conjugate **2** were examined by absorption and emission spectroscopy. The absorption spectrum of perylene conjugate **2** indicated a band at 305 nm, due to the pyrrole moieties, together with bands at 431 and 458 nm that were 20 nm longer than those of free perylene (Fig. 1a). It was reported that similar alkynyl perylene oligonucleotide (ODN) conjugate also has an absorption maximum at 454 nm.[20] As polyamide **2** possesses *N*-methyl pyrrole and β-alanine-moieties, this compound would preferentially bind to AT-rich sequences through a 1:1 binding mode (Fig. 1b).[21] In fact, upon addition of ODN-1/2, strong emission at 463 nm appeared with excitation of the perylene at 425 nm, suggesting DNA binding by polyamide **2**. Analogous alkynyl perylene ODN conjugate also has an emission maximum at 468 nm (λ_{ex} = 430 nm).[20] Interestingly, the excitation spectrum clearly indicated that excitation at 313 nm also provided perylene emission at an extent similar to that of direct excitation of perylene at 425 nm (Fig. 1c). Because free perylene with ODN-1/2 did not show such an excitation spectrum, these results suggest the excitation band at 313 nm is derived from the Py moiety of the polyamide. As the emission band of the Py moiety largely overlaps the absorption band of perylene,[22] energy transfer would occur from the Py moiety to the perylene.

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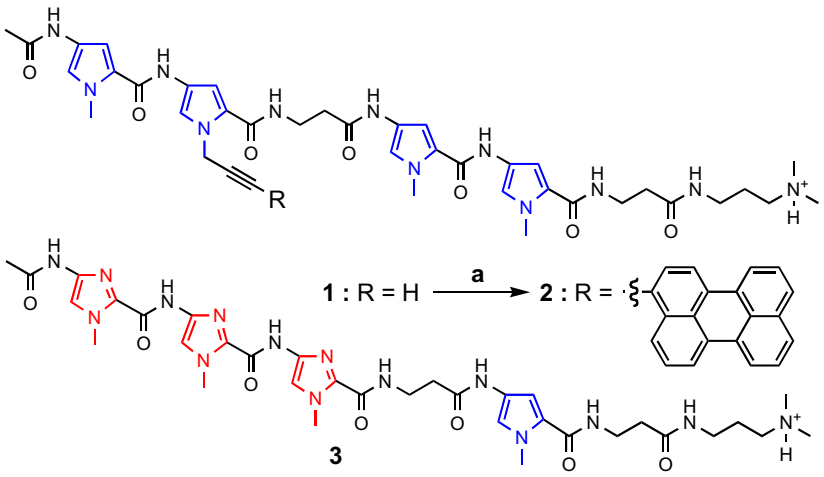

Scheme 1. Synthesis scheme for the preparation of conjugate **2** and the chemical structure of partner **3** (a) 3-bromoperylene, [$Pd(PPh_3)_4$], CuI, triethylamine, DMF.

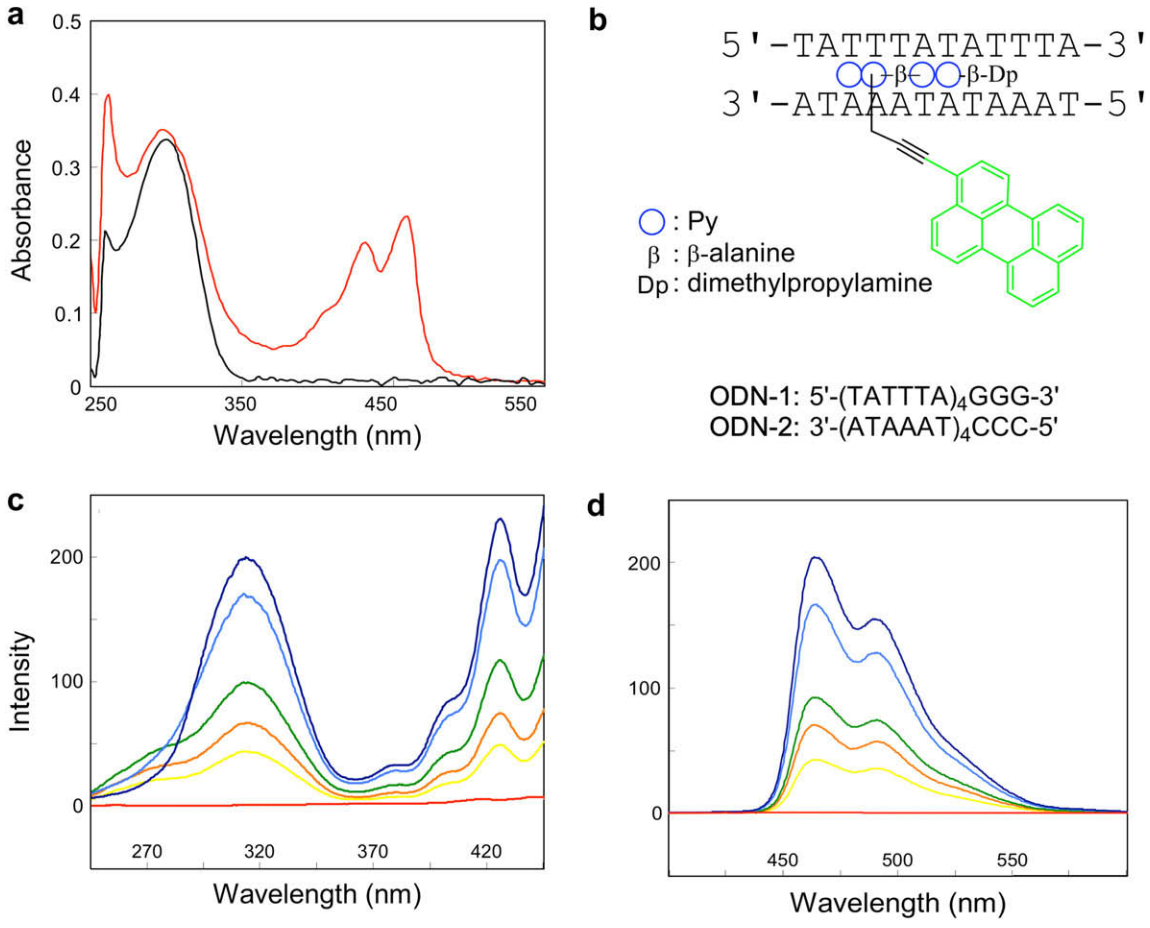

Figure 1. (a) The UV–visible absorption spectra of **1** (black line) and **2** (red line) in DMF solution ([**1**] = [**2**] = 100 μM). (b) The proposed interaction model for conjugate **2** with the target AT-rich sequences. (c) The excitation spectra of conjugate **2** (10 μM) in a 5 mM Na phosphate buffer (pH 7.0, containing 2%, v/v, DMF) in the absence (red) and presence of ODN-1/2, 250 nM (yellow), 500 nM (orange), 1 μM (green), 3 μM (light blue) and 10 μM (blue); λ_{em} = 463 nm. (d) The emission spectra of conjugate **2** (10 μM) in a 5 mM Na phosphate buffer (pH 7.0, containing 2%, v/v, DMF) with ODN-1/2 (0–10 μM); λ_{ex} = 313 nm.

The steady-state fluorescence titration spectra of ODN-1/2 (0–10 μM) with conjugate **2** with irradiation at 313 nm exhibited strong fluorescence at 462 and 494 nm, as shown in Figure 1d. Importantly, the fluorescence intensity of **2** ([ODN-1/2] = 10 μM) was almost 250 times greater than that of **2** in the absence of ODN-1/2.[23] Fluorescence quantum yields of conjugate **2** was 0.59 in DMF and 0.04 in H_2O containing 2%, v/v, DMF (λ_{ex} = 423 nm). Conjugate **2** did not luminesce in water but was "turned on" in the presence of DNA, and showed strong emission with increasing DNA concentration. Similar enhancement of the fluorescence of TMR upon binding to the target dsDNA was reported by Dervan and coworkers.[3–5] It was suggested that the fluorescence of TMR

would be quenched by the covalent link to the ring nitrogen of the pyrrole in the absence of matched dsDNA, and as such quenching was prevented upon binding to dsDNA and the fluorescence of TMR was restored. A similar dramatic change in the quenching process upon DNA binding may occur in the present system.

We next investigated the detection of a telomere sequence by conjugate **2** with partner **3** using ODN-3/4. Conjugate **2** with partner **3** is assumed to bind to the target telomere sequences through heterodimer formation (Fig. 2a).[24] Steady-state fluorescence spectra of conjugate **2** with partner **3** ([2] = [3] = 10 μM) were measured with excitation at 313 nm in the presence of different concentrations of ODN-3/4. Intense emission was observed at 462 and 494 nm, even at the ODN-3/4 concentration of 3 μM (Fig. 2b). The existence of the ODN-3/4 in aqueous solution was visible, as shown in the inset of Fig. 2b. It is significant to note that the observed fluorescence intensity of **2** and **3** with ODN-3/4 was almost 7 times greater than that of **2** with ODN-1/2 under the same conditions.

The excitation spectrum showed that the excitation band at 313 nm was greater than that at 425 nm (Fig. 2c), indicating efficient energy transfer from multiple pyrrole and imidazole rings. These results suggest that the fluorescent DNA binding complex between **2** and **3** was efficiently formed in the minor groove of ODN-3/4. In clear contrast, almost no fluorescence was observed in the absence of **3** or in the presence of the mismatched ODN-5/6 (Fig. 2d). These results suggest the versatility of perylene-conjugated Py polyamides as sequence-specific fluorescence probes for duplex DNA.

4. Conclusions

We have developed a new type of perylene-conjugated Py polyamide that emits strong fluorescence only in the presence of duplex DNA containing the target sequence. The excitation spectrum of **2** clearly indicates that the Py and Im moieties in the polyamide effectively sensitize the perylene moiety to give rise to fluorescence emission. Thus, screening of perylene-conjugates will allow us to develop a novel "molecular light switch" with sequence-specificity.

5. Experiments

5.1. General

5.1.1. Materials

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

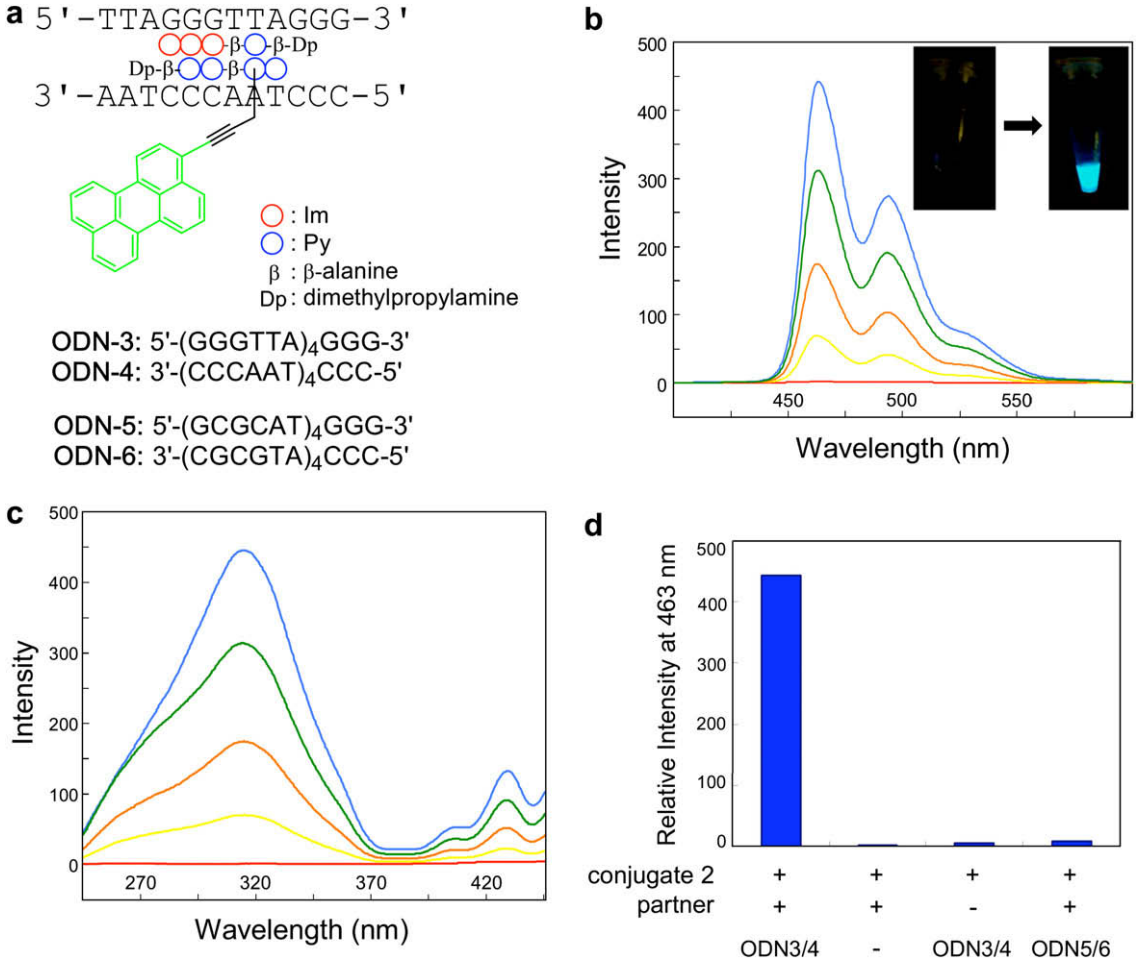

Figure 2. (a) The proposed interaction model of conjugate **2** and partner **3** with the target telomere repeat sequences. (b) The fluorescence at 463 nm of **2** (10 μM) + partner **3** (10 μM) in a 5 mM Na phosphate buffer (pH 7.0, containing 2%, v/v, DMF) as a function of increasing concentrations of ODN-3/4 at 50 nM (red), 250 nM (yellow), 500 nM (orange), 1 μM (green) and 3 μM (light blue); λ_{ex} = 313 nm. The inset is a fluorescence image of solutions **2** + **3** in the absence (left) and presence (right) of ODN-3/4 (50 ng/μL) illuminated by using a 302 nm transilluminator. (c) The excitation spectra of **2** (10 μM) + partner **3** (10 μM) in a 5 mM Na phosphate buffer (pH 7.0, containing 2%, v/v, DMF) in the presence of ODN-3/4 (50 nM–3 μM); λ_{em} = 463 nm. (d) The fluorescence intensity at 463 nm of conjugate **2** (10 μM) in the absence or presence of partner **3** (10 μM), ODN-3/4 (3 μM) and ODN-5/6 (3 μM); λ_{ex} = 313 nm.

ylformamide. 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), t (triplet), qu (quintet), m (multiplet), br (broad). Electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS) was performed on BioTOF II (Bruker Daltonics) mass spectrometers. DNA oligonucleotides were purchased from Sigma–Aldrich Co. UV–visible spectra were measured with a NanoDrop spectrometer (NanoDrop Technologies). Fluorescence measurements were performed on Spectrofluorometer FP-6300 (JASCO). Fluorescence quantum yields in degassed solvent were determined by using perylene[25] as a standard with a known quantum yield in EtOH of 0.92 at room temperature. All Py–Im polyamide syntheses were performed on manual at a 0.10 mmol scale (250 mg of CLEAR resin, 0.4 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 3.0 mL/min, appropriate gradient elution conditions, and detection at 254 nm.

5.2. AcPyPy^{propynyl}-β-PyPy-β-Dp (1)

Fmoc-β-Ala-CLEAR-acid resin (250 mg, 0.10 mmol) was swollen in 2 mL of DMF in a 50-mL plastic centrifuge tube for 30 min. The solid-phase synthesis was followed by manual using the established procedure. The reactions were performed using Fmoc deprotection steps of 10 min by 20% piperidine in DMF (25 °C), and coupling steps of 60 min by corresponding Fmoc-carboxylic acid (0.4 mmol), HCTU (0.4 mmol), $^{i}Pr_2NEt$ (0.4 mmol) in DMF (25 °C), and a capping step of 30 min by 20% Ac_2O, 4-dimethylaminopyridine in DMF (25 °C). All couplings were carried out with single-couple cycles. After the completion of the solid-phase synthesis, the resin was washed with DMF, methanol, and dichloromethane, respectively, and dried *in vacuo*. The dried resin was then placed in a 10-mL glass scintillation vial, 2 mL of *N,N*-(dimethylamino)propylamine was added, and the solution was stirred at 55 °C overnight. The resin was removed by filtration through a Celite, and washed thoroughly with methanol, DMF. The filtrates was concentrated *in vacuo*, and triturated by diethyl ether. The dried crude **1** was used in the next step without further purification. Product **1** was analyzed after the further purification by HPLC using a Chemcobond 5-ODS-H column (0.1% AcOH/CH_3CN 10–50% linear gradient, 0–20 min). ^{1}H NMR (400 MHz, DMSO-d_6) δ9.95 (s, 2H; NH), 9.89 (s, 1H; NH), 9.85 (s, 1H; NH), 8.23 (brs, 1H; NH), 8.02 (brs, 1H; NH), 7.90 (brs, 1H; NH), 7.43 (s, 1H; Py-H), 7.28 (s, 1H; Py-H), 7.22 (s, 1H; Py-H), 7.18 (s, 1H; Py-H), 6.93 (s, 2H; Py-H), 6.89 (s, 1H; Py-H), 6.84 (s, 1H; Py-H), 5.28 (s, 2H; propynyl-CH_2), 3.86 (s, 3H; Py-CH_3), 3.83 (s, 3H; Py-CH_3), 3.78 (s, 3H; Py-CH_3), 3.48 (m, 4H; CH_2), 3.09 (m, 2H; CH_2), 2.71 (s, 1H; propynyl-CH), 2.54 (2H, eclipsed by DMSO), 2.36 (m, 2H; CH_2), 2.21 (t, J = 7.0 Hz, 2H, NCH_2), 2.12 (s, 6H; NCH_3), 2.01 (s, 3H; $COCH_3$), 1.54 (qu, J = 7.0 Hz, 2H, CH_2); ESI-TOF-MS: m/z calcd for $C_{39}H_{51}N_{12}O_7$: $[M+H]^+$ 799.40. Found: 799.56.

5.3. AcPyPy^{propynylperylene}-β-PyPy-β-Dp (2)

To a solution of crude polyamide **1** (20 μmol) in triethylamine–DMF (2:1, 900 μL), 3-bromoperylene (10.0 mg, 30 μmol), $Pd(PPh_3)_4$ (2.3 mg, 2.0 μmol), and CuI (0.8 mg, 4.0 μmol) were added. The reaction mixture was stirred at 80 °C for 6 h under argon gas. The solvent was removed *in vacuo*. The residue was washed with diethyl ether. After the further purification by HPLC using a Chemcobond 5-ODS-H column (0.1% AcOH/CH_3CN 30–70% linear gradient, 0–40 min), to give the perylene-modified polyamide **2** (0.2 mg, 1.1% yield for seven steps) as a yellow powder. ESI-TOF-MS: m/z calcd for $C_{59}H_{61}N_{12}O_7$: $[M+H]^+$ 1049.48. Found: 1049.73.

5.4. AcImImIm-β-Py-β-Dp (3)

Compound **3** was prepared using a synthetic procedure similar to that used for the preparation of compound **1**. The crude was purified by HPLC using a Chemcobond 5-ODS-H column (0.1% AcOH/CH_3CN 10–50% linear gradient, 0–20 min), to give the partner polyamide **3**. ^{1}H NMR (400 MHz, DMSO-d_6) δ10.44 (s, 1H; NH), 9.95 (s, 1H; NH), 9.76 (s, 1H; NH), 9.65 (s, 1H; NH), 8.23 (brs, 1H; NH), 8.05 (brs, 1H; NH), 7.90 (brs, 1H; NH), 7.68 (s, 1H; Im-H), 7.57 (s, 1H; Im-H), 7.54 (s, 2H; Im-H), 7.17 (s, 1H; Py-H), 6.69 (s, 1H; Py-H), 4.05 (s, 3H; Im-CH_3), 4.01 (s, 6H; Im-CH_3), 3.83 (s, 3H; Py-CH_3), 3.55 (m, 4H; CH_2), 3.07 (m, 2H; CH_2), 2.54 (2H, eclipsed by DMSO), 2.34 (s, 2H; CH_2), 2.21 (s, 2H, NCH_2), 2.12 (s, 6H; NCH_3), 2.08 (s, 3H; $COCH_3$), 1.53 (brs, 2H, CH_2); ESI-TOF-MS m/z calcd for $C_{34}H_{48}N_{15}O_7$: $[M+H]^+$ 778.39. Found: 778.59.

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