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Design of a New Fluorescent Probe: Pyrrole/Imidazole Hairpin Polyamides with Pyrene Conjugation at their γ-Turn
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Design of a New Fluorescent Probe: Pyrrole/Imidazole Hairpin Polyamides with Pyrene Conjugation at their γ-Turn

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Fluorophores that are conjugated with N-methylpyrrole-N-methylimidazole (Py–Im) polyamides postulates versatile applications in biological and physicochemical studies. Here, we show the design and synthesis of new types of pyrene-conjugated hairpin Py–Im polyamides (I–5). We evaluated the steady state fluorescence of the synthesized conjugates (I–5) in the presence and absence of oligodeoxynucleotides 5'-CGATATGACTCGG-3' (ODN 1) and 5'-CCGAGTCCATACG-3' (ODN 2) and observed a distinct increase in emission at 386 nm with conjugates 4 and 5. Notably, conjugate 5 that contains a β-alanine linker had a stronger binding affinity (K_D = 1.73 × 10^{-5} M) than that of conjugate 4 (K_D = 1.74 × 10^{-6} M). Our data suggests that Py–Im polyamides containing pyrene fluorophore with a β-alanine linker at the γ-turn NH2 position can be developed as the competent fluorescent DNA-binding probes.

1. Introduction

In recent years, development of methods to diagnose the hereditary diseases in their early stages is of increasing demand. DNA carries the genetic information specifying the structure and function of the cells across generations. Hence, diagnostic molecules that could directly detect specific double-stranded DNA (dsDNA) sequences obviating the need for denaturation, hybridization, washing, or labeling of the DNA samples have been gaining immense interest owing to their potential clinical applications. Accordingly, molecular beacons and DNA-binding molecules have been demonstrated as powerful tools to probe for the specific DNA sequences.

Among the DNA-binding molecules, N-methylpyrrole (Py)-N-methylimidazole (Im) polyamides have been extensively studied for their ability to recognize each of the four Watson–Crick base pairs. Moreover, Py–Im polyamides could be altered to achieve sequence-specific DNA bindings and could be conjugated to a wide variety of functional groups. Accordingly, Dervan and coworkers explored Py–Im polyamide–fluorophore conjugates and novel fluorescent scaffolds of Py–Im polyamides without dye molecules as fluorescent probes. We have also been developing various types of sequence-specific alkylating Py–Im polyamides and have investigated their chemical and biological properties.

In addition to such basic applications pertaining to gene regulation, the sequence-specific binding properties of Py–Im polyamides have been exploited to detect precise sequences of duplex DNA for potential diagnostic applications. Previously, we have demonstrated recognition of the target and CAG-repeated DNA sequences by Py–Im polyamide-pyrene and perylene fluorophores. Pyrene-based fluorophores are attractive molecules that could be developed into efficient DNA binding probes. Because it is known that pyrene-based fluorescence exhibits large extinction coefficients, excellent quantum yields, and good stability in aqueous solution.

Design and synthesis of novel Py–Im conjugates that causes notable shift in the fluorescence upon binding to the minor groove of duplex DNA could lead to the development of effective sequence-specific fluorescence-based probe(s). Here, we report the molecular design of new types of pyrene-based hairpin Py–Im conjugates I–5, synthesized by Fmoc solid-phase methods. Also, through studies with the steady state fluorescence we suggest the vital role of β-alanine linker at the γ-turn NH2 position in improving the DNA binding affinity. Our data could be used in the field of chemical biosensors for dsDNA.

2. Results and discussion

2.1. Synthesis of pyrene conjugates (I–5)

The pyrene-conjugated Py–Im polyamides 1–5 were synthesized following the previously reported procedures (Figure 1). The corresponding Py–Im polyamide units were synthesized using an Fmoc solid-phase synthesis method using HCTU as the coupling reagent. For polyamide 9, R-Boc-Dab(Fmoc)-OH was used instead of γ-butyric acid at the position of the γ-turn. On completion of the solid-phase synthesis, Boc-protected polyamides 6, 7, and 9 were obtained by cleaving from their corresponding β-wang resins using aminolysis with N,N-

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dimethyl-1,3-propanediamine (Dp). 8 was obtained by treatment with methyl (aminopropyl)amine. The desired conjugates 1–4 were produced by the deprotection of Boc with TFA and subsequent coupling with 1-pyrene butyric acid N-hydroxy succinimide ester (Pyrb-OSu). Conjugate 5 was obtained by coupling with Pyb-β-CO₂H, which was synthesized by the coupling of Pyb-OSu with ethyl β-alanine, followed by hydrolysis. HPLC purification yielded the polyamide conjugates 1–5 as pale-yellow powders with >95% purity. The purity and identity of pyrene conjugates 1–5 were verified by reversed-phase analytical HPLC and electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS).

2.2. Fluorescence emission of conjugates (1–5) in the presence of ODNs 1/2

To demonstrate the ability of the synthesized conjugates 1–5 to precisely bind to the target sequences, their fluorescence emission was examined in both the absence (I) and presence (II) of 13-mer DNA: 5′-CGTATGGACTCGG-3′ (ODN 1) and 5′-CCGAGTCCATACG-3′ (ODN 2), as shown in Figure 2. Conjugates 1 and 2 showed about a threefold increase in fluorescence intensity emission centered at ~430 nm in the presence of ODNs 1/2 (Figure 2a, Dark blue and Orange lines respectively). DNA interaction of the pyrene moiety located at the N-terminus position of conjugates 1 and 2 may have caused this increase in emission. The increase in fluorescence emission with a bathochromic shift of ~45 nm of conjugates 1 and 2 might have been caused by the aqueous phase charge/electron transfer from pyrrole to pyrene. Hence, conjugates 1 and 2 with an incorporation of the pyrene fluorophore at the N-terminal position did not have suitable properties to act as DNA-binding probes. In contrast, a weak increase in fluorescence emission by using conjugate 3 was observed at 385 nm in the presence of ODNs 1 and 2 (Figure 2a, Red line).

To design Py-Im polyamides as efficient fluorescent probes, pyrene moieties were attached to the NH₂ of the γ-turn. Interestingly, FITC and BoFl-polyamides linked at the γ-turn were reported to have better nuclear uptake profiles than C-tail-linked polyamides.²² In our results, conjugate 4 showed ~7-fold increase in fluorescence emission compared with 5, which showed ~4-fold increase in fluorescence emission (Figure 2b, Red and Green lines respectively). It is important to note here that Py-Im polyamides conjugated with the fluorophores such as Bis-Pyrene, Perylene, Tetramethyl rhodamine and Thiazole orange were known to have an extremely weak or almost no fluorescence emission in the presence of the mismatched ODNs even at higher concentrations.¹, ⁹, ¹⁶-¹⁸ Hence, it is reasonable to assume that our conjugates (1-5) might have poorer binding affinity with mismatched ODNs and give weak fluorescence emission.

2.3. Binding affinity of the conjugates (4–5)

To acquire additional insight into the interactions of conjugates 4 and 5 with target binding sequences, their binding affinities were investigated using SPR methods and biotinylated hairpin DNA (Figures 3a and 3b). The dissociation equilibrium constant (Kₒ) was obtained by fitting the resulting sensograms.
the potential utility of conjugate 5 as a fluorescent probe to recognize specific DNA sequences.

3. Conclusions

Cell permeable and nuclear localizing hairpin Py-Im polyamides have been successfully employed in gene regulation to selectively switch ‘On’ and ‘OFF’ the gene(s) of interest. Biologically active hairpin Py-Im polyamides could also be employed to accumulate a huge wealth of biological information when they could be conjugated with effective fluorescent dyes. Pyrene is known to possess unique spectral properties that have been exploited in wide range of biological applications. Hence, we have conjugated our hairpin polyamides with pyrene to synthesize a new type of pyrene-conjugated hairpin Py-Im polyamide (1–5) using Fmoc solid-phase synthesis. Steady state fluorescence studies carried out to investigate the photophysical properties of the conjugates 1–5 showed that the fluorescence emission of Py–Im polyamides (possessing a pyrenylbutyl group at the γ-NH2 position in the hairpin) 4 and 5 is dramatically increased in the presence of ODNs 1 and 2. This result clearly demonstrates that the Py and Im moieties present in the polyamide successfully induce the pyrene moiety to generate fluorescence emissions. Also, it is shown that the presence of β-alanine linker in conjugate 5 could enable its good affinity for binding with target sequences. Our results suggest the critical role of γ-turn position and β-alanine linker in improving the sequence-specificity which encourages us to explore and develop compounds harboring them as a new type of DNA minor-groove binding molecule. Efficient recognition of the selective sequences could contribute to the development of potent diagnostic tools for detecting genetic diseases.

4. Experimental

4.1. Materials

Reagents and solvents were purchased from standard suppliers and used without further purification. Abbreviations of some reagents: DMF, N,N-dimethylformamide; DCM, dichloromethane; TFA, trifluoroacetic acid, CH, CN, acetonitrile, PyBOP, (Benzotriazol-1-yl)-oxytripyrrolidinophosphonium hexafluorophosphate; DIEA, disopropylethylamine; Pyb-OSu, 1-Pyrenebutyric acid N-hydroxysuccinimide ester. Pyb-β-CO-H was synthesized by the coupling with Pyb-OSu and Ethyl β-alanine then hydrolysis. 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. Fluorescence measurements were performed on Spectrofluorometer FP-6300 (JASCO). All machine-assisted polyamide syntheses were performed on PSSM-8 peptide synthesizer (Shimadzu) with a computer-assisted operation system. HPLC purification was performed with a Chemcobond 5-ODS-H reversed-phase column (10 × 150 mm) in 0.1% TFA with acetonitrile as eluent at a flow rate of 1.0 mL/min, appropriate gradient elution conditions, and detection at 254 nm.

4.2. Synthesis of Conjugates (1–5)

**Pyrb-(β)-ImImPyPy-γ-ImPyPyPyβ-Dp** [1 (n=1) and 2 (n=2)]: Boc-(β)-ImImPyPy-γ-ImPyPyPyβ-wang resin (n=1 and 2) were programmed to synthesize in a stepwise reaction by Fmoc solid-phase protocol. Each resin was cleaved with 500 μL of Dp at 55 °C for 4 h. The resin was removed by filtration,
washed with DCM, concentrated in vacuo, and then triturated with diethyl ether. These crude Boc-protected PI polyamides 6, 7 were used in the next step without further purification. After deprotection of Boc with TFA, the crude polyamides were coupled with Pyrb-OSu and DIEA in DMF at room temperature to yield pyrene conjugates 1 and 2 and were purified by HPLC using a Chemocobond 5-ODS-H column (0.1% TFA/CH₂CN 0-100% linear gradient, 0-20 min, 254 nm). 1: ESI-TOF-MS (positive) m/z calcd for C₆H₄O₂N₅O₁₂ [M+2H]⁺ 790.36; found 790.38. 2: ESI-TOF-MS (positive) m/z calcd for C₆H₄O₂N₅O₁₂ [M+2H]⁺ 825.88; found 825.90.

AcImPyPyγIm-PyPy-β-Dp-Pyrb (3): AcImPyPyγIm-PyPy-β-wang resin was programmed to synthesize in a stepwise reaction by Fmoc solid-phase protocol. The resin was cleaved with 500 μL of methyl di(aminopropyl)amine at 55 °C for 4 h. The resin was removed by filtration, washed with DCM, concentrated in vacuo, and then triturated with diethyl ether and DCM. The crude polyamide 8 was used in the next step without further purification. The crude polyamide was coupled with Pyrb-OSu and DIEA in DMF at room temperature to yield pyrene conjugate 3, was purified by HPLC using a Chemocobond 5-ODS-H column (0.1% TFA/CH₂CN 0-100% linear gradient, 0-20 min, 254 nm). 3: ESI-TOF-MS (positive) m/z calcd for C₄₃H₄O₄N₂₃O₁₂ [M+2H]⁺ 797.38; found 797.45.

AcImPyPyγ{Pyrb-(β)}ₐ₋ₜ₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋･･･...


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References and notes