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Synthesis and optical properties of pyrrolidinyl peptide nucleic acid bearing a base discriminating fluorescence nucleobase 8-(pyrene-1-yl)-ethynyladenine

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ABSTRACT

A combination of fluorophore and nucleobase through a π-conjugated rigid linker integrates the base pairing and the fluorescence change into a single event. Such base discriminating fluorophore can change its fluorescence as a direct response to the base pairing event and therefore have advantages over tethered labels or base surrogates lacking the hydrogen-bonding ability. 8-(Pyrene-1-yl)ethynyl-adenine (A^{PyE}) has been extensively used as fluorescence labels in DNA and LNA, but it showed little discrimination between different nucleobases. Herein we investigated the synthesis, base pairing ability and optical properties of A^{PyE} in pyrrolidinyl peptide nucleic acid – a DNA mimic that shows much stronger affinity and specificity towards DNA than natural oligonucleotides. The A^{PyE} in PNA pairs specifically with thymine in the DNA strand, and resulted in 1.5–5.2-fold enhanced and blue-shifted fluorescence emission. Fluorescence quenching was observed in the presence of mismatched base or abasic site directly opposite to the A^{PyE}. The behavior of A^{PyE} in acpcPNA is distinctively different from DNA whereby a fluorescence was increased selectively upon duplex formation with complementary DNA and therefore emphasizing the unique advantages of using PNA as alternative oligonucleotide probes. Applications as color-shifting probe for detection of trinucleotide repeats in DNA were demonstrated, and the performance of the probe was further improved by combination with reduced graphene oxide as an external nanoquencher.

1. Introduction

Self-reporting fluorescence oligonucleotide probes have been of considerable interest due to their potential applications for direct nucleic acid sequence analysis in homogeneous format without requiring washing or additional treatment step,[1] which are essential features for cellular applications or in vivo experiments.[2] A number of strategies have been developed in order to design probes that can change their fluorescence upon hybridization with the correct nucleic acid targets. One notable example is molecular beacons,[3] which are long strands of oligonucleotide labeled at one end with a fluorophore and the other end with a quencher. Classical molecular beacons were designed to have a partial self-complementary region so that they could form a hairpin structure. The fluorophore and the quencher were forced to intimately contact and thus the fluorescence was effectively quenched in the free state. Binding of the target at the loop region destroyed the original hairpin structure, resulting in separation of the fluorophore and quencher, and a fluorescence increases. Many variations of molecular beacon design have been reported to improve certain properties such as the difficulty in design and synthesis as well as the trade-off between the background fluorescence signal and the kinetics/thermodynamics of the hybridization. Employing graphene oxide (GO) as an external quencher allows the use of singly-labeled linear oligonucleotide probes as a simplified molecular beacon.[4–6] Alternatively, the linear probe may carry a single fluorophore that is responsive to microenvironment change such as pyrene (polarity)[7] or thiazole orange (intercalation/groove binding).[8–10] Multiple fluorophores may be used in combination to improve the performance of these linear probes further.[11–13] The fluorophore and nucleobase may be combined to form a base discriminating fluorophore that integrates the base pairing and the fluorescence change in one single entity thereby enabling precise control of the location and orientation of the fluorophore,[14] and also reducing non-specific fluorescence response often observed when using tethered labels or base surrogates lacking the hydrogen-bonding ability.[15]

Several pyrene-modified nucleobases have been successfully incorporated into oligonucleotides and their utilities as fluorescence probe have been demonstrated.[16] The performances of these

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linear probes bearing pyrene-modified nucleobases are variable, depending on the design. DNA and LNA containing pyrimidine bases modified at C5 position with ethynylpyrene show excellent base discrimination properties and fluorescence response to the base pairing.[17–20] On the other hand, the analogous C8 modification of purine bases often gave low affinity and non-specific binding. These modified purine bases tend to adopt a *syn* conformation that allows partial stacking of the hydrophobic pyrene in the DNA·DNA duplex, thereby reducing the affinity and specificity of its pairing with base T.[21,22] Hence the utilization of the fluorescence nucleobase 8-(pyrene-1-yl)ethynyl-adenine (abbreviated as A^{PyE}) as base discriminating fluorescence nucleobase was limited to excimer or exciplex formation between two pyrene-labeled nucleobases[23–25] or between A^{PyE} and other nucleobases,[26,27] and thus its pairing ability has not been fully utilized. Moreover, the DNA carrying multiple A^{PyE} can form self-aggregates that may further complicate the probe design.[28,29]

The modest binding affinity and limited biological stability of DNA and RNA oligonucleotides prompted the development of alternative oligonucleotide probes.[30] Peptide nucleic acid (PNA) is one of the most interesting DNA analogues since it shows stronger binding affinity and greater base discriminating ability (i.e. higher specificity) than natural oligonucleotides.[31] In addition, PNA also possesses several unique features not observed in other DNA analogues such as the independency of hybrid stability to ionic strength, the ability to directly bind to dsDNA by duplex or triplex invasions and the complete stability to nucleases. Several linear fluorescence PNA probes have been developed and they often show better performances than the corresponding linear DNA probes.[32–35] Along this line, we have introduced a new class of conformational constrained pyrrolidinyl peptide nucleic acid showing even better base discrimination ability than the original PNA and have demonstrated their potential applications in various areas,[36] most notably as a self-reporting probe for DNA sequence determination.[37–39]

It was proposed that the stronger and higher base pairing specificity of PNA might overcome the low base discriminating ability of A^{PyE} observed in the DNA stated above. Herein, we aim to incorporate the fluorescence nucleobase A^{PyE} into pyrrolidinyl peptide nucleic acid bearing prolyl-2-aminocyclopentanecarboxylic acid backbone (acpcPNA)[40,41] and investigate its base pairing as well as optical properties in order to evaluate the potential of using A^{PyE}-modified acpcPNA as self-reporting fluorescence probes for DNA sequence analysis.

2. Results and discussion

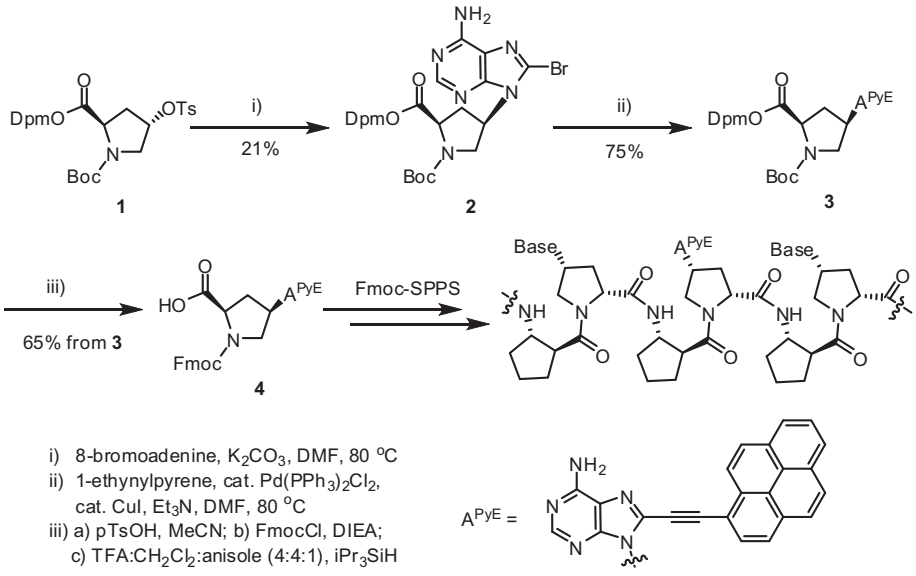
2.1. Synthesis of ethynylpyrene-labeled adenine (A^{PyE}) acpcPNA monomer and acpcPNA carrying A^{PyE} base

The Fmoc-protected ethynylpyrene-labeled adenine acpcPNA monomer **4** was synthesized in 48% yield over 4 steps from 8-bromoadenine precursor **2** and 1-ethynylpyrene via Sonogashira cross-coupling reaction following a protocol previously developed for DNA[18] as shown in Scheme 1. The intermediate 8-bromoadenine derivative **2** was obtained from the reaction between 8-bromoadenine[42] and tosylate **1**[43] in 21% yield.

Eleven acpcPNA sequences carrying one or two A^{PyE} bases were synthesized following the standard pyrrolidinyl PNA synthesis protocol.[40,41] These include a test sequence with one or two (adjacent and separated) internal A^{PyE} units [**T4(A^{PyE})T4**, **T8(A^{PyE})2_0B**, **T8(A^{PyE})2_3B**], mix base 12mer sequences carrying one A^{PyE} unit with different neighboring nucleobases [**M12T(A^{PyE})T**, **M12A(A^{PyE})A**, **M12G(A^{PyE})G**, **M12C(A^{PyE})C**], a mix base 12mer sequence with terminal A^{PyE} base (**A^{PyE}M12**), two mix base 10mer sequences [**M10A(A^{PyE})C** and **M10G(A^{PyE})T**], and a 12mer probe for detection of trinucleotide repeats [**CAG(A^{PyE})**]. All PNA sequences were purified by reverse phase HPLC to >90% purity and their identities were confirmed by MALDI-TOF mass spectrometry (Table 1).

2.2. DNA binding and optical properties of A^{PyE} acpcPNA

Thermal stability data of hybrids between the model A^{PyE}-containing acpcPNA sequence **T4(A^{PyE})T4** and DNA are shown by T_m curves in Fig. 1A. In case of complementary DNA, the hybrid was almost as stable as the unmodified acpcPNA T_4AT_4 (T_m = 61.6 °C vs 66.8 °C).[41] Other mismatched PNA-DNA hybrids showed much decreased T_m (mismatched G: 28.3; mismatched C: 35.0; mismatched A: 36.4 °C) (Fig. 1A). The data suggest that the base pairing of A^{PyE} with thymine is somewhat less stable than unmodified adenine, presumably due to steric effects of the ethynylpyrene substituent. Nevertheless, the specificity in recognition of base T is fully retained as shown by the large decrease of T_m (−25.2 to



Scheme 1. Synthesis of ethynylpyrene-labeled adenine (A^{PyE}) acpcPNA monomer (**4**) and the structure of acpcPNA bearing A^{PyE} base.