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Pyrrolidinyl peptide nucleic acid terminally labeled with fluorophore and end-stacking quencher as a probe for highly specific DNA sequence discrimination☆

Nattawut Yotapan, Duangrat Nim-anussornkul, Tirayut Vilaivan *

Organic Synthesis Research Unit, Department of Chemistry, Faculty of Science, Chulalongkorn University, Phayathai Road, Patumwan, Bangkok 10330, Thailand

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ABSTRACT

Peptide nucleic acid (PNA) labeled with a dye/quencher pair is useful as a high-performance fluorescence probe for DNA sequence determination. Previous work has mainly focused on placing the dye/quencher at different ends of the PNA strand. In this work, we propose a new pyrrolidinyl PNA probe bearing a fluorescent dye and an anthraquinone quencher at the same end of the PNA molecule. The single-stranded PNA probe exhibits a weak fluorescence as a result of close contact between the dye and the quencher. End-stacking of the anthraquinone to the terminal base pair of the PNA·DNA duplex disrupts the fluorophore-quencher interaction, resulting in a large fluorescence increase in the presence of complementary DNA. The high specificity of the present PNA probe allows multicolor, multiplex detection of single mismatched DNA targets. In addition, direct detection of double stranded DNA target by double duplex invasion was demonstrated.

1. Introduction

Fluorescence oligonucleotides are useful as probes for molecular diagnostic applications such as the determination of DNA or RNA sequences[1,2] as well as other targets such as proteins[3,4] and small molecules.[5–7] To enable the detection of these targets in homogeneous format which does not require immobilization or washing and thus applicable for in vivo detection, a mechanism that can modulate the fluorescence properties of the probe in response to the target binding must be introduced. A classic example of such probes is molecular beacon[8]—an oligonucleotide carrying two labels at the opposite end with a partial self-complementary sequence so that it can form a stem-loop structure in the free state. Binding with the correct target DNA at the loop region leads to a conformational change that result in different interactions between the two labels when compared to the free probes,[9] leading to a fluorescence change. Limitations of this probe design are the requirement of a rather long sequence to form the stem-loop structure which is expensive to make, slow reaction kinetics and further functionalization being difficult since both ends are occupied. Several alternative designs to overcome one or more these limitations have been proposed such as in-stem molecular beacons,[10,11] strand displacement probes,[12] binary probes,[13] and quencher-free molecular beacons.[14,15] The performance of these probes could be improved further by the use of oligonucleotide analogues that offer stronger binding affinity/specificity such as locked nucleic acid (LNA) or peptide nucleic acid (PNA).[16] The latter is particularly promising because the design of PNA beacons does not generally require the stem-loop structure.[17,18] The potential problem of non-specific binding in unstructured probes[19] is compensated by the high specificity of PNA probes, and thus several impressive examples of linear PNA beacons have been reported.[20,21] Most of these PNA beacons carry either an environment sensitive label or two labels placed at different positions in the PNA strand.

Linear oligonucleotide probes bearing two labels attached to the same end or adjacent positions have been reported as early as 1994.[22] Instead of relying on conformational change as in classical molecular beacons, the mechanisms of fluorescence change in these linear probes upon hybridization with the target was due to the change in the interactions between the two labels as a result of end stacking, intercalation,[23–25] or uncoupling of excitons.[26,27] This probe design tends to give a lower background signal than linear DNA probes in general due to the more efficient quenching as a results of a better contact between the dye and the quencher. Inspired by this promising probe design and our long interest in the development of self-reporting PNA probes,[28–30] we proposed to

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* Corresponding author. E-mail address: vtirayut@chula.ac.th (T. Vilaivan).

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combine the concept of a doubly-end-labeled linear probe with our new conformationally constrained acpcPNA.[31] In this report, we synthesize and evaluate the performance for DNA detection of some doubly-end-labeled linear PNA probes bearing a fluorophore (FAM or TMR) and a quencher with end-stacking ability (anthraquinone) at the same end of the molecule. The anthraquinone is expected to stack on top of terminal base pairs of the PNA·DNA duplexes. As a result, hybridization with the correct DNA target is expected to produce a signal by fluorescence change.

2. Results and discussion

2.1. Design and synthesis of doubly-end-labeled acpcPNA probes

The present doubly-end-labeled PNA probe design consists of a fluorophore and a quencher (denoted X and Y). Both of which are attached to a diamine linker (lysine or APC) at the *N*-termini of the acpcPNA probes (Fig. 1). One or more lysine residues were incorporated at the *C*-termini to improve water solubility and stability of the PNA·DNA hybrids. Anthraquinone (AQ) was chosen as the quencher because of its known ability to intercalate and/or end-stack with DNA duplexes.[24] The previously observed increased in T_m of duplexes with terminally anthraquinone-labeled acpcPNA suggests that the end stacking should also be possible in acpcPNA·DNA duplexes.[32]

The strategy for end-labeling of acpcPNA with the fluorophore-quencher pair involves attachment of the orthogonally protected (Fmoc/Tfa) diamine linker (lysine or 3-amino-4-pyrrolidine carboxylic acid, APC) at the *N*-terminus of the PNA. The amino groups were selectively deprotected and modified with the label (FAM or TMR) or the quencher (AQ or Dab). The crude PNA was purified by reversed phase HPLC (to >90% purity) and characterized by MALDI-TOF mass spectrometry. Characterization data of all PNA probes, as well as selected thermal stability data of their DNA hybrids are shown in Table 1.

2.2. Fluorescence properties of acpcPNA probes bearing various end-labeling groups and liker chemistry

The performance of several end-labeled acpcPNA probe designs was first evaluated by measuring fluorescence spectra of the probes

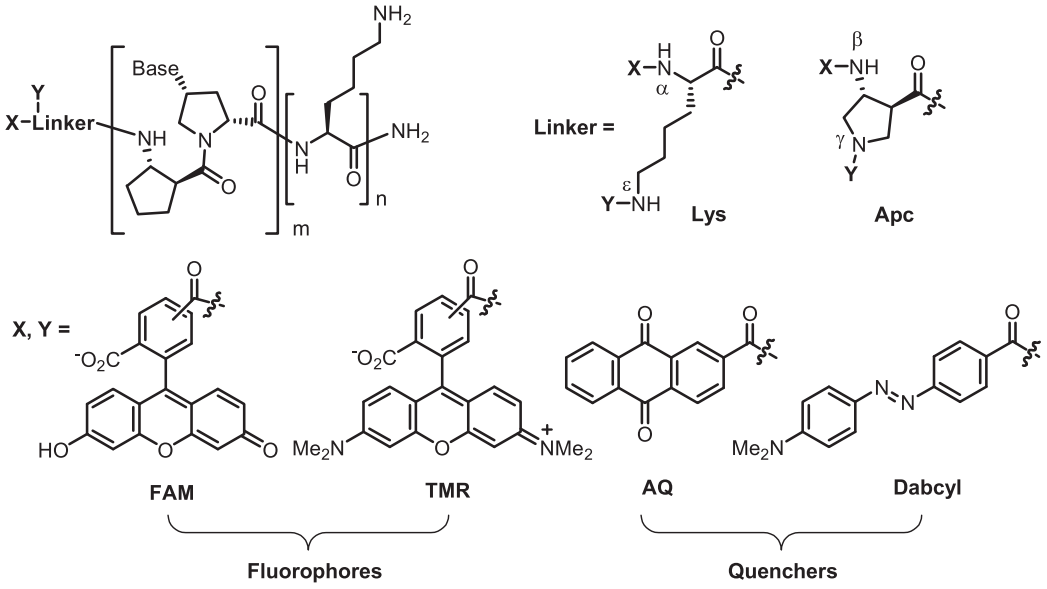

Fig. 1. Chemical structure of doubly-end-labeled acpcPNA probes described in this study.

Table 1
Sequence and characterization data of end-labeled acpcPNA probes synthesized in this study

PNA	Sequence (N→C)	t_R (min)[a]	Yield (%)[b]	*m/z* (calcd)[c]	*m/z* (found)[d]	T_m (°C)[e]
(AQ/FAM)Lys-P1	$N_{\varepsilon}^{AQ}N_{\alpha}^{FAM}$Lys-GTAGATCACT-LysNH$_2$	31.2	3.0	4239.5	4237.6	58
(AQ/TMR)Lys-P1	$N_{\varepsilon}^{AQ}N_{\alpha}^{TMR}$Lys-GTAGATCACT-LysNH$_2$	33.8, 34.3	8.3	4293.6	4294.6	55
(AQ/FAM)Apc-P1	$N_{\gamma}^{AQ}N_{\beta}^{FAM}$Apc-GTAGATCACT-LysNH$_2$	30.5	6.9	4111.3	4111.1	N.D.
(FAM/AQ)Lys-P1	$N_{\varepsilon}^{FAM}N_{\alpha}^{AQ}$Lys-GTAGATCACT-LysNH$_2$	30.8	6.8	4239.5	4237.6	N.D.
(FAM)Lys-P1	N_{α}^{FAM}Lys-GTAGATCACT-LysNH$_2$	31.2, 31.5	12.0	4004.2	4002.5	65
(Dab/FAM)Lys-P1	$N_{\varepsilon}^{Dab}N_{\alpha}^{FAM}$Lys-GTAGATCACT-LysNH$_2$	31.2	5.5	4256.5	4253.6	N.D.
(AQ/TMR)Lys-P2	$N_{\varepsilon}^{AQ}N_{\alpha}^{TMR}$Lys-GTACATCACT-LysNH$_2$	34.0	8.2	4253.6	4250.5	N.D.
(AQ/FAM)Lys-P3	$N_{\varepsilon}^{AQ}N_{\alpha}^{FAM}$Lys-TACAGACATC-LysNH$_2$	34.1, 34.3	9.1	4208.5	4205.9	50
(AQ/FAM)Lys-P4	$N_{\varepsilon}^{AQ}N_{\alpha}^{FAM}$Lys-CTAAATTCAGA-LysNH$_2$	34.4, 34.5	3.8	4564.9	4564.1	74
(AQ/FAM)Lys-P5	$N_{\varepsilon}^{AQ}N_{\alpha}^{FAM}$Lys-AGTTATCCCTGC-LysNH$_2$	34.5, 34.8	4.1	4865.2	4863.6	71
(AQ/FAM)Lys-P6	$N_{\varepsilon}^{AQ}N_{\alpha}^{FAM}$Lys-GAACAAGCTGGGAA-LysNH$_2$	33.1, 33.4	2.0	5671.0	5673.0	84
(AQ/FAM)Lys-P1-Lys5	$N_{\varepsilon}^{AQ}N_{\alpha}^{FAM}$Lys-GTAGATCACT-Lys$_5NH_2$	33.1, 33.5	5.4	4752.2	4752.7	66

[a] HPLC conditions: flow rate 0.5 mL/min, The gradient consists of two solvent systems: A (0.1% TFA in MilliQ water) and B (0.1% TFA in methanol). The elution started with 90:10 A:B for 5 min, followed by a linear gradient to 10:90 A:B over 35 min. Some peaks are doubled due to the presence of two fluorescein isomers from the commercially available 5(6)-carboxyfluorescein used.
[b] Isolated yield after HPLC.
[c] Average mass ($M+H^+$).
[d] MALDI-TOF.
[e] T_m with complementary DNA as determined by fluorescence spectrophotometry; conditions: 10 mM phosphate buffer pH 7.0; [PNA]=1.0 μM, [DNA]=1.2 μM, λ_{ex}=490 nm, λ_{em}=520 nm.