Available online at www.sciencedirect.com
SCIENCE DIRECT
Tetrahedron Letters
Tetrahedron Letters 47 (2006) 4037–4039

Quencher-free molecular beacon systems with two pyrene units in the stem region

Young Jun Seo, Gil Tae Hwang and Byeang Hyean Kim*

National Research Laboratory, Department of Chemistry, Division of Molecular and Life Sciences, Pohang University of Science and Technology, Pohang 790-784, Republic of Korea

Received 6 March 2006; revised 30 March 2006; accepted 4 April 2006
Available online 27 April 2006

Abstract—We appended pyrene units covalently onto adenosine and uridine nucleosides (forming $\mathbf{A^P}$ and $\mathbf{U^P}$ units, respectively) and then incorporated them into oligonucleotides such that they were positioned in complementary locations in opposite strands in the middle positions of hairpin stems. Systems **1** ($\mathbf{A^PU^P}$) and **3** ($\mathbf{A^PA^P}$) individually exhibit aromatic stacking between the opposing pyrene units in the stems of their hairpins and display in their spectra the photophysical properties of strongly red-shifted bands; in contrast, the $\mathbf{U^PU^P}$ system **2** exhibits quenching spectra. Systems **1** ($\mathbf{A^PU^P}$) and **3** ($\mathbf{A^PA^P}$) behave as effective molecular beacons (MBs) that change color from green to blue upon duplex formation, whereas **2** ($\mathbf{U^PU^P}$) is an effective MB that changes the intensity of its fluorescence upon forming its perfectly matched duplex.

Fluorescence biosensors are powerful tools for probing the structures and interactions of biomolecules, but a strong demand exists for increasingly more advanced biomolecular recognition probes.[1] Among the most useful probe systems for detecting molecular dynamics are the molecular beacons (MBs).[2] MBs are being used in a variety of biotechnology applications, for example, as biosensors,[3a–d] for monitoring enzymatic cleavage,[3e] and for monitoring of the polymerase chain reaction.[2a] We are interested in preparing chemically modified fluorescence biosensors, especially those exploiting π–π stacking interactions, that can also be used to investigate nucleic acid interactions and structures.[4] Recently, we reported that pyrene-labeled deoxyuracil and deoxyadenine units are novel unnatural nucleobases.[5] These fluorescent nucleobases exhibit a range of different emission intensities when they form duplexes with one another. In this letter, we describe how this property can be utilized in the preparation of quencher-free molecular beacon (MB) probes. These new types of MBs might be of use as probes in various biosensor applications.[6]

We synthesized pyrene-modified nucleoside building blocks through Sonogashira coupling of pyrene units to the 5-position of a uracil base [i.e., to form 5-(1-ethynylpyrenyl)-2′-deoxyuridine; $\mathbf{U^P}$] and to the 8-position of a 2′-deoxyadenosine base [i.e., to form 8-(1-ethynylpyrenyl)-2′-deoxyadenosine; $\mathbf{A^P}$].[7] The quantum yields of $\mathbf{U^P}$ and $\mathbf{A^P}$ are very high: 81% and 89% in $CHCl_3$ and 12% and 73% in MeOH,[8] respectively. We incorporated $\mathbf{U^P}$ and $\mathbf{A^P}$ into oligodeoxynucleotides (ODNs) by using the phosphoramidite method and the aid of a DNA synthesizer. We constructed three kinds of modified single-stranded ODNs (**1**–**3**) in which the two fluorophores were positioned in opposite strands of the stem of each hairpin. We also synthesized a perfectly matched sequence (**4**) and a one-base-mismatched sequence (**5**) for hybridizing to the loop regions of ODNs **1**–**3** (Fig. 1).

Table 1 shows the fluorescence data of **1** ($\mathbf{A^PU^P}$), **2** ($\mathbf{U^PU^P}$), and **3** ($\mathbf{A^PA^P}$). Interestingly, **1** (existing in its hairpin state) emits a band at $\lambda_{max} = 505$ nm, whereas the λ_{max} of the **1**·**4** duplex (in which **1** exists in an open state) occurs at 443 nm. We hypothesized in the previous study[5] that the strongly red-shifted band at 505 nm must arise from stacking interactions between the two pyrene units of $\mathbf{A^P}$ and $\mathbf{U^P}$, which would preclude hydrogen bonding from occurring between these bases.

To confirm that a red-shifted band of **1** arises from a state that does not feature hydrogen bonding between

Keywords: Fluorescence; Quencher-free molecular beacon; π–π stacking; Nucleotides.
* Corresponding author. Tel.: +82 54 279 2115; fax: +82 54 279 3399; e-mail: bhkim@postech.ac.kr

0040-4039/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tetlet.2006.04.002

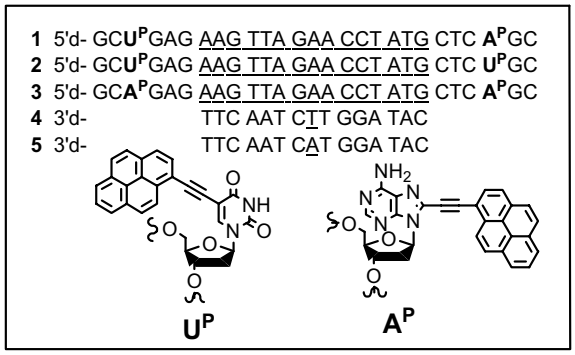

Figure 1. Designed fluorescent unnatural nucleobases and oligonucleotides.

its two pyrene-labeled bases, we analyzed the fluorescence spectra of ODNs **2** ($\mathbf{U^PU^P}$) and **3** ($\mathbf{A^PA^P}$), which are hairpin-mismatched systems that cannot possibly possess such hydrogen bonds. As expected, **3** also exhibits a strongly red-shifted band [$\lambda_{max} = 521$ nm]; in comparison, the value of λ_{max} of the duplex **3·4** (i.e., in which **3** exists in the open state) is 450 nm, which is different from that of pyrene. In contrast,**2** did not exhibit a strongly red-shifted band either alone [i.e., in a hairpin state: $\lambda_{max} = 435$ nm] or in the duplex **2·4** [i.e., in an open state: $\lambda_{max} = 430$ nm] (Fig. 2).

Unfortunately, we are unable to confirm whether the specific origin of this red-shifted band is a property of an exiplex or merely that of a dimer of the two pyrene units.[9]

We tested the potential application of our modified ODNs as stable optical probes that can be used as biosensors. Initially, our attention was focused on testing these systems as fluorophore probes that selectively sense complementary sequences. To apply these hairpins as target sequence-sensitive probes, it is very important that they be able to discriminate a difference between the stabilities of the duplexes of the matched and one-base-mismatched sequences in the hairpin loop.

From the data in Table 1, we find that the optimized range of temperatures for the performance of our MBs is between 33 and 48 °C. Thus, we chose a temperature of 37 °C for our MB experiments. Figure 3A indicates that at 37 °C the perfectly matched duplex **3·4** (blue color) is discriminated clearly from the corresponding one-base-mismatched duplex **3·5** (green color). Thus, this system can be used as an effective color-changing MB.

We also tested the hybridization properties of **2** ($\mathbf{U^PU^P}$) by means of fluorescence measurements (Fig. 4A). The

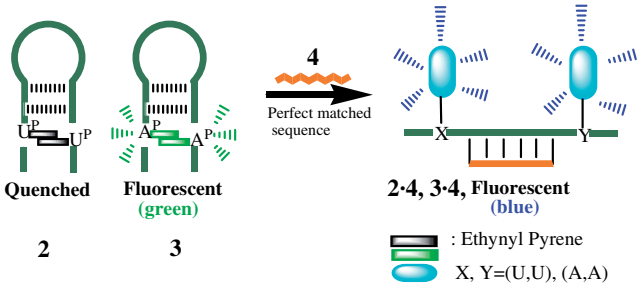

Figure 2. Designed quencher-free molecular beacon systems.

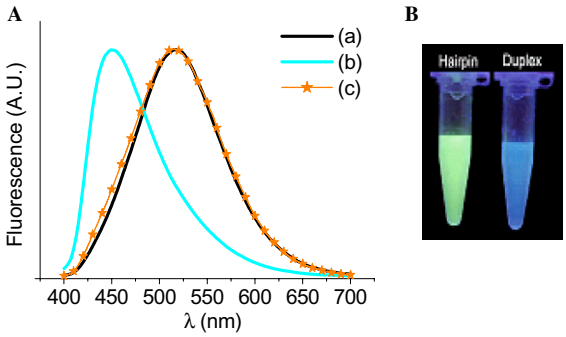

Figure 3. (A) Fluorescence spectra of (a) ODN **3** ($\lambda_{max} = 521$ nm), (b) the perfectly matched duplex **3·4** (443 nm), and (c) the one-base-mismatched duplex **3·5** (520 nm). (B) Photographic image of solutions of ODN **3** and the perfectly matched duplex **3·4**. Fluorescence spectroscopic data were recorded using 1.5 μM solutions in buffer (100 mM Tris–HCl/1 mM $MgCl_2$; pH 8) upon excitation at 386 nm after annealing at 80 °C, 30 min.

hybridization of ODN **2** with its perfectly matched ODN **4** led to a dramatic enhancement in its emission intensity, relative to that observed for the one-base-mismatched duplex **2·5**. Therefore, this $\mathbf{U^PU^P}$-based system can be used as an 'on/off' molecular beacon.

The photographic images in Figures 3B and 4B demonstrate dramatically that the naked eye can distinguish significant changes in both the color and intensity. We believe that such visual color change phenomena have great potential for determining the presence of perfectly matched sequences.

We used gel electrophoresis to confirm the operation of the MB (Fig. 5). The gel images indicate that ODN **3** does not bind with the one-base-mismatched strand **5** at 37 °C, but after addition of the perfectly matched strand **4**, the system forms the duplex **3·4** at 37 °C. Thus, we can discriminate the matched sequence from the mismatched sequence at 37 °C.

Table 1. Fluorescence spectroscopic data and thermal melting temperatures (T_m, °C) of ODNs

Hairpin sequence	λ_{max} (nm)[a]	λ_{max} (nm)[b]	T_m (°C)	Perfectly matched sequence	T_m (°C)	One-base-mismatched sequence	T_m (°C)
1	505	443	58	**1·4**	47	**1·5**	33
2	435	430	62	**2·4**	47	**2·5**	33
3	516	450	58	**3·4**	48	**3·5**	33

[a] Closed hairpin states of sequences **1**–**3**.

[b] Open states of sequences **1**–**3** formed in the presence of their respective matched sequences. Thermal melting temperatures (T_m) were determined from UV spectra using 1.5 μM solutions prepared in buffer (100 mM Tris–HCl/1 mM $MgCl_2$; pH 8). Fluorescence spectroscopic data were recorded using 1.5 μM solutions in buffer (100 mM Tris–HCl/1 mM $MgCl_2$; pH 8) upon excitation at 386 nm.

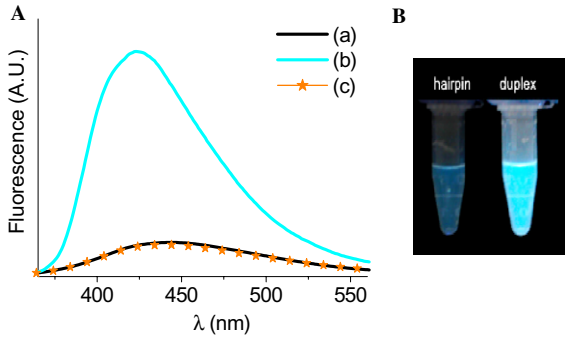

Figure 4. (A) Fluorescence spectra of (a) ODN **2** ($\lambda_{max} = 435$ nm), (b) the perfectly matched duplex **2**·**4** (430 nm), and (c) the one-base-mismatched duplex **2**·**5** (435 nm). (B) Photographic image of ODN **2** and the perfectly matched duplex **2**·**4**. All conditions were the same as those described in Figure 3, except that the systems were excited at 356 nm.

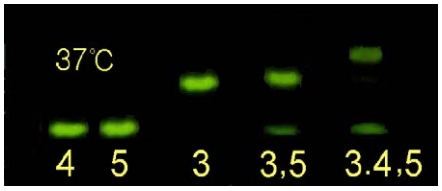

Figure 5. Gel electropherograms demonstrating the operation of the MB. ODN **3** is a hairpin strand. ODNs **4** and **5** are, respectively, the perfectly matched and one-base-mismatched single strands for the pyrene-modified ODN **3**. The state '**3**,**5**' is the hairpin state obtained after adding **5** into a solution of **3**. The state '**3**·**4**,**5**' contains the perfectly matched duplex **3**·**4**; it was obtained after adding **4** into solution '**3**,**5**' (see Supplementary data for details of sample preparation).

In conclusion, systems **1** ($\mathbf{A^PU^P}$) and **3** ($\mathbf{A^PA^P}$) individually exhibit aromatic stacking between the opposing pyrene units in the stems of their hairpins; their spectra display the characteristics of π-stacked pyrene units. In contrast, the $\mathbf{U^PU^P}$ system **2** exhibits quenching spectra. Systems **1** ($\mathbf{A^PU^P}$) and **3** ($\mathbf{A^PA^P}$) can be used as effective MBs that change color from green to blue upon duplex formation, whereas **2** ($\mathbf{U^PU^P}$) is an effective MB that operates through changes in the intensity of its fluorescence. These novel types of MBs are relatively simple to synthesize, and their termini remain free for the introduction of other useful functionalities.

Acknowledgments

We are grateful to KOSEF for financial support through the National Research Laboratory Program (Laboratory for Modified Nucleic Acid Systems), Gene Therapy R&D program (M10534000011-05N3400-01110), and KNRRC program.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2006.04.002.

References and notes

1. Mcdonald, D.; Vodicka, M. A.; Lucero, G.; Svitkins, T. M.; Borisy, G. G.; Emerman, M.; Hope, T. J. *J. Cell Biol.* **2002**, *159*, 441–452.
2. (a) Tyagi, S.; Bratu, D.; Kramer, F. R. *Nat. Biotechnol.* **1998**, *16*, 49–53; (b) Steemers, F. J.; Ferguson, J. A.; Walt, D. R. *Nat. Biotechnol* **2000**, *18*, 91–94(c) Li, J. J.; Geyer, R.; Tan, W. *Nucleic Acids Res.* **2000**, *28*, 1–5.
3. (a) Fang, X.; Liu, X.; Schuster, S.; Tan, W. *J. Am. Chem. Soc.* **1999**, *121*, 2921–2922; (b) Chen, W.; Martinez, G.; Mulchandani, A. *Anal. Biochem.* **2000**, *280*, 166–172; (c) Steemers, F. J.; Ferguson, J. A.; Walt, D. R. *Nat. Biotechnol.* **2000**, *18*, 91–94; (d) Li, J. J.; Fang, X.; Schuster, S. M.; Tan, W. *Angew. Chem., Int. Ed.* **2000**, *112*, 1049–1052; (e) Li, J. J.; Geyer, R.; Tan, W. *Nucleic Acids Res.* **2000**, *28*, 1–5.
4. (a) Guckian, K. M.; Schweitzer, B. A.; Ren, R. X.-F.; Sheils, C. J.; Paris, P. L.; Tahmassebi, D. C.; Kool, E. T. *J. Am. Chem. Soc.* **1996**, *118*, 8182–8183; (b) Paris, P. L.; Langenhan, J. M.; Kool, E. T. *Nucleic Acids Res.* **1998**, *26*, 3789–3793; (c) Matray, T. J.; Kool, E. T. *J. Am. Chem. Soc.* **1998**, *120*, 6191–6192.
5. Hwang, G. T.; Seo, Y. J.; Kim, B. H. *Tetrahedron Lett.* **2005**, *46*, 1475–1477.
6. (a) Okamoto, A.; Kanatani, K.; Saito, I. *J. Am. Chem. Soc.* **2004**, *126*, 4820–4827; (b) Wagner, C.; Rist, M.; Elke, M. E.; Wagenknecht, H. A. *Org. Biomol. Chem.* **2005**, *3*, 2062–2063; (c) Fujimoto, K.; Shimizu, H.; Inouye, M. *J. Org. Chem.* **2004**, *69*, 3271–3275.
7. (a) Hwang, G. T.; Son, H. S.; Ku, J. K.; Kim, B. H. *Org. Lett.* **2001**, *3*, 2469–2471; (b) Hwang, G. T.; Son, H. S.; Ku, J. K.; Kim, B. H. *J. Am. Chem. Soc.* **2003**, *125*, 11241–11248; (c) Hwang, G. T.; Seo, Y. J.; Kim, B. H. *J. Am. Chem. Soc.* **2004**, *126*, 6528–6529; (d) Hwang, G. T.; Seo, Y. J.; Kim, S. J.; Kim, B. H. *Tetrahedron Lett.* **2004**, *45*, 3543–3546; (e) Seo, Y. J.; Kim, B. H. *Chem. Commun.* **2006**, 150–152; (f) Seo, Y. J.; Ryu, J. H.; Kim, B. H. *Org. Lett.* **2005**, *7*, 4931–4933.
8. (a) Rist, M.; Amann, N.; Wagenknecht, H.-A. *Eur. J. Org. Chem.* **2003**, 2498–2504; (b) Netzel, T. L.; Zhao, M.; Nafisi, K.; Headrick, J.; Sigman, M. S.; Eaton, B. E. *J. Am. Chem. Soc.* **1995**, *117*, 9119–9128; (c) Amann, N.; Pandurski, E.; Fiebig, T.; Wagenknecht, H.-A. *Angew. Chem., Int. Ed.* **2002**, *41*, 2978–2980.
9. (a) Förster, Th. *Angew. Chem., Int. Ed. Engl.* **1969**, *8*, 333–343; (b) Kawai, T.; Ikegami, M.; Arai, T. *Chem. Commun.* **2004**, 824–825; (c) Fiebig, T.; Stock, K.; Lochbrunner, S.; Riedle, E. *Chem. Phys. Lett.* **2001**, *345*, 81–88; (d) Raytchev, M.; Pandurski, E.; Buchvarov, I.; Modrakowski, C.; Fiebig, T. *J. Phys. Chem. A* **2003**, *107*, 4592–4600.