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Bis-pyrene-labeled molecular beacon: A monomer–excimer switching probe for the detection of DNA base alteration

Kazushige Yamana,[a,c,*] Yoshikazu Ohshita,[a] Yudai Fukunaga,[a] Mitsunobu Nakamura[a] and Atsushi Maruyama[b,c]

[a]*Department of Materials Science and Chemistry, Graduate School of Engineering, University of Hyogo, 2167 Shosha, Himeji, Hyogo 671-2201, Japan*
[b]*Institute of Materials Chemistry and Engineering, Kyusyu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan*
[c]*CREST of JST, Japan*

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Abstract—A new bis-pyrene-labeled oligonucleotide probe (BP-probe) has been designed for the detection of a single base mismatch in single strand (ss) DNA as a target. The sequence of BP-probe was chosen to form stem-loop structure similar to a molecular beacon (MB-probe), yielding bis-pyrene-labeled molecular beacon (BP–MB-probe). Partially double stranded (ds) BP–MB-probes were prepared by complexation with oligonucleotides whose sequences are complementary to the loop segment but not to the stem and exchangeable with the target DNA. The partially ds BP–MB-probes were shown to exhibit monomer fluorescence as major fluorescence, while the ss BP–MB-probe in the stem-loop form displays strong excimer fluorescence. The strand exchange reactions between partially ds BP–MB-probe and target ss DNA in the presence of cationic comb-type copolymer as a catalyst were monitored by the excimer fluorescence changes. The existence of a mismatched base can be determined by the slower PASE rates compared with fully matched DNA.

1. Introduction

Fluorescence detection of a single base alteration in DNA using hybridization probes is the current subject of intense research, because it is important in many molecular genetics and diagnostics. Heterogeneous hybridization assays on DNA micro-array chips are widely used in large-scale DNA mutation detection.[1,2] Homogeneous mutation assays based on hybridization are an attractive alternative, and have advantages over heterogeneous assays such as fast hybridization kinetics and no washing steps. These advantages facilitate automation of the assays.

One possible method for homogeneous mutation assays is the use of molecular beacon probe (MB-probe) that displays the strong emission only upon binding to a target DNA sequence.[3–5] The multi-colored MB-probes have been used to detect DNA mismatch, where the mismatch discrimination is largely dependent on the *thermodynamic differences* in the probe hybridizations between fully matched and mismatched DNAs.[6–9] However, these differences are generally small and vary according to DNA sequence, which make the thermodynamic approach necessary to optimize hybridization conditions and probe design. The other approach is the use of an oligonucleotide containing a base-discriminating fluorescent nucleoside that binds to a target DNA.[10,11] Due to the fluorescence change in the hybridization of the probe to target DNA, the bases at the target site of the complementary strands can be fluorometrically read out. Although this approach should be promising, the appropriate design and synthesis of all four fluorescent nucleosides are necessary for the detection of mismatched bases at the target site of DNA.

In contrast to these hybridization assays, DNA mutation detection has been achieved by monitoring the *kinetic differences* in the strand exchange reactions be-

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* Corresponding author. Fax: +81 79 267 4895; e-mail: yamana@eng.u-hyogo.ac.jp

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tween a fluorescent DNA probe and target DNA.[12–14] In this mutation detection, a cationic comb-type copolymer (CCC) was found to be a good catalyst for acceleration of DNA strand exchange reactions (we term this particular reaction as PASE) and for differentiation of the kinetic barrier between a perfect match DNA and a mismatch-containing DNA. The existence of mismatches can therefore be determined from the different PASE rates compared with those for fully matched targets without careful optimization process.[12] In the initial assays, the probes labeled with a pair of FRET fluorophores were used to detect the PASE. Because the synthesis of FRET probes is tedious and costly, the development of superior and inexpensive probes has been a key issue in order to make the mismatch detection by PASEs more practical.

The pyrene excimer fluorescence has attracted much attention in the development of DNA and RNA probes.[15–25] We have recently disclosed a 5′-bis-pyrene-labeled oligonucleotide probe (BP-probe).[26] This probe can be prepared by using a 2,2-bis-pyrene-modified-1,3-propanediolphosphoramidite in an automated DNA synthesizer. The important characteristics of the BP-probes are that they exhibit the weak monomer fluorescence in the single strand form, while in the duplex structure, the strong excimer emission becomes a major fluorescence. The single strand (ss) BP-probes therefore provided a useful tool for monitoring the PASE between the probe and ds target DNA.[27] By the use of the probe sequence exchangeable to the ds target DNA, a single base mismatch can be detected from the faster PASE rates for a mismatch-containing DNA than a perfect match DNA.

We now describe a new bis-pyrene-labeled oligonucleotide probe (BP-probe) for the PASE detection of a single base mismatch that is present in ss DNA as a target. The probe sequence was chosen to form stem-loop structure similar to a molecular beacon probe (MB-probe). We thus named the present probe as BP–MB-probe. The hybrid formation of the BP–MB-probe with oligonucleotides provided the partially ds BP–MB-probe, in which the oligonucleotide sequences are complementary to the loop segment but not to the stem sequence and exchangeable with the target DNA. It was shown that the partial ds BP–MB-probes exhibit monomer fluorescence as major fluorescence, while the BP–MB-probe in the stem-loop structure displays strong excimer fluorescence. We used the partial ds BP–MB-probe to monitor the PASE between the probe and single strand DNA as a target. The presence of a mismatched base in ss DNA can be detected based on the different PASE rates from fully matched DNA, which could not be achieved by the use of ss BP-probes.

2. Results

2.1. Probe design

The detection format of a mismatched base in DNA using BP–MB-probe is depicted in Figure 2. The

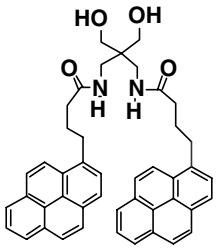

Figure 1. Chemical structure of bis-pyrene-label.

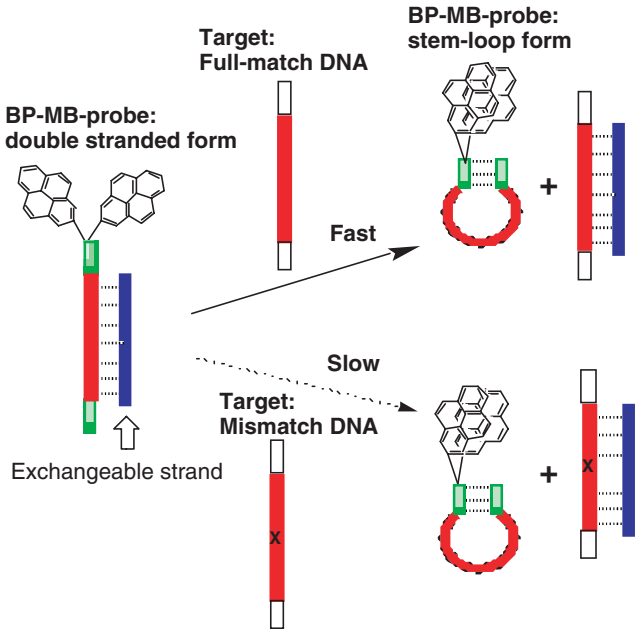

Figure 2. Detection of a single base mismatch in DNA using bis-pyrene-labeled oligonucleotide probe (BP–MB-probe).

sequences of BP–MB-probe and target DNA for the present studies are shown in Table 1. The BP–MB-probe has the sequence forming loop-stem structure in the absence of complementary DNA sequences or in the ss form. The partially double stranded BP–MB-probes were used in the mutation detection. The oligonucleotide strands of the partially ds BP–MB-probes are fully complementary to the loop but not to the stem and exchangeable with the target DNA. Therefore, in the partially ds BP–MB-probes, the bis-pyrene residue is stayed at the terminus of the single strand region. As a result, the probes should exhibit monomer emission as major fluorescence. In contrast, because the bis-pyrene-labeled strand spontaneously forms the loop-stem structure, the bis-pyrene unit is placed at the edge of the double stranded region of the stem. Therefore, the BP–MB-probe in the ss form should emit strong excimer. The monomer–excimer switching would thus facilitate to monitor the PASE between the partially ds probe and ss DNA.

2.2. Partially double stranded probe and its fluorescence

We prepared three kinds of partially ds BP–MB-probes. The BP–MB-probe **1** contains the counter strand (24-

Table 1. Sequence and T_m value of double strand bis-pyrene oligonucleotide probe (BP-probe) and target DNA

	Oligonucleotide[a]	T_m[b] (°C)
BP–MB-probe 1	**I**: 5′-(BP)TCCTCG TAGGAAACACCAAAGATGATATTT CGAGGA + **II**: 3′-ATCCTTTGTGGTTTCTACTATAAA-5′	58.8
BP–MB-probe 2	**I** + **III**: 3′-ATAATAATCCTTTGTGGTTTCTACTATAAA-5′	56.8
BP–MB-probe 3	**I** + **IV**: 3′-ATCCTTTGTGGTTTCTACTATAAAAACATA-5′	56.8
	II + **V**: 5′-GATATTATTAGGAAACACCAAAGATGATATTTTTGTATAG-3′	59.8
	II + **VI**: 5′-GATATTATTAGGAAACAC***A***AAAGATGATATTTTTGTATAG-3′	52.8
	II + **VII**: 5′-GATATTATTAGGAAACAC***T***AAAGATGATATTTTTGTATAG-3′	53.8
	II + **VIII**: 5′-GATATTATTAGGAAACAC***G***AAAGATGATATTTTTGTATAG-3′	52.9
	II + **IX**: 5′-GATATTATTAGGAA***C***CACCAAAGATGATATTTTTGTATAG-3′	50.9
	II + **X**: 5′-GATATTATTAGGAA***G***CACAAAAGATGATATTTTTGTATAG-3′	54.0
	II + **XI**: 5′-GATATTATTAGGAA***T***CACAAAAGATGATATTTTTGTATAG-3′	52.0
	III + **V**	59.7
	III + **VI**	54.7
	III + **VII**	55.8
	III + **VIII**	54.8
	III + **IX**	53.9
	III + **X**	57.0
	III + **XI**	55.1
	IV + **V**	62.6
	IV + **VI**	57.7
	IV + **VII**	58.7
	IV + **VIII**	57.8

[a] The sequence corresponding to the loop region in the probe is shown by box. BP denotes the bis-pyrene label whose structure is indicated in Figure 1.

[b] T_m values were determined from the UV-melting curves that were obtained at 2 μM of strand concentration in a pH 7 buffer containing 100 mM NaCl and 10 mM sodium phosphate.

mer) whose length is equal to the loop segment of the probe. The counter strands of the BP–MB-probes **2** and **3** have the extra six bases at the 3′-terminal or 5′-terminal end of the 24-mer strand, respectively. The double strand formation and fluorescence properties of BP–MB-probes **1**–**3** were investigated by UV-melting studies and fluorescence spectra. Figure 3 shows the melting curves and the emission spectra of **1**–**3** and the single strand BP-labeled sequence **I**. The BP–MB-probes **1**–**3** exhibited the sharp melting profiles as expected for the duplex melting. The sequence **I** showed the relatively broad melting transition at ~35 °C, which is consistent with the loop-stem structure at the low temperature. Importantly, all the BP–MB-probes **1**–**3** at 22 °C exhibited the pyrene monomer emission as major fluorescence, while **I** displayed the pyrene excimer as major emission. The ca. 10 times larger intensity at 480 nm for **I** was observed when compared with **1**–**3** under the same conditions. These results clearly indicate that the partially ds BP–MB-probes have useful properties to monitor the PASE between the probes and a target DNA by the intensified excimer fluorescence. It is noted that this is the first example for the monomer to excimer switching upon the structural transformation of the singly labeled bis-pyrenyl oligomers.[15]

2.3. Detection of a single base mismatch in DNA

The partially ds BP–MB-probes in the detection of a single base mismatch were tested by using 40-mer DNAs (**VI**–**XI**) containing six different mismatch bases. As described, BP–MB-probe **1** has the 24-mer strand (**II**) that

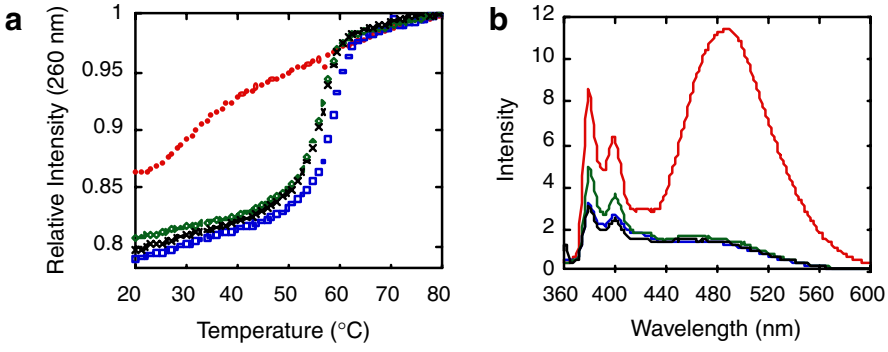

Figure 3. (a) UV-melting curves for the BP-probes (**I**, red; BP–MB-probe 1, blue; BP–MB-probe 2, black; BP–MB-probe 3, green). (b) Fluorescence spectra at 22 °C for the BP-probes (**I**, red; BP–MB-probe 1, blue; BP–MB-probe 2, black; BP–MB-probe 3, green). The measurements were carried out for 2 μM of probe in a pH 7 buffer containing 0.1 M NaCl and 0.01 M sodium phosphate.

is exchangeable with the target and BP–MB-probes **2** and **3** have the exchangeable 30-mer strand (**III** and **IV**). Table 1 summarised T_m values of the partially ds BP–MB-probes, and the DNA duplexes that should be formed after the strand exchange reaction. The partially double stranded BP–MB-probes have the T_m values of 56–58 °C, while the final DNA duplexes show the T_ms ranging from 50 to 63 °C that are dependent on the sequence and length of DNA.

We carried out the PASE assays in a pH 7 buffer containing 0.1 M NaCl at 22 °C that is well below the T_m values of the partially ds probes and the final duplexes. To the mixture containing target DNA (0.4 μM) and probe (0.2 μM), dextran-grafted poly-L-lysine as cationic comb-type copolymer was added in cation (copolymer) and anion (DNA) ratio of 0.2. Then the time-dependent fluorescence changes at 480 nm were monitored. The fluorescence response curves for the fully matched DNA **V** and DNA (**VI**–**VIII**) containing a single base mismatch at the position of 19 are shown in Figure 4. Under the conditions above, the use of BP–MB-probe **1** resulted in little or no fluorescence changes in the PASE for DNA **V**–**VIII**, indicating that the PASE of **1** with fully matched DNA (**V**) as well as with mismatched DNA (**VI**–**VIII**) did not proceed (Fig. 4a). On the other hand, the increase of the excimer fluorescence was observed for the PASE between the BP–MB-probes **2** and **3** and the fully matched DNA (**V**), while the small changes in the fluorescence were observed for the mismatch-containing DNA (**VI**–**VIII**) (Fig. 4b and c). These fluorescence changes indicated that almost 100% of the used probe had been displaced for the exchangeable strand of the fully matched DNA within 10 min, but less than 30% for the mismatched DNA. By using the probes having the long exchangeable strand, the existence of single base mismatches was clearly identifiable from the slower fluorescence changes observed for them, compared to that of fully matched DNA. In these assays, better discrimination was observed for BP–MB-probe **2** compared to probe **3**. We further tested the utility of the BP–MB-probe **2** in the PASE detection of DNA mismatches at the different position (position 15). The results are shown in Figure 4d. The almost complete fluorescent discrimination of the fully matched DNA (**V**) from mismatch-containing DNA (**IX**–**XI**) can be achieved.

3. Discussion

We have designed oligonucleotide probes on the basis of our recent findings that the bis-pyrene (BP) placed at the edge of duplex DNA exhibits strong excimer, while it shows monomer fluorescence in the ss oligomers. By the choice of the sequence of BP-probes to form the stem-loop structure like a molecular beacon, ss BP–MB-probes were expected to exhibit the strong excimer emission. For PASE assays, ds BP–MB-probes consisting of the exchangeable strand to a target ss DNA were necessary. We prepared three probes one of which has the counter strand or exchangeable strand

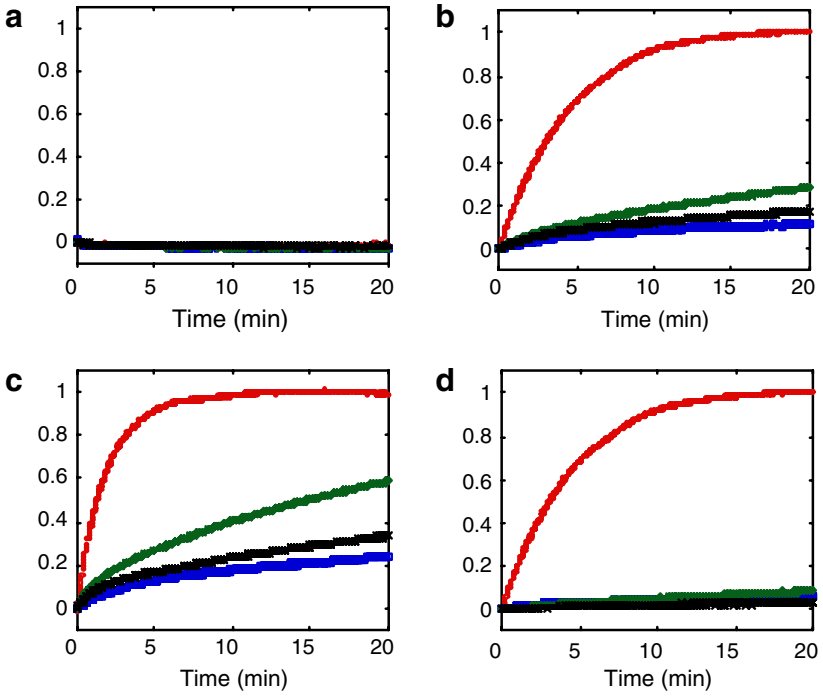

Figure 4. Time course of PASE between ds BP-probe (total strand conc.: 0.2 μM) and ss target DNA (strand conc.: 0.4 μM), monitored by the excimer fluorescence. (a) PASE between BP–MB-probe 1 and DNA (**V**, red; **VI**, green; **VII**, black; **VIII**, blue). (b) PASE between BP–MB-probe 2 and DNA (**V**, red; **VI**, green; **VII**, black; **VIII**, blue). (c) PASE between BP–MB-probe 3 and DNA (**V**, red; **VI**, green; **VII**, black; **VIII**, blue). (d) PASE between BP–MB-probe 2 and DNA (**V**, red; **IX**, green; **X**, black; **XI**, blue). The assays were carried out at 22 °C in 0.1 M NaCl and 0.01 M sodium phosphate, adjusted to pH 7 in the presence of the polycation (cation/DNA anion charge ratio = 0.2:1). The polycation used was dextran (M.W. 5300) grafted poly-L-lysine (M.W. 14400).

whose length is the same as that of the loop segment of the probe. The other two probes consist of 30-mer of exchangeable sequence, in which the extra six bases are complementary to a part of the target DNA but not to the stem region of the probe. According to this design, the bis-pyrene can be placed at the region of the single strand, resulting in monomer emission as a major fluorescence. The melting and fluorescence studies indeed indicated that all the BP–MB-probes possess the monomer–excimer switching properties upon structural changes from ds- to ss-forms. The intensity of the excimer fluorescence was found to be 10 times stronger than that of the monomer emission. Notably, this fluorescence change is reverse from that of the pyrene-labeled beacon at both terminals.[15] In addition, unlike FRET-based molecular beacons[3–9] and the dual pyrene-labeled beacon,[15] our BP–MB-probes have the single fluorescence label at the terminus, which made the probe synthesis simple and easy.

The partially ds BP–MB-probes have been used in the detection of a single base mismatch in DNA based on PASE. The assays were carried out at room temperature that is considerably lower than the T_ms of the ds probes and the duplexes after the exchange. We used the polycation whose charge is 0.2 equiv to the total anion of DNA (probe and target), since it was found that the less amount of polycation gives the better discrimination of mismatch-containing DNA from fully matched DNA in the PASE assay.[16] Moreover, the excimer intensity of the ss BP–MB-probe decreased with the increased amount of the polycation used, which caused the negative effect on the fluorescence monitoring of the PASE (data not shown). However, under the above conditions, when the ds BP–MB-probe possessing the exchangeable strand whose length is same as that of the loop segment of the probe (BP–MB-probe **1**) was used, the PASE with fully matched and mismatched DNA did not occur. Then, for the exchangeable strand of ds BP–MB-probes, we have chosen the longer sequence than the loop segment. The extra bases in this sequence should be involved in the formation of nucleation complex between the exchangeable strand and the target DNA sequence. The complex formation would thus facilitate the strand exchange reactions (SER). With these BP–MB-probes (**2** and **3**), we observed the significant difference in the SER between the fully matched target and the mismatch-containing DNA. These observations are well consistent with that a base mismatch is an effective block for the displacement pathway in the SER.[12,14] We have thus achieved the almost complete discrimination of mismatch-containing DNA from fully matched DNA by the PASE.

4. Conclusion

In summary, we have developed a pyrene-fluorescence-switching probe for the detection of single base mismatches in DNA, based on PASE reactions. The probe design is based on some aspects of a molecular beacon. In contrast to a usual FRET-based beacon, our bis-pyrene molecular beacon offers some advantages such as an easy synthesis of the probes. In addition, the assay format does not require any special equipment for mismatch detection. We therefore anticipate that a bis-pyrene-labeled molecular beacon will provide a superior and inexpensive probe in PASE assay of DNA mutation.

5. Experimental

5.1. The preparation of BP–MB-probes

The modified and unmodified oligonucleotides used in the present studies were synthesized on an automated DNA synthesizer (ABI 394) by using commercially available nucleoside phosphoramidites (Transgenomic Inc.) and the bis-pyrene-modified propanediol phosphoramidite.[26] The oligonucleotides were purified with a reversed-phase HPLC and the purified oligomers were characterized as described.[26]

5.2. Procedure for mismatch detection

All the PASE experiments were carried out in 10 mM phosphate buffer (pH 7.0) containing 100 mM NaCl. A buffer solution (1 mL) containing a single strand target oligonucleotide and a bis-pyrene-probe, whose final concentrations are given in Figure 4, was held at the desired temperature. After that an aliquot (10–20 μL) of solution containing an appropriate amount of cationic comb-type copolymer (poly-L-lysine (MW 14400) containing dextran (MW 5300) grafted at the ε-amino residue (grafting 14.4%)) was added. The cation (polycation) to anion (DNA) concentration ratios were maintained at 0.2 in the PASE experiments. The fluorescence intensity at 480 nm was recorded at 5–10 s intervals on a Hitach F-2500 spectrophotometer equipped with a thermo-controller to maintain cell temperature. The excitation and emission slits of 5 nm and excitation at 350 nm were used for the fluorescence measurements.

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