DNA mismatch detection using a pyrene–excimer-forming probe†‡

Kazushige Yamana,*[a] Yudai Fukunaga,[a] Yusuke Ohtani,[a] Sayaka Sato,[a] Mitsunobu Nakamura,[a] Won Jong Kim,[b] Toshihiro Akaike[b] and Atsushi Maruyama[c]

Received (in Cambridge, UK) 10th February 2005, Accepted 11th March 2005
First published as an Advance Article on the web 1st April 2005
DOI: 10.1039/b502033f

A pyrene–excimer-forming probe allowed the easy and sensitive detection of a single base mismatch in target DNA. This was due to the faster strand exchange rate compared to a fully-matched target.

The detection of single base mismatches in DNA is important for diagnostics, treatment of genetic diseases and the identification of single nucleotide polymorphisms. Of the many assays proposed for large-scale mutation analysis, heterogeneous hybridization assays on DNA micro-array chips are widely used.[1] Homogeneous mutation assays based on hybridization are an attractive alternative[2] and have advantages over heterogeneous assays, such as fast hybridization kinetics and the absence of washing steps. These advantages facilitate the automation of assays.

Homogeneous DNA mutation analysis is performed by using molecular beacon probes that exhibit intense fluorescence when bound to target DNA sequences.[3] Multi-colored beacon probes are used to discriminate a sequence variant in DNA, amplified by polymerase chain reactions.[4] In these assays, DNA mutation discrimination is largely dependent on the thermodynamic differences between fully-matched and mismatched DNA. These differences are generally small and vary according to the DNA sequence—making this approach necessary in order to optimize hybridization conditions and probe design.

In contrast to the thermodynamic approach, DNA mutation detection is achieved by monitoring the kinetic differences in the strand exchange reactions (SERs) between double-strand (ds) DNA probes and single-strand (ss) target DNA.[5] It is possible to determine the existence of mismatches from the slower SER rates, compared to those for fully-matched targets. In these assays, a cationic com-type copolymer (CCC) acting as an SER accelerator allows the effective differentiation of the kinetic barrier between a perfect match DNA and a mismatch-containing DNA without careful optimizing processes.[5a] However, these assays use the ds probes labeled with a pair of FRET fluorophores to detect the SERs.[5] Because the preparation of the doubly-labeled probes is tedious and costly, there is a high demand for superior and inexpensive probes to make mismatch detection using SERs more practical.

We describe here a new format for DNA mutation detection based on polycation-accelerated SER (PASE). The systematic representation of mutation detection is depicted in Fig. 1. The important feature of the present detection format is the use of a bis-pyrene-labeled oligonucleotide probe. The bis-pyrene probe can be easily synthesized by using the phosphoramidite method and displays weak monomer emission as a major fluorescence in its single-stranded form, but strongly enhanced excimer fluorescence upon duplex formation.[6] We therefore anticipated that the excimer-forming probe could be used as an exchangeable, homologous strand to monitor the PASE between the probe and a ds target DNA by intensified excimer fluorescence. The excimer-forming probes[7–9] in combination with a PASE would thus make mutation detection possible with unlabeled ds DNA targets—something which could not be achieved by using doubly-labeled FRET probes.

In order to establish the assay format of mismatch detection using a pyrene–excimer-forming probe, we have used 20-mer ds DNA as a target, containing a single base mismatch in different sequences and positions (Systems I and II). The sequences and T_m values of the targets are shown in Table 1. The probe sequence used was homologous to the exchangeable strand in the ds DNA, containing a single base mismatch. Therefore after the strand exchange, the probe duplex shown in Table 1 for each system was formed. The T_m values for the mismatch-containing DNA duplexes are lower by 5–10 °C than that for the fully-matched duplex. The T_m value for the duplex of the probe does not significantly differ from that of the initial fully-matched duplex.

Upon mixing of the ds DNA of System I with the probe at 50 °C close to the duplex melting temperatures, the enhanced excimer fluorescence at 480 nm was observed for all the target ds DNA. However, the excimer intensity did not significantly differ between the fully-matched and mismatched DNA as shown in Fig. 2A. At this high temperature a similar result was also obtained for System II. Therefore, the discrimination of the mismatched

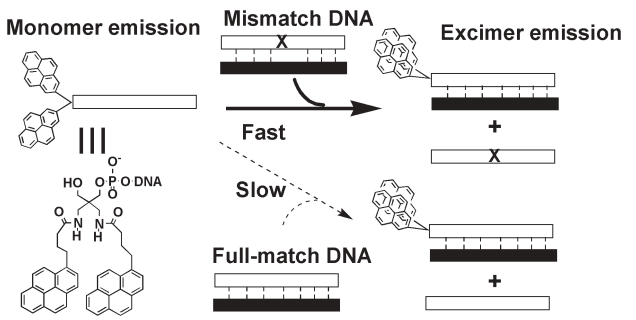

Fig. 1 A schematic representation of mismatch detection using a pyrene–excimer-forming probe based on a polycation-accelerated strand exchange (PASE) reaction.

† Electronic Supplementary Information (ESI) available: Arrhenius plot for the PASE reaction, and the time course of the PASE between the bis-pyrene probe and DNA of various sequences and lengths. See http://www.rsc.org/suppdata/cc/b5/b502033f/

‡ This research was partly supported by SORST of JST (K.Y.).

*yamana@eng.u-hyogo.ac.jp

Table 1 Sequences and T_m values of DNA duplexes used for the mismatch detection

DNA	T_m[a] (ΔT_m)/°C
System I	
FULL (X = G)	
5′-TGTXGTATGGCTGATTATAG	60
3′-ACACCATACCGACTAATATC	
CC-MIS (X = C)	50 (−10)
AC-MIS (X = A)	51 (−9)
TC-MIS (X = T)	52 (−8)
BP-Probe I	
5′-(BP)TGTGGTATGGCTGATTATAG	62 (+2)
3′-ACACCATACCGACTAATATC	
System II	
FULL (X = T)	
5′-TCCTCGCCCXTGCTCACCAT	68
3′-AGGAGCGGGAACGAGTGGTA	
CA-MIS (X = C)	59 (−9)
AA-MIS (X = A)	60 (−8)
GA-MIS (X = G)	63 (−5)
BP-Probe II	
5′-(BP)TCCTCGCCCTTGCTCACCAT	69 (+1)
3′-AGGAGCGGGAACGAGTGGTA	

[a] T_m values were determined from UV-melting curves obtained for duplexes (total strand concentration = 2.5 μM) in 0.1 M NaCl and 0.01 M sodium phosphate adjusted to pH 7.0. BP denotes a bis-pyrenyl unit whose structure is shown in Fig. 1.

duplexes from the fully-matched DNA cannot be realized by the thermally-induced strand exchange reaction. This is owing to the small differences in the T_m values between the mismatched and fully-matched duplexes.

We carried out the PASE experiments in a pH 7 buffer containing 0.1 M NaCl at a temperature well below the T_m values for the initial mismatched and fully-matched DNA, as well as the final duplex of the probe after the exchange. The exchangeable strand of ds DNA and probe were used in equal amounts. 2 and 1.3 equivalents of the cationic com-type copolymer (CCC)[10] per one of DNA anion were used for Systems I and II respectively. Under these conditions, after the mixing of probe and DNA, the time-dependent changes in excimer fluorescence (480 nm) were monitored. Significant increases in the excimer intensity for all the mismatched duplexes were observed, but little for the fully-matched duplex. The resulting fluorescence response curves are shown in Figs. 2B and 2C for Systems I and II respectively. These fluorescence changes indicated that 10–60% of the used probe had been displaced for the exchangeable strand of the mismatched DNA, but less than 3% for the fully-matched DNA.[11] In both systems, the existence of single base mismatches was clearly identifiable from the faster fluorescence changes observed for them, compared to those of fully-matched DNA. Slower PASE rates yet better fluorescence discrimination between the mismatched targets were observed for System II compared to System I, due to the reduced amount of the polycation used in System II.[12] Without use of the polycation, mixing of the probe and target DNA at lower temperatures resulted in little or no changes to the fluorescence for either the mismatched or fully-matched DNA. These important observations indicate that the presence and quantity of the polycation are essential to allow mismatch detection by SERs.

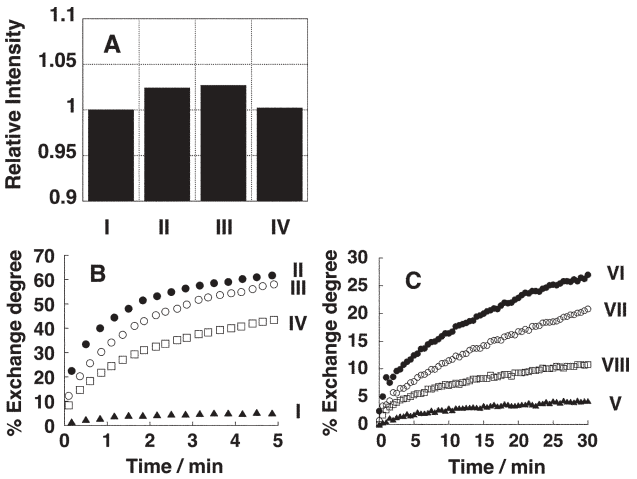

Fig. 2 A: Relative fluorescence intensity (480 nm) measured at 23 °C after the mixing of 0.5 μM DNA duplexes [FULL (I), CC-MIS (II), AC-MIS (III), TC-MIS (IV)] with 0.24 μM probe at 50°C for 5 min in the absence of cationic com-type copolymer (CCC). B: Time course of the PASE between 0.5 μM DNA duplexes [FULL (I), CC-MIS (II), AC-MIS (III), TC-MIS 3 (IV)] and 0.24 μM probe at 10 °C in the presence of CCC (CCC cation/DNA anion charge ratio = 2 : 1). C: Time course of the PASE between 0.5 μM DNA duplexes [FULL (V), CA-MIS (VI), AA-MIS (VII), GA-MIS 3 (VIII)] and 0.24 μM probe at 13 °C in the presence of CCC (CCC cation/DNA anion charge ratio = 1.3 : 1). The values of percentage exchange degree were calculated using the reported procedure.[5b] The sequences of DNA and probes used are shown in Table 1. All the experiments were performed in 10 mM phosphate buffer (pH 7.0) containing 100 mM NaCl.

Table 2 The relative SER rates and activation energies in PASE reactions

DNA	Relative SER rate[a]	Activation energy[b]/kJ mol^{-1}
CC–MIS	26.8	52
AC–MIS	26.1	56
TC–MIS	14.5	60
FULL	1	105

[a] The rate constants at 10 °C for the SERs between ds DNA and the probe were obtained by pseudo-first-order analyses in the presence of CCC (CCC cation/DNA anion charge ratio = 2 : 1). [b] The activation energies for the SER reactions were estimated from their Arrhenius plots.

Table 2 shows the relative rates and the activation energies[13] estimated for the PASE between the 20-mer DNA of System I and the probe. The PASE rates for the mismatched duplexes were more than 14 times faster than that for the fully-matched duplex. The activation energies for the PASE between the mismatched duplexes and the probe (52–60 kJ mol^{-1}) were significantly lower than that for the PASE between the fully-matched duplex and the probe (105 kJ mol^{-1}). The magnitude of the activation energy for the fully-matched duplex is consistent with the reported value.[5,14] This is indicative that the strand displacement between the ds DNA and the ss probe is initiated by nucleation of a hetero-duplex (from a terminally uncoiled ds DNA) and a homologous single strand.[5,14] The very low activation energy required for the mismatch DNA strongly suggests that the mismatch site is involved as the additional site of nucleation complex formation. It is therefore very likely that the polycation stabilizes the nucleation

complex or lowers the energy level of the transition state complex consisting of the mismatch site. The existence of single-base mismatches in target DNA can thus be easily discriminated from fully-matched targets by their faster PASE rates.

In summary, we have developed an assay for the detection of single base mismatches by using a pyrene–excimer-forming probe, based on PASE reactions. The probe design and synthesis is straightforward and the assay format does not require any special equipment for mismatch detection. In addition, the mismatch discrimination by PASE can be performed at the wide range of the temperatures below the T_ms of the target ds DNA.[13] Since the PASE reaction is tunable, especially by the choice of polycation, the present method would also be applicable for longer strand target DNA.[5a] We therefore anticipate that PASE, in combination with an excimer-forming probe, will provide a simple and sensitive method for DNA mutation analysis.

Kazushige Yamana,*[a] Yudai Fukunaga,[a] Yusuke Ohtani,[a] Sayaka Sato,[a] Mitsunobu Nakamura,[a] Won Jong Kim,[b] Toshihiro Akaike[b] and Atsushi Maruyama[c]

[a]Department of Materials Science and Chemistry, University of Hyogo, 2167 Shosha, Himeji, Hyogo 671-2201, Japan. E-mail: yamana@eng.u-hyogo.ac.jp; Fax: 81-792-67-4895

[b]Department of Biomolecular Engineering, Tokyo Institute of Technology, 4259 Nagatsuda, Midori-Ku, Yokohama 226-8501, Japan

[c]Institute for Materials Chemistry and Engineering, Kyushu University and CREST of JST, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan. E-mail: maruyama@ms.ifoc.kyushu-u.ac.jp

Notes and references

1 (*a*) A. Marshall and J. Hodgson, *Nat. Biotechnol.*, 1998, **16**, 27–31; (*b*) D. G. Wang, J.-B. Fan, C.-J. Siao, A. Berno, P. Young, R. Sapolsky, G. Ghandour, N. Perkins, E. Winchester, J. Spencer, L. Kruglyak, L. Stein, L. Hsie, T. Topaloglou, E. Hubbell, E. Robinson, M. Mittmann, M. S. Morris, N. Shen, D. Kilburn, J. Rioux, C. Nusbaum, S. Rozen, T. J. Hudson, R. Lipshutz, M. Chee and E. S. Lander, *Science*, 1998, **280**, 1077–1082.

2 (*a*) A. Okamoto, K. Tainaka and I. Saito, *J. Am. Chem. Soc.*, 2003, **125**, 4972–4973; (*b*) A. Okamoto, K. Tanaka, T. Fukuta and I. Saito, *J. Am. Chem. Soc.*, 2003, **125**, 9296–9297; (*c*) H. Asanuma, H. Kashida, X. Liang and M. Komiyama, *Chem. Commun.*, 2003, 1536–1537; (*d*) K. Yamana, H. Zako, K. Asazuma, R. Iwase, H. Nakano and A. Murakami, *Angew. Chem., Int. Ed.*, 2001, **40**, 1104–1106.

3 (*a*) S. Tyagi and F. R. Kramer, *Nat. Biotechnol.*, 1996, **14**, 303–307; (*b*) S. Tyagi, S. A. E. Marras and F. R. Kramer, *Nat. Biotechnol.*, 2000, **18**, 1191–1196; (*c*) P. Zang, T. Beck and W. Tan, *Angew. Chem., Int. Ed. Engl.*, 2001, **40**, 402–405.

4 (*a*) S. Tyagi, D. P. Bratu and F. R. Kramer, *Nat. Biotechnol.*, 1998, **16**, 49–53; (*b*) L. G. Kostriks, S. Tyagi, M. M. Mhlanda, D. D. Ho and F. R. Kramer, *Science*, 1998, **279**, 1228–1229; (*c*) S. A. E. Marras, F. R. Kramer and S. Tyagi, *Genet. Anal. Biomol. Eng.*, 1999, **14**, 151–156; (*d*) I. Tapp, L. Malmberg, E. Rennel, M. Wik and A.-C. Syvanen, *BioTechniques*, 2000, **28**, 732–738.

5 (*a*) W. J. Kim, Y. Sato, T. Akaike and A. Maruyama, *Nat. Mater.*, 2003, **2**, 815–820; (*b*) W. J. Kim, T. Akaike and A. Maruyama, *J. Am. Chem. Soc.*, 2002, **124**, 12676–12677; (*c*) Q. Li, G. Luan, Q. Guo and J. Liang, *Nucleic Acids Res.*, 2002, **30**, e5.

6 K. Yamana, T. Iwai, Y. Ohtani, S. Sato, M. Nakamura and H. Nakano, *Bioconjugate Chem.*, 2002, **13**, 1266–1273.

7 S. M. Langenegger and R. Häner, *Chem. Commun.*, 2004, 2792–2793.

8 M. N. Dioubankova, A. D. Malakhov, D. A. Stetsenko, M. J. Gait, P. E. Volynsky, R. G. Efremov and V. A. Korshun, *ChemBioChem*, 2003, **4**, 841–847.

9 P. J. Hrdlicka, B. R. Babu, M. D. Sorensen and J. Wengel, *Chem. Commun.*, 2004, 1478–1479.

10 Poly-L-lysine (MW = 14400) containing dextran (MW = 5300) grafted at the ε-amino residue (grafting = 14.4%).

11 Since the amount of probe used was equivalent to the exchangeable strand, and the T_m of the probe duplex was 1–2 °C higher than that of the initial duplex, a slight excess of probe duplex over the initial target DNA should be present, even after the completion of the SERs. Therefore, the maximum degree of percentage strand exchanges based on the probe used should not significantly exceed 50%.

12 The quantity of polycation has a large effect on the rates of the PASE. See ref. 5*b*.

13 Activation energies were obtained from the analysis of the PASE rates, measured in the temperature range 10–30 °C. Within this range for all experiments, faster rates for the mismatched DNA than the fully-matched target were observed. See the ESI (http://www.rsc.org/suppdata/cc/b5/b502033f/).

14 L. P. Reynaldo, A. V. Vologodskii, B. P. Neri and V. I. Lyamichev, *J. Mol. Biol.*, 2000, **297**, 511–520.