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Fluorescence detection of single-nucleotide polymorphisms using a thymidine-based molecular beacon

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ABSTRACT

We have developed a universal molecular beacon (T_7-MB-T_7) for the detection of single-nucleotide polymorphisms (SNPs). The beacon, which contains a 19-mer loop and a stem comprising a pair of seven thymidine (T) bases, forms double-stranded structures with target DNA molecules, leading to increases in the fluorescence of ethidium bromide (EthBr) as a result of intercalation. The interactions of the beacon with perfectly matched (DNA_{pm}) and single-base mismatched (DNA_{mm}) DNA strands are stronger and weaker, respectively, than those with Hg^{2+} ions. As a result, the fluorescence of a solution containing T_7-MB-T_7, DNA_{pm}, EthBr, and Hg^{2+} is higher than that of a corresponding solution containing T_7-MB-T_7, DNA_{mm}, EthBr, and Hg^{2+}, because the former has a greater number of intercalation sites for EthBr. Under the optimal conditions (100 nM T_7-MB-T_7, 20 mM NaCl, 5.0 μM Hg^{2+}, and 300 nM EthBr in 5.0 mM Tris–HCl solution, pH 7.4), the plot of the fluorescence intensity against the concentration of DNA_{pm} was linear over the range 5.0–100 nM ($R^2 = 0.98$). A similar probe, T_7-MB_t-T_7, is sensitive and selective for the detection of a gene associated with hereditary tyrosinemia type I. Relative to conventional MBs, our new probe offers the advantages of higher selectivity toward DNA, less nonspecific binding toward single-stranded-DNA-binding protein, greater resistance to nuclease digestion, and low cost; therefore, we suspect that this system holds great potential for practical studies of SNPs.

1. Introduction

Single-nucleotide polymorphisms (SNPs), the most common form of variation in the human genome, are important markers for the diagnosis of disease, for studies of the genome, and for the synthesis of new medicines (Gray et al., 2000). The last few years have witnessed significant progress in the development of optical and electrochemical techniques for the detection of DNA molecules that have sequences differing in (or missing) a single base, employing, for example, molecular beacons (MBs) (Tyagi and Kramer, 1996), DNA-modified nanoparticles (Storhoff et al., 2004; Nam et al., 2004), conjugated polymers (Gaylord et al., 2002; Liu and Bazan, 2004), DNA-specific redox indicators and conjugated mediators (Kelley et al., 1999; Drummond et al., 2003), and DNA-conjugated enzymes (Li et al., 2005; Kolpashchikov, 2008). Although they are sensitive and specific, many of these systems have features that limit their practical use, such as tedious bioconjugation and labeling processes, the need for expensive reagents and biomolecules (e.g., enzymes and fluorescent dye-labeled DNA molecules), and the need for tight control over the experimental conditions (e.g., temperature).

Fluorescence spectroscopy using MBs that form stem-and-loop structures to recognize targeted DNA molecules is particularly interesting in the study of SNPs, mainly because they offer the advantages of simplicity and sensitivity. In a common MB, the loop contains a probe sequence that is complementary to a target sequence, and the termini of the stem are modified with a donor (fluorophore) and an acceptor (quencher), respectively. MBs act as fluorescence resonance energy transfer (FRET)-based switches that are normally in the closed or "fluorescence off" state, but switch to the open or "fluorescence on" state in the presence of target (complimentary) DNA strands.

When MBs are used for the detection of SNPs, problems occur that are associated with their nonspecific binding to single-stranded-DNA-binding protein (SSB) and endogenous nuclease degradation, leading to false-positive signals and, hence, their limited applicability in complex biological samples (Leonetti et al., 1991; Fisher et al., 1993). Another major drawback of MBs is that they are usually expensive because the two ends of the stem and the sequence of MBs must be modified with signal generators and nuclease-resistant backbone chemistries, respectively (Tsourkas et al., 2002; Kuhn et al., 2002, Kim et al., 2007). We have previously unveiled a universal MB in the presence of Hg^{2+} for the

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detection of SNPs based on Hg^{2+}–DNA complexes inducing a conformational change in the MB. The specificity of SNPs was enhanced 10-fold or more toward normal MBs and problems associated with interferences of SSB and endogenous nuclease degradation were minimized (Lin et al., 2008). However, expensive fluorescent MBs were employed.

In this paper, we present a fluorescence assay—employing a thymidine-based MB (T_7-MB-T_7) in the presence of Hg^{2+} and ethidium bromide (EthBr)—for the detection of SNPs. The T_7-MB-T_7 contains a stem comprising a pair of 7-mer T bases, which interact with Hg^{2+} ions, and a loop of 19-mer DNA bases, which recognize the targeted DNA. Because of the T–Hg^{2+}–T interactions (Ono and Togashi, 2004; Liu et al., 2008), the Hg^{2+}–DNA complexes in the hairpin structure are more resistant to nuclease degradation and less likely to form complexes with SSB. We investigated the roles that the pH, the nature of the dye, and the concentrations of Hg^{2+} and NaCl in the probe solutions play in determining the sensitivity and selectivity of the T_7-MB-T_7 probe toward perfectly matched (DNA_{pm}) and single-base mismatched (DNA_{mm}) DNA strands. In addition, we evaluated the performance of several different DNA probes to confirm the advantageous features of the T_7-MB-T_7 probe for SNPs studies.

2. Experimental

2.1. Chemicals

Tris(hydroxymethyl)aminomethane (Tris), mercury chloride ($HgCl_2$), magnesium chloride ($MgCl_2$), and deoxyribonuclease I (DNase I) were purchased from Aldrich (Milwaukee, WI, USA). The MBs and DNA samples (see Table 1 for sequences) were purchased from Integrated DNA Technology, Inc. (Coralville, IA, USA); SSB was purchased from Promega (Madison, WI, USA). EthBr, YOYO-3, TOTO-3, and OliGreen were obtained from Molecular Probes (Portland, OR). Milli-Q ultrapure water was used in all experiments.

2.2. Analysis of samples

Aliquots (350.0 μL) of 5.0 mM Tris–HCl buffer (pH 7.4) containing NaCl (0–100.0 mM) and one of the tested MBs (100.0 nM) were maintained at ambient temperature for 5 min. Aliquots (50.0 μL) of the target DNA (1.0 μM) were added separately to each of the probe solutions, which were then incubated for 1 h. The final ratio of the concentrations of the MBs to the targeted DNA was 1:1. An aliquot (50 μL) of Hg^{2+} (0–10.0 μM) was added to each solution, which was incubated for 1 h and then EthBr (0.1–5.0 μM) or another dye (1.0 μM) was added. The mixtures were further equilibrated for 10 min prior to fluorescence measurements (Cary Eclipse; Varian, CA, USA).

To evaluate the degree of nonspecific binding of the MB probes to SSB, 5.0 mM Tris–HCl buffer (pH 7.4, 350.0 μL) solutions containing NaCl (20.0 mM), SSB (100.0 nM), one of the MBs (100.0 nM), and the targeted DNA (0 and 100.0 nM) were maintained at ambient temperature for 60 min. An aliquot (50 μL) of Hg^{2+} (5.0 μM) was added to each solution, which was then incubated for 60 min before EthBr (0.3 μM) was added. The mixtures were further equilibrated for 10 min prior to fluorescence measurement.

To evaluate the resistance of the MBs toward endogenous nuclease degradation, aliquots (400.0 μL) of 5.0 mM Tris–HCl buffer (pH 7.4) containing NaCl (20.0 mM), $MgCl_2$ (5.0 mM), one of the MBs (100.0 nM), Hg^{2+} (5.0 μM), and EthBr (0.3 μM) were maintained at ambient temperature for 1 h and then an aliquot (50.0 μL) of DNase I (final concentration: 2.5 μg/mL) was added to each solution. The mixtures were then subjected to fluorescence measurements while they were equilibrated for certain periods of time, as indicated in Section 3. Finally, the target DNA (final concentration 100.0 nM) was added to each mixture and its fluorescence was continually recorded.

Table 1
DNA sequences of MBs and Target DNA.

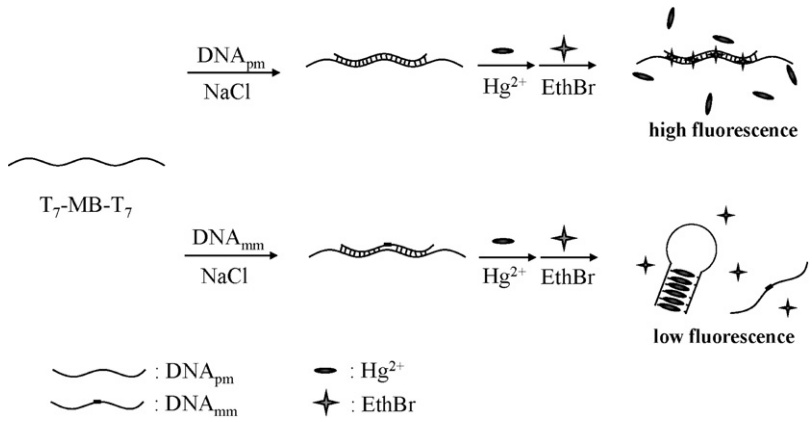
name	sequence (5′ – 3′)
T_6-MB-T_6	TTTTTTTCTAAATCACTATGGTCGCTTTTTT
T_7-MB-T_7	TTTTTTTTCTAAATCACTATGGTCGCTTTTTTT
T_8-MB-T_8	TTTTTTTTTCTAAATCACTATGGTCGCTTTTTTTT
MB_{C1}	ACCTAGCTCTAAATCACTATGGTCGCGCTAGGT
MB_{C2}	FAM-ACCTAGCTCTAAATCACTATGGTCGCGCTAGGT-DABCYL
MB_{C3}	FAM-TTTTTTTTCTAAATCACTATGGTCGCAAAAAAA-DABCYL
MB_{C4}	FAM-GGGGGGGTCTAAATCACTATGGTCGCCCCCCCC-DABCYL
MB_{C5}	FAM-ACTTAGTTCTAAATCACTATGGTCGCACTAAGT-DABCYL
MB_{C6}	FAM-GCCGAGCTCTAAATCACTATGGTCGCGCTCGGC-DABCYL
DNA_{pm1}	GCGACCATAGTGATTTAGA
DNA_{mm1}	GCGACCATAATGATTTAGA
DNA_{mm2}	GCGACCATACTGATTTAGA
DNA_{mm3}	GCGACCATATTGATTTAGA
DNA_{mm4}	GCAACCATAGTGATTTAGA
DNA_{mm5}	GCTACCATAGTGATTTAGA
DNA_{mm6}	GCCACCATAGTGATTTAGA
DNA_{mm7}	GCGACCATAGTAATTTAGA
DNA_{mm8}	GCGACCATAGTTTATTTAGA
DNA_{mm9}	GCGACCATAGTCATTTAGA
T_7-MB_1-T_7	TTTTTTTTCTAAATTACTATTGTTGTTTTTTTT
DNA_{pm2}	ACAACAATAGTAATTTAGA
DNA_{mm10}	ACAACAATAATAATTTAGA
T_7-MB_2-T_7	TTTTTTTCCTAACCCACTACGGTCGCTTTTTTT
DNA_{pm3}	GCGACCGTAGTGGGTTAGG
DNA_{mm11}	GCGACCGTAATGGGTTAGG
T_7-MB_t-T_7	TTTTTTTCCAGATACTCACCGGTTTTTTT
DNA_{pmt}	CCGGTGAGTATCTGG
DNA_{mmt}	CCGGTGAATATCTGG

3. Results and discussion

3.1. Sensing strategy

Scheme 1 displays the sensing strategy of the T_7-MB-T_7 probe toward target DNA molecules. Because the T_7-MB-T_7 probe possesses seven pairs of T bases, it exists in a random coiled structure, but forms folded structures in the presence of Hg^{2+} ions through T–Hg^{2+}–T binding. Under optimal conditions, the interactions of T_7-MB-T_7 with DNA_{pm} and DNA_{mm} are stronger and weaker, respectively, than those with Hg^{2+}. In other words, the T_7-MB-T_7 forms dsDNA complexes in the presence of Hg^{2+} and DNA_{pm}, but a folded structure in the presence of Hg^{2+} and DNA_{mm}. As a result, the fluorescence of a solution containing T_7-MB-T_7, DNA_{pm}, EthBr, and Hg^{2+} is higher than that of a corresponding solution containing T_7-MB-T_7, DNA_{mm}, EthBr, and Hg^{2+}, mainly because the dsDNA complex formed in the former solution has a greater number of intercalation sites for EthBr.

We conducted proof-of-concept experiments using the T_7-MB-T_7 probe and two different sequences of target DNA (Table 1): the perfectly matched DNA (DNA_{pm1}) and a single-base-mismatched DNA (DNA_{mm1}). First, we prepared solutions consisting of the



Scheme 1. Schematic representation of the mechanism of action of the T_7-MBs probe for the detection of DNA_{pm} and DNA_{mm} in the presence of Hg^{2+} and EthBr.

T_7-MB-T_7 (100.0 nM), Tris–HCl (5.0 mM; pH 7.4), NaCl (20.0 mM), Hg^{2+} (5.0 μM), and EthBr (300.0 nM) in the absence and presence of DNA_{pm1} (100 nM). In the absence of DNA_{pm1}, the fluorescence intensity of EthBr at 605 nm (excited at 520 nm) was low (spectrum a, Fig. 1). In the presence of DNA_{pm1}, the fluorescence intensity (spectrum b) of EthBr was much higher than that in the absence of the target DNA. These results support the sensing mechanism outlined in Scheme 1. In contrast, in the presence of DNA_{mm1}, the fluorescence intensity of EthBr (spectrum c) in the T_7-MB-T_7 probe solution increased only slightly, showing high specificity of the T_7-MB-T_7 probe toward its target DNA. As a reference, we did not observe any difference in the fluorescence spectrum of an EthBr (300.0 nM) solution lacking the T_7-MB-T_7 after the addition of either DNA_{pm1} or DNA_{mm1}. We note that EthBr intercalates much more strongly with dsDNA (or folded DNA) than it does with ssDNA.

3.2. Optimal conditions

The sensing capability of our T_7-MB-T_7 probe toward DNA depends on the relative strength of the complexes of the T_7-MB-T_7 stem with Hg^{2+} and of its loop region with targeted DNA strands. Because the stability of the folded structure of T_7-MB-T_7 with Hg^{2+} is dependent on the Hg^{2+} concentration, we expected that the concentration of Hg^{2+} would play a role in determining the specificity and sensitivity of our T_7-MB-T_7 probe. We investigated (Fig. 2A) the effect of Hg^{2+} at various concentrations (0–10 μM) on the value of $(I_F - I_{F0})/I_{F0}$ of EthBr (1.0 μM) in the presence of DNA molecules and T_7-MB-T_7 (100 nM), where I_{F0} and I_F are the fluorescence intensities of the complexes of EthBr with T_7-MB-T_7 in the absence and presence of the targeted DNA, respectively. Upon increasing the Hg^{2+} concentration, the fluorescence intensity decreased, suggesting that the hairpin DNA structure was more stable in the presence of higher concentrations of Hg^{2+}. As a control, we used a traditional DNA-MB (MB_{C1}) to detect the target DNA under the same experimental conditions. MB_{C1} exhibited a high fluorescence background and, thus, its fluorescence enhancement upon binding the targeted DNA was relatively low (Figure S1, Supporting Information). When compared with the fluorescence behavior of the T_7-MB-T_7 probe solution in the presence of Hg^{2+}, the higher fluorescent background in the MB_{C1} system arose because more EthBr molecules intercalated with MB_{C1} in the stem region. We note that Hg^{2+} competes with EthBr to interact with DNA. The curves in Fig. 2A also suggest that the concentration of Hg^{2+} is an important factor determining the specificity of the T_7-MB-T_7. To determine the optimal Hg^{2+} concentration under the tested conditions, we plotted $(I_{F1} - I_{F0})/(I_{F2} - I_{F0})$ against the Hg^{2+} concentration, where I_{F0}, I_{F1}, and I_{F2} are the fluorescence intensities of the solutions in the absence of targeted DNA, the presence of DNA_{pm1}, and the presence of DNA_{mm1}, respectively. A higher value of this ratio indicates a greater specificity of the T_7-MB-T_7 probe toward DNA_{pm1} over DNA_{mm1}. The curve (open circles) in Fig. 2A displays that the value of $(I_{F1} - I_{F0})/(I_{F2} - I_{F0})$ reached a maximum at a Hg^{2+} concentration of 5.0 μM. At higher concentrations (e.g., 7.5 μM), the interactions of T_7-MB-T_7 with Hg^{2+} are stronger than those with the target DNA, thereby reducing its ability to recognize the target DNA.

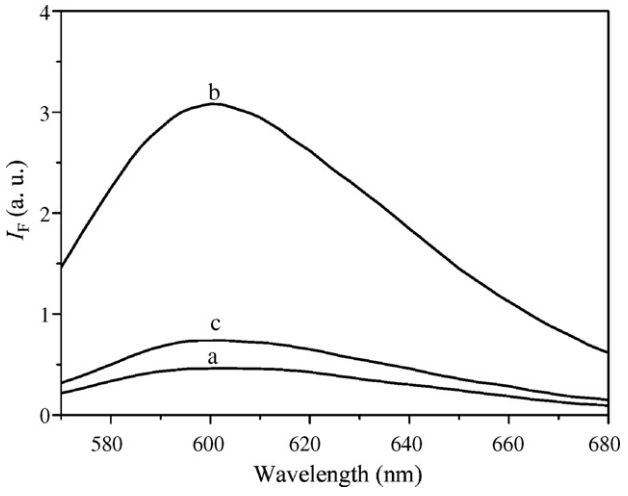


Fig. 1. Fluorescence spectra of T_7-MB-T_7 (100.0 nM) in the (a) absence of target DNA and (b and c) presence of (b) DNA_{pm1} (100.0 nM) and (c) DNA_{mm1} (100.0 nM) in 5.0 mM Tris–HCl (pH 7.4) containing 20.0 mM NaCl, 5.0 μM Hg^{2+}, and 300.0 nM EthBr.

Next, we investigated the impact of the pH and the concentration of NaCl on the performance of our T_7-MB-T_7 probe toward DNA_{pm1}. Fig. 2B suggests that the value of $(I_F - I_{F0})/I_{F0}$—and, hence, the specificity of the T_7-MB-T_7 probe toward DNA_{pm} over DNA_{mm1}—was optimal at pH 7.4, mainly because T–Hg^{2+}–T bonding occurs preferably at pH 6.0–8.0 (Miyake et al., 2006). We note that Hg^{2+} binds directly to N3 of the T moiety and bridges two T residues to form a T–Hg^{2+}–T pair. Upon increasing the concentration of NaCl from 0 to 50 mM, the efficiency of hybridization increased and, thus, the value of $(I_F - I_{F0})/I_{F0}$ increased (Figure S2). Further increases in the NaCl concentration (>50 mM) led to the efficiency of the T_7-MB-T_7 probe decreasing as a result of salt screening. From the curve of $(I_F - I_{F0})/I_{F0}$ plotted against the NaCl concentration, we determined that the optimal NaCl concentration was 20 mM (Figure S2, Supporting Information).

We also compared the performance of EthBr with some other DNA-binding dyes, namely TOTO-3, YOYO-3, and OliGreen (OG), for the determination of target DNA molecules using the T_7-MB-T_7

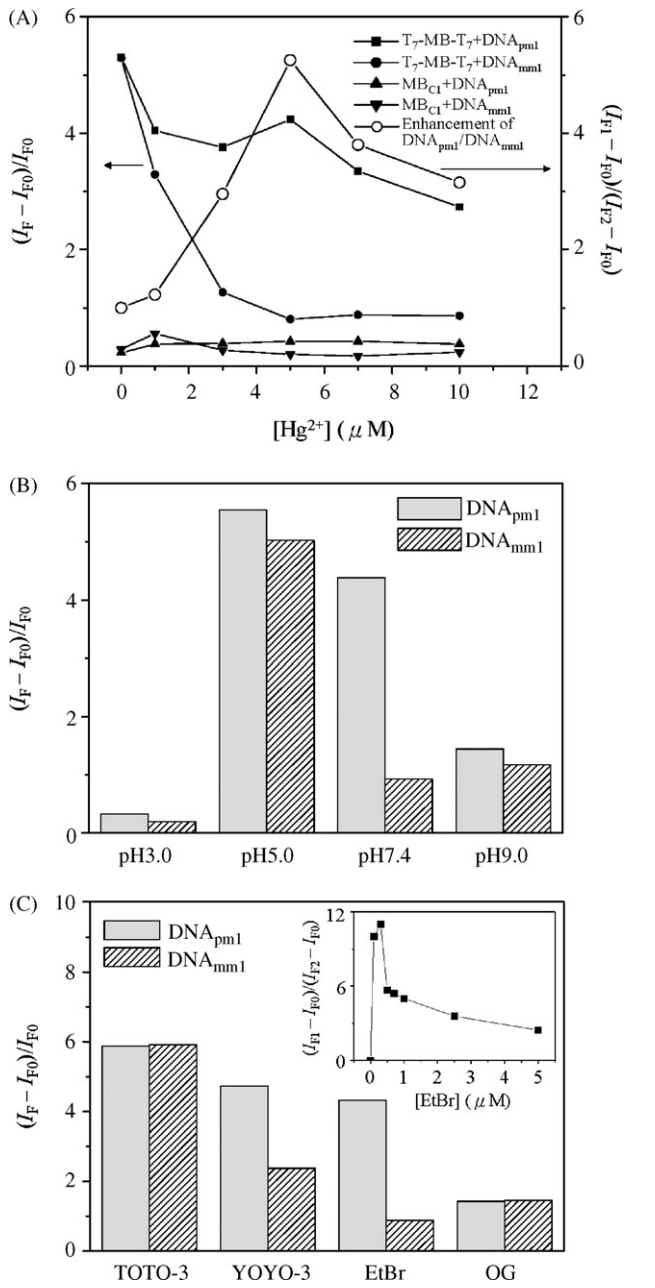


Fig. 2. (A) Plots of the values of $(I_F - I_{F0})/I_{F0}$, with respect to the concentration of Hg^{2+}, of T_7-MB-T_7 and MB_{C1} solutions containing EthBr (1.0 μM) in the presence of DNA_{pm1} (100 nM) or DNA_{mm1} (100 nM) and of $(I_{F1} - I_{F0})/(I_{F2} - I_{F0})$ of T_7-MB-T_7 probe solutions (100 nM) containing EthBr in the presence of DNA_{pm1} (I_{F1}) or DNA_{mm1} (I_{F2}). I_{F0} and I_F are the fluorescence intensities of the solutions in the absence and presence of the targeted DNA, respectively. (B) Values of $(I_F - I_{F0})/I_{F0}$ of T_7-MB-T_7 in the presence of either DNA_{pm1} or DNA_{mm1} plotted with respect to the pH. (C) Plots of the values of $(I_F - I_{F0})/I_{F0}$ of T_7-MB-T_7 solutions containing various fluorophores (1.0 μM) in the presence of DNA_{pm1} or DNA_{mm1}. Inset: plot of the values of $(I_{F1} - I_{F0})/(I_{F2} - I_{F0})$ for T_7-MB-T_7 in the presence of DNA_{pm1} and DNA_{mm1} with respect to the concentration of EthBr (0–5.0 μM).

probe. Of these dyes, the specificity of the T_7-MB-T_7 probe toward DNA_{pm} over DNA_{mm} was best when using EthBr (Fig. 2C). We note that both TOTO-3 and YOYO-3 bind to DNA with greater affinity (K_a = ca. 10^9 and ca. 10^7 M^{-1}, respectively) than does EthBr (K_a = ca. 10^6 M^{-1}) (Bowen and Woodbury, 2003; Mecklenburg et al., 1997; Joseph et al., 1996).

The lower specificity of the T_7-MB-T_7 probe toward DNA_{pm} over DNA_{mm} when using the higher-affinity intercalating dyes suggests that stronger intercalators stabilize the hybridization of T_7-MB-T_7 with its targeted DNA and, therefore, decrease the specificity of the T_7-MB-T_7 probe (Boger et al., 2001; Wang et al., 2005; Chen et al., 2008). We further investigated the effect of the EthBr concentration on the specificity of the T_7-MB-T_7 probe. The fluorescence intensities of the solutions increased (spectra not shown) upon increasing the EthBr concentration either in the absence or in the presence of its targeted DNA. The inset to Fig. 2C indicates that the specificity of T_7-MB-T_7 reached a maximum at 300.0 nM EthBr. At higher EthBr concentrations, fluorescence background was greater; poor specificity occurred, mainly because greater numbers of intercalated EthBr molecules further stabilized the hybridization of T_7-MB-T_7 with DNA_{pm} or with DNA_{mm}; the use of too much carcinogenic EthBr might also be problematic from a practical point of view. Thus, the T_7-MB-T_7 probe providing optimal specificity comprised 100.0 nM T_7-MB-T_7 in 5.0 mM Tris–HCl solution (pH 7.4) containing 5.0 μM Hg^{2+}, 20.0 mM NaCl, and 300.0 nM EthBr.

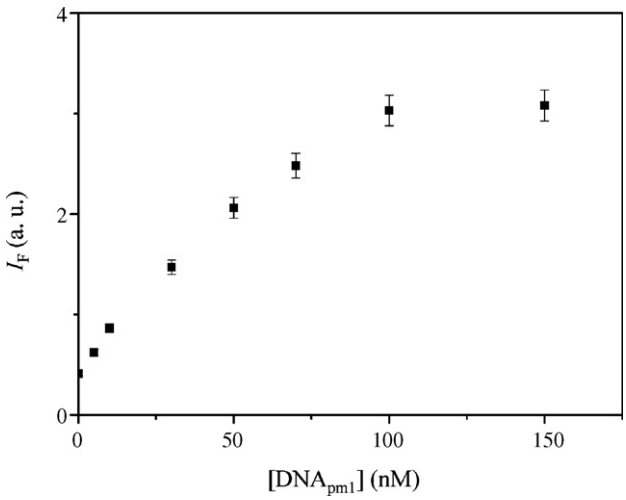


Fig. 3. Fluorescence intensity of EthBr at 605 nm in T_7-MB-T_7 probe solutions plotted with respect to the DNA_{pm1} concentration. 10 measurements were conducted at each DNA_{pm1} concentration. Other conditions were the same as those described in Fig. 1.

3.3. Sensitivity and specificity

Under the optimal conditions, we investigated the sensitivity of the T_7-MB-T_7 probe toward DNA_{pm}. We obtained a linear response of the fluorescence intensity with respect to the concentration of DNA_{pm} (Fig. 3) over the range 5.0–100 nM (R^2 = 0.98). Then, we calculated the limit of detection (LOD) using the equation, $S_m = S_{b1} + ks_{bl}$, in which S_{b1} is the average blank (10 measurements) and s_{bl} is the standard deviation of the blank. The values of S_{b1} and s_{bl} were 41 and 1.4, respectively. We then calculated S_m to be 45.2, based on 3-times of s_{bl} (k = 3). Finally, we obtained the LOD to be 1.0 nM by using the linear equation (y = 4.42 x + 40.8). Fig. 4A indicates that the selectivity of T_7-MB-T_7 and MB_{C2} toward DNA_{pm1} over DNA_{mm1} at ambient temperature were 9.8- and 1.3-fold, respectively. When conducting the assay at 35 °C (optimal conditions), the selectivity of MB_{C2} toward DNA_{pm1} over DNA_{mm1} was 3.6-fold. We also modified the fluorophores (FAM and DABCYL) on the two termini of $MB_{C3\text{-}6}$ (Table 1) to compare the performance of the standard MBs with T_7-MB-T_7 in SNPs study. We changed the concentrations of NaCl (5–100 mM) and temperatures (25–75 °C) to obtain the optimal conditions for these standard beacons. Because the T_m values of MB_{C3} and MB_{C5} (the contents of GC pairs in the stem are 0 and 29%, respectively) are both lower than 10 °C, there is thus no need to conduct the sensing at higher temperature. The optimum conditions for MB_{C4} and MB_{C6} (GC pairs in stem: 100 and 86%, respectively) were 80 and 50 mM NaCl at 55 and

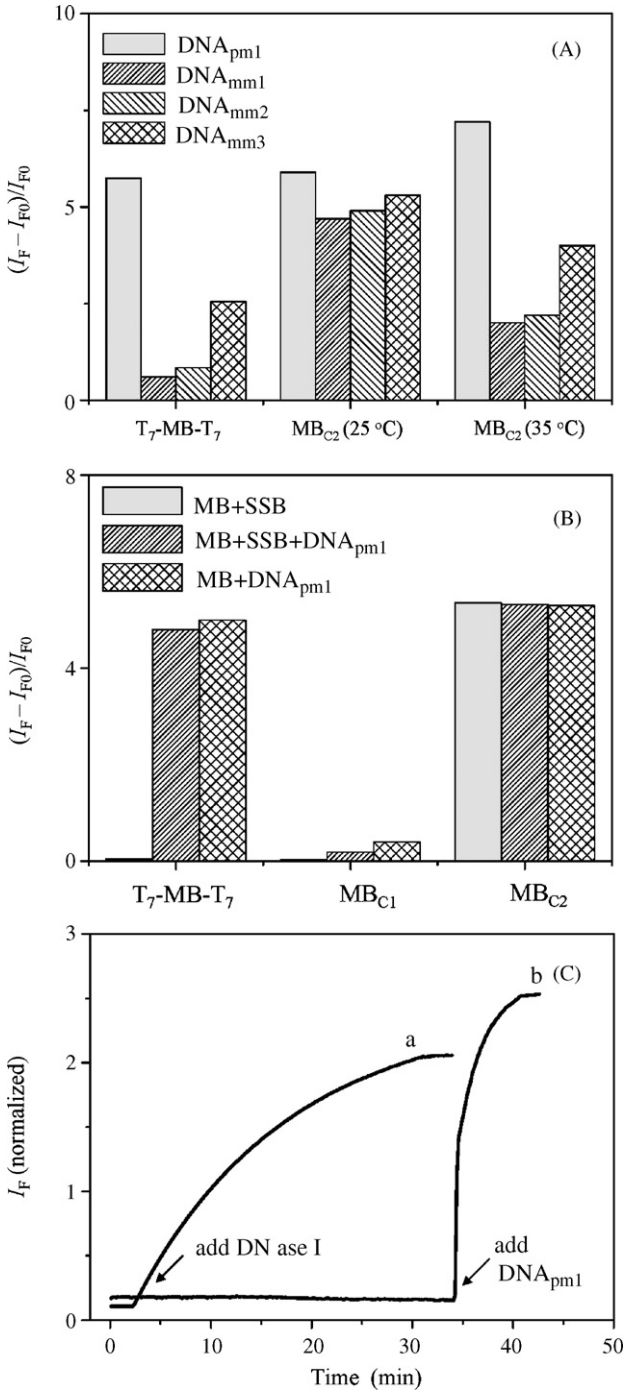


Fig. 4. (A) Fluorescence enhancements of T_7-MB-T_7 probe solutions containing EthBr and MB_{C2} in the presence of DNA_{pm1}, DNA_{mm1}, DNA_{mm2}, and DNA_{mm3} (100.0 nM) at ambient temperature and 35 °C, respectively. (B) Fluorescence responses of T_7-MB-T_7, MB_{C1}, and MB_{C2} (100.0 nM) solutions containing EthBr in the presence and absence of SSB (100.0 nM). (C) Resistance to DNase I (2.5 μg/mL)-mediated digestion of (a) MB_{C2} and (b) T_7-MB-T_7 (100 nM) in the presence of Hg^{2+} and 5.0 mM $MgCl_2$. Other conditions were the same as those described in Fig. 1.

50 °C, respectively. Their selectivity values were 2.8 and 4.1, respectively. These results indicated the selectivity of the standard MBs was highly dependent on the DNA sequences and temperature. When compared to those of the standard MBs, the selectivity of T_7-MB-T_7 was less dependent on temperature, mainly because the T–Hg^{2+}–T complex was more stable than the double stranded DNA at high temperature. We further tested our probe toward other target DNAs (DNA_{mm4-9}; Table 1) that have different mismatched sequences. The selectivity values of T_7-MB-T_7 toward DNA_{pm1} over DNA_{mm4-9} at ambient temperature were 4.5, 4.9, 4.2, 8.4, 5.9, and 14.8, respectively. Because Hg^{2+} and EthBr both interacted with the single stranded DNA and doubled stranded DNA (DNA probe and target DNA), they played some roles in determining the selectivity of our probe. When compared to the normal MBs, the T_7-MB-T_7 is advantageous; without conducting the assay at high temperature. We compared the resistance of T_7-MB-T_7 and the conventional MBs (MB_{C1} and MB_{C2}) to interactions with the nonspecific binding protein SSB. The presence of SSB did not cause significant changes in the fluorescence intensities of EthBr in solutions containing MB_{C1} in the presence or absence of DNA_{pm1}, suggesting that no interactions occur between MB_{C1} and DNA_{pm1} in the presence of SSB. In contrast, SSB caused increases in the fluorescence intensity of MB_{C2} in the presence and absence of DNA_{pm1}, mainly because MB_{C2} forms a random coiled structure upon its interaction with SSB (Leonetti et al., 1991; Fisher et al., 1993), leading to the false-positive signal in Fig. 4B. In contrast, the T_7-MB-T_7 did not respond to the addition of excess SSB, but the value of $(I_F - I_{F0})/I_{F0}$ increased once DNA_{pm} was added, indicating that our probe should be superior to conventional MBs for the detection of its target DNA in biological samples. We also compared the stabilities of the T_7-MB-T_7 and conventional MB probes in the presence of DNase I. Whereas MB_{C2} degraded rapidly once DNase I was added (curve a, Fig. 4C), the T_7-MB-T_7 degraded only slightly during the first 30 min (curve b), mainly because of the greater stability of folded DNA relative to that of ssDNA (Kim et al., 2007). Nevertheless, the use of toxic Hg^{2+} ions, albeit in small amounts, in our probe system is a disadvantageous feature. This disadvantage can be overcome by using different DNA sequences that respond to the presence of lower toxicity metal ions such as Ag^+, Cu^{2+}, and K^+ ions (Ono and Miyake, 2001; Meggers et al., 2000; Huang and Chang, 2008). Furthermore, we also believe that this approach can serve as a foundation for the development of practical DNA chip for high throughput SNPs screen.

We further tested the impacts that the DNA sequences in the MBs have on SNPs study. To study the impact of the length of stem (number of thymidine) on SNPs study, T_6-MB-T_6 and T_8-MB-T_8 were tested. The optimal concentrations of Hg^{2+} for T_6-MB-T_6 and T_8-MB-T_8 were 3.0 and 7.0 μM, respectively (Figure S3A). The LODs and specificities of T_8-MB-T_8 for the targeted DNA (DNA_{pm1}, Table 1) were close to those of T_7-MB-T_7, which were better than those of T_6-MB-T_6 for the targeted DNA (DNA_{pm1}). When thymidine chain in the stem was too short, the stability of hairpin structure is too weak to compete with the hybridization of the probe to the target DNA. On the other hand, when the chain length is too long, it is difficult for the hybridization of the probe with the target DNA to occur. We also discussed the impact of GC contents in the stems on the specificity of our approach toward target DNA. The percentages of GC pairs in the stems of MB_{C1}, MB_{C3}, MB_{C4}, MB_{C5}, and MB_{C6} were 57, 0, 100, 29, and 86%. The selectivity values for these five probes toward the perfect match DNAs (DNA_{pm1}) over mismatch DNAs (DNA_{mm1}) were 2.4, 2.3, 1.9, 2.4, and 2.1, respectively, all providing poor selectivity when compared to the thymidine-based MBs. Next, we kept the stem as the same (T_7), but changed the GC contents in the loop of MBs (T_7-MB_1-T_7 and T_7-MB_2-T_7). We point out that the percentages of GC pairs in the loops of T_7-MB-T_7, T_7-MB_1-T_7, andT_7-MB_2-T_7 were 42, 21, and 63%, respectively. We observed that the specificity and sensitivity for the target DNA are also dependent on the contents of GC pairs in the loop of the MBs (Figure S3B). The optimal concentrations of Hg^{2+} with respects to the specificity and sensitivity of the T_7-MB-T_7, T_7-MB_1-T_7 and T_7-MB_2-T_7 for the DNA_{pm} were 5.0, 1.0 and 20 μM Hg^{2+}, respectively. The results suggest that higher concentrations of Hg^{2+} are required when the GC contents in the MBs are higher. The selectivity values for these three probes toward the perfect match DNAs (DNA_{pm1}, DNA_{pm2}, and DNA_{pm3}) over mismatch DNAs (DNA_{mm1}, DNA_{mm10}, and DNA_{mm11}) were 9.8,

4.4, and 8.2 and LODs for the perfect match DNAs were 1.0, 3.5, and 2.1, respectively. Our results suggest that in our MBs the length of stem plays more important roles than that of the sequence in the loop in determining their specificity and sensitivity for DNA. We also suggest that MBs having high GC contents are not proper from the view point of safety because greater amounts of Hg^{2+} are required.

To test the practicality, we used another DNA probe (T_7-MB_t-T_7) that can recognize the fumarylacetoacetate hydrolase (FAH) gene. Mutation of this gene is associated with a human genetic disease, hereditary tyrosinemia type I (St-Louis and Tanguay, 1997; Phaneuf et al., 1992). The sequence of the new probe T_7-MB_t-T_7 is complementary to the wild type of this gene (DNA_{pmt}: 5′-CCGGTGA*G*TATCTGG-3′). Figure S4 reveals that our probe is sensitive to this gene, with an LOD of 1.0 nM. Like T_7-MB-T_7, the T_7-MB_t-T_7 is resistant to nonspecific interactions with SSB and stable in the presence of DNase I. We tested the selectivity of our T_7-MB_t-T_7 probe by measuring its affinity toward a mutated DNA (DNA_{mmt}: 5′-CCGGTGA*A*TATCTGG-3′). The inset to Figure S4 displays that the selectivity of our probe toward DNA_{pmt} over DNA_{mmt} was greater than 4.0-fold.

4. Conclusion

We have developed a simple, cost-effective, sensitive, and selective assay—using T_7-MB-T_7 and T_7-MB_t-T_7 probes in the presence of Hg^{2+} and EthBr—for studying SNPs. The sensing specificity is dependent on a competition between the target DNA strand and Hg^{2+} ions for interaction with the T_7-MB-T_7. We have demonstrated that the T_7-MB_t-T_7 is sensitive and selective, with an LOD of 1.0 nM, for the detection of a gene associated with hereditary tyrosinemia type I. Unlike conventional approaches, there is no need to use fluorescently labeled MBs in our system. In addition, the T_7-MBs probes are much more resistant to nonspecific binding with SSB and to nuclease digestion. We envision that T_7-MB probes will be useful for practical studies of SNPs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2009.01.003.

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