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

Chi-Wei Liu [a], Yang-Wei Lin [a], Chih-Ching Huang [b], Huan-Tsung Chang [a,*]

[a] Department of Chemistry, National Taiwan University, 1, Section 4, Roosevelt Road, Taipei 106, Taiwan
[b] Institute of Bioscience and Biotechnology, National Taiwan Ocean University, 2, Beining Road, Jhongjheng District, Keelung 202, Taiwan

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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

* Corresponding author. Tel.: +11 886 2 33661171; fax: +11 886 2 33661171.
E-mail address: changht@ntu.edu.tw (H.-T. Chang).

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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