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Visual detection of gene mutations based on isothermal strand-displacement polymerase reaction and lateral flow strip

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ABSTRACT

Here, we describe a simple and sensitive approach for visual detection of gene mutations based on isothermal strand-displacement polymerase reactions (ISDPR) and lateral flow strip (LFS). The concept was first demonstrated by detecting the R156H-mutant gene of keratin 10 in Epidermolytic hyperkeratosis (EHK). In the presence of biotin-modified hairpin DNA and digoxin-modified primer, the R156H-mutant DNA triggered the ISDPR to produce numerous digoxin- and biotin-attached duplex DNA products. The product was detected on the LFS through dual immunoreactions (anti-digoxin antibody on the gold nanoparticle (Au-NP) and digoxin on the duplex, anti-biotin antibody on the LFS test zone and biotin on the duplex). The accumulation of Au-NPs produced the characteristic red band, enabling visual detection of the mutant gene without instrumentation. After systematic optimization of the ISDPR experimental conditions and the parameters of the assay, the current approach was capable of detecting as low as 1-fM R156H-mutant DNA within 75 min without instrumentation. Differentiation of R156H- and R156C-mutant DNA on the R156 mutation site was realized by using fluorescein- and biotin-modified hairpin probes in the ISDPR process. The approach thus provides a simple, sensitive, and low-cost tool for the detection of gene mutations.

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1. Introduction

The detection of gene mutations and specific DNA sequences is of central importance for the diagnosis and treatment of genetic diseases, for the detection of infectious agents, and for reliable forensic analysis (Palecek and Fojta, 2001). Various methods and technologies, such as Northern, Southern and Western blotting; agarose and polyacrylamide gel electrophoresis (Nelson and Cox, 2000); DNA biosensors (Leung et al., 2007); DNA biochips (Brown and Botstein, 1999); polymerase chain reactions (PCR) (Kaltenboeck and Wang, 2005); ligation (Toubanaki et al., 2009); primer extension (Hoogendoorn et al., 1999); and endonuclease digestion (Lyamichev et al., 1999), have been developed for this purpose. Because of high sensitivity and specificity, PCR has become the gold standard in clinical diagnostics and the food industry. However PCR requires precise control of temperature cycling for successful DNA amplification, and the resultant instrumental restraint has been hampering its wider and more versatile applications (point-of-care use in hospitals and miniaturized systems for high-throughput analysis) (Gill and Ghaemi, 2008). Therefore, there is a need for a suitable and cost-effective analytical technology to carry out a rapid, simple, and sensitive analysis of gene mutations and DNA sequences.

Isothermal nucleic acid amplification technologies have been developed to overcome the disadvantages of PCR (Hellyer and Nadeau, 2004). Particularly, isothermal strand-displacement polymerase reaction (ISDPR) in connection with different detection platforms (fluorescence and electrochemistry) has attracted considerable interest (Guo et al., 2009; He et al., 2010a). ISDPR is based on an isothermal amplification process which yields large amounts of labeled DNA products to enhance the signal and the sensitivity of DNA detection (Hellyer and Nadeau, 2004). Although the ISDPR-based fluorescent and electrochemical DNA assays offer high specificity and sensitivity, there are still many challenges. For example, the detection still needs expensive instruments or skilled personnel, preventing its in-field or point-of-care applications, particularly in developing countries. In addition, the false initiation of ISDPRs would result in a high background signal.

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Recently, we reported a sensitive, portable nucleic-acid biosensor based on oligonucleotide-modified gold nanoparticle (Au-NP) and a conventional lateral flow device for visual detection of DNA (Mao et al., 2009); the sensitivity and ability to discriminate single base-mismatched DNA and perfect-matched DNA with the device have been explored by using enzyme-Au-NP dual label and hairpin oligonucleotide-modified Au-NPs, respectively (He et al., 2010b, 2011). However, the sensitivities of such visual detection are still relatively low compared with PCR-based and fluorescence-based isothermal nucleic acid amplification methods. In this article, we report a simple and sensitive approach based on ISDPR and the lateral flow strip (LFS) for the determination of R156 mutations (R156H and R156C) of keratin 10 in Epidermolytic hyperkeratosis (EHK, Mendelian Inheritance in Man no. 113800). EHK is an autosomal dominant skin disease which is caused by mutations in the genes encoding the keratin 1 gene (KRT 1) or the keratin 10 gene (KRT 10) (Rothnagel et al., 1992; Syder et al., 1994). Recent results from the pertinent published work and the Human Gene Mutation Database have shown that the 156 site of the KRT 10 gene is a mutation hot spot. R156H (CGC to CAC, arginine to histidine) and R156C (CGC to TGC, arginine to cysteine) are the most frequent mutations of KRT 10 (Haruna et al., 2007). The concept was first demonstrated by using a biotin-modified hairpin probe and a digoxin-modified primer to determine the R156H-mutant DNA. Visual differentiation of R156H- and R156C-mutant DNA was realized by using fluorescein- and biotin-modified hairpin probes. The attractive characteristics of the new approach are reported in the following sections.

2. Materials and methods

2.1. Materials

The Airjet AJQ 3000 dispenser, Biojet BJQ 3000 dispenser, Clamshell Laminator, and Guillotine cutting module CM 4000 were from Biodot, Ltd. (Irvine, CA). A portable strip reader (DT1030) was purchased from Shanghai Goldbio Tech. Co., Ltd. (Shanghai, China). The polymerase Klenow fragment exo$^-$ was purchased from New England Biolabs, Inc. The deoxynucleotide solution mixture (dNTPs), dithiothreitol (DTT), dimethyl-sulfoxide (DMSO), anti-biotin antibody, anti-fluorescein antibody, anti-digoxin antibody, human serum albumin (HSA), sucrose, hydroxylamine, Tween 20, Triton X-100, trisodium citrate, bovine serum albumin (BSA), sodium chloride–sodium citrate (SSC) Buffer 20× concentrate (pH 7.0), Tris/Borate/EDTA (TBE) buffer, $HAuCl_4$, and phosphate buffer saline (PBS, pH 7.4, 0.01 M) were purchased from Sigma–Aldrich (St. Louis, MO). Glass fibers (GFCP000800), cellulose fiber sample pads (CFSP001700), laminated cards (HF000MC100), and nitrocellulose membranes (HFB18004) were purchased from Millipore (Billerica, MA). NuSeive® GTG® agarose was purchased from Cambrex Bio Science (Rockland, ME). The DNA oligonucleotide probes and digoxin-modified primers were obtained from Integrated DNA Technologies, Inc. (Coralville, IA). The oligonucleotide sequences were as follows:

R156H-mutant DNA: 5′-CTGAATGAC**CAC**CTGGCTGTGTCC-3′

R156C-mutant DNA: 5′-CTGAATGAC**TGC**CTGGCTGTGTCC-3′

Wild-type DNA: 5′-CTGAATGAC**CGC**CTGGCTGTGTCC-3′

Noncomplementay DNA: 5′-TGCAAGGTGTCAGTATAATCCGAC-GTTTT-3′

Hairpin probe to R156H-mutant DNA:

5′-Bio-TCTTGGACACAGCCAGGTGGTCATTCAGTGTGTCCAAGA-3′

Hairpin probe to R156C-mutant DNA:

5′-Flu-TCTTG GACACAGCCAGGCAGTCATTCAGTGTGTCCAAGA-3′

Primer: 5′-/5DigN/TCTTGGAC-3′

All chemicals used were analytical reagent grade. Other solutions were prepared with ultrapure (>18 MΩ) water from a Millipore Milli-Q water purification system (Billerica, MA).

2.2. Preparation of anti-digoxin–Au-NP conjugates and lateral flow strip (LFS)

Au-NPs with average diameter 15 ± 3.5 nm were prepared according to the reported methods with slight modifications (He et al., 2011, also see the details in supporting information). Conjugation reactions were conducted by adding 5 μL of 10 mg mL^{-1} anti-digoxin antibody into 1 mL of a tenfold-concentrated Au-NP solution (pH 8.4) followed by incubation at room temperature with periodic gentle mixing for 1 h. Then, a certain volume of 10% BSA was slowly added to the mixture solution to obtain a final concentration of 1%. After gentle stirring for 30 min, the solution was centrifuged at 13,000 × *g* for 15 min. Two phases can be obtained: a clear to pink supernatant of unbound antibodies and a dark red, loosely packed sediment of the anti-digoxin–Au-NP conjugates. The supernatant was discarded, and the soft sediment of anti-digoxin–Au-NP conjugates was rinsed by resuspending in 1 mL of PBS–BSA and collected after a second centrifugation at 13,000 × *g* for 15 min. Finally, the conjugate was resuspended in a 1 mL buffer containing 20 mM of sodium phosphate, 0.25% Tween-20, 10% sucrose, and 5% BSA, and the conjugate was stored at 4 °C before further use. LFS was prepared according to the reported methods with slight modifications (He et al., 2011, also see the details in supporting information).

2.3. Preparation of digoxin- and biotin-attached duplex DNA complexes with ISDPRs

Briefly, ISDPR was performed in a 100-μL, 50-mM Tris–HCl (pH 8.0) buffer consisting of 5.0×10^{-8} M biotin-modified hairpin probe, 5.0×10^{-8} M digoxin-modified primer, 3 U polymerase Klenow fragment exo$^-$, 50 μM dNTPs, 6% DMSO, 0.1% BSA, 1 mM DTT, and 5 mM $MgCl_2$. R156H-mutant DNA at different concentrations was then added to every mixture sample solution and incubated at 42 °C for 1 h.

2.4. Gel electrophoresis

Gel electrophoresis of the ISDPR product was conducted with a 4% gel (3% NuSeive GTG agarose + 1% agrose) prepared in 1× TBE (pH 8.3) at 65 V constant voltage for about 4.5 h. After ethidium bromide staining, gels were scanned using the Chemi Genius Bio Imaging System (Syngene, NJ).

2.5. Visual detection of ISDPR products with LFS

A 100-μL running buffer (10 mM Tris–HCl solution (pH 8.0) containing 5 mM $MgCl_2$, 80× SSC, and 0.5% BSA) was mixed with 10 μL of ISDPR product solution in a 1.5-mLcentrifuge tube; then, a LFS was dipped into the mixture. After waiting for 10 min, 60 μL of running buffer were added to wash the LFS. The bands were visualized within 5 min. The intensities of the red bands on the LFS were quantified with a portable strip reader. The optical intensities of the test and the control lines were recorded simultaneously by using "Gold-Bio strip reader" software, which could search the red bands in a fixed reaction area automatically and then figure out parameters such as peak height and area integral.

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