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Fluorometric detection of mutant DNA oligonucleotide based on toehold strand displacement-driving target recycling strategy and exonuclease III-assisted suppression

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ABSTRACT

We describe here a fluorometric assay for sensitive detection of oligonucleotides, based on a target recycling amplification strategy driven by toehold-mediated strand displacement reaction and on exonuclease III (Exo III)-assisted fluorescence background suppression strategy. The network consists of a pair of partially complementary DNA hairpins (HP_1 and HP_2) with 3′ overhang ends, between which the spontaneous hybridization is kinetically hindered by the stems. The target DNA is repeatedly used to trigger a recycling progress between the hairpins, generating numerous HP_1–HP_2 duplex complexes. Exo III was then employed to digest the double strand parts of the residual hairpins and the intermediate products. The fluorescent dye, SYBR Green I, binds to the double-strand DNA products and emits strong fluorescence to achieve sensitive detection of the target DNA with the detection limit of 5.34 pM. Moreover, this proposed strategy showed high discrimination efficiency towards target DNA against mismatched DNA and was successfully applied in the analysis of human serum sample.

1. Introduction

Amplified detection of DNA oligonucleotide has recently attracted substantial research interest in the field of bioanalytical science due to its wide applications in molecular diagnostics, genetics therapy, forensic investigations, and biomedical development (Willner et al., 2009; Bi et al., 2012). The sensitivity-improved sensors reported so far fall into two categories: amplified signal and restrained background (Lu et al., 2011).

In the first case, great efforts have been expended to develop various signal amplified methods. A splendid instance of signal amplification is the employment of various protein enzymes (Freeman et al., 2011; Lin et al., 2011; Liu et al., 2012a, 2012b; Ma et al., 2014; Wang et al., 2014a; Ren et al., 2015; Sun et al., 2015) as well as DNAzymes (Liu and Lu, 2007; Teller et al., 2009; Zhao et al., 2011; Wang et al., 2014b; Lu et al., 2015) to attain large signal-to-noise ratios by enhancing optical or electrochemical signal. Besides, amplification strategies are widely applied in the area of nucleic acid research (Wolcott, 1992; Abramson and Myers, 1993; Zhang et al., 2013; Jung and Ellington, 2014; Li et al., 2014a, 2015d), such as traditional polymerasechain reaction (PCR) (Mullis and Faloona, 1987), ligase chain reaction (LCR) (Barany, 1991), rolling circle amplification (RCA) (Fire and Xu, 1995), and recently developed strand displacement reaction (SDR) (Walker et al., 1992a, 1992b). The main advantage of SDR lies in its being robust, easy-to-use and inexpensive, in comparison with the other three methods which always involve multiple assay steps and require many additional reagents. One kind of SDR strategies that are widely adopted recently is the one known as toehold-mediated strand displacement. Toehold is a short single-strand stretching region that leads up to the SDR (Zhang and Winfree, 2009). The toehold initiated hybridization, which can enhance the rate of the SDR by a fold of 10^6, is triggered by the overhang toehold, to which the target sequence attaches and then compels the former matched branch migrate away, forming a more thermodynamically stable duplex (Genot et al., 2011). To realize the recycling amplification, a pair of partially complementary DNA hairpins (HP_1 and HP_2) is admirably designed and applied (Feng et al., 2013; Zhang et al., 2013; Li et al., 2015a). Briefly, the spontaneous hybridization between the paired hairpins is kinetically blocked by the stems. But the hindrance is dissipated in the presence of an inducing strand and two toehold-mediated SDRs could be initiated

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successively between the inducing strand and HP_1, as well as unfolded-HP_1 and folded-HP_2, respectively. Thus, the inducing strand is released for further recycle. Wonderful although the invention is, it relies on a labeled dsDNA to sense the signal. Thus, further study could be done to make the strategy simpler and cheaper, where unmodified oligonucleotides that lack covalently fluorophore and/or quencher units are adopted. Recently, many research efforts have been made to develop approaches for the fluorescence detection of various target molecules, based on oligonucleotides that are free from covalently modification of fluorophore and/or quencher units (Jiang et al., 2004; Zayats et al., 2006; Li et al., 2013; Ma et al., 2013). But these signal amplified strategies may suffer from elevated background fluorescence signals

In the second case, nanomaterials such as magnetic particles (Luo et al., 2012a; Deng et al., 2013; Persano et al., 2013; Li et al., 2015b), graphene oxide (Lu et al., 2009; Wu et al., 2009; Dong et al., 2010; Li et al., 2010, 2014b, 2015c; Wang et al., 2010; Hong et al., 2015; Zhu et al., 2015), nanoparticles (Shi et al., 2013; Luo et al., 2012b; Qiang et al., 2014, 2015; Torabi and Lu, 2014; Wei et al., 2015), are generally applied as quenchers to obtain lower background. Fascinating as developing a zero-background biosensor is, it remains a challenge. To our best knowledge, few research efforts are poured into employing enzymes to lower the background of fluorescent biosensors and thus make the sensitivity higher.

For all we know, approaches combining these two strategies within one system, i.e., to amplify the signal and suppress the background simultaneously, have been attractive but still need more study. Here, our work makes a step toward this attraction by coupling SDR mediated signal amplifying strategy with exonuclease Ⅲ-assisted background signal rejecting method, where an approach has been established for fluorescence detection of oligonucleotide, taking tumor-related p53 (the source of the target oligonucleotide is given in Supplementary material) gene as a model (Hollstein et al., 1991; Kern et al., 1992; Aguilar et al., 1993; Kirby et al., 1996; Liu et al., 2014). We chose SYBR Green I (SG) as the fluorescent dye, which binds to the SDR-produced duplexes and emits greatly enhanced fluorescence signals for sensitive detection of p53 gene.

2. Experimental section

2.1. Materials and reagents

2-Amino-2-(hydroxymethyl)-1.3-propanediol (Tris) was purchased from Aladdin Chemistry Co., Ltd., (Shanghai, China). Exo Ⅲ (specific activity, 100,000 units) was purchased from Invitrogen Biotechnology Co., Ltd., Shanghai, China. 6 × DNA loading buffer was bought from Solarbio Co., Ltd., Beijing, China. 10,000 × SYBR Green I (SG) was provided by Sangon Biotech Co., Ltd., Shanghai, China. The as-received SG stocking solution was about 19.8 mM, based on the Lambert–Beer measurement (Lin et al., 2015). A 19.8 μM SG dyeing solution was obtained by diluting the stocking solution with dimethyl sulfoxide (DMSO) and stored in darkness at −20 °C for use.

The DNA oligonucleotides were synthesized and HPLC-purified by Invitrogen Biotechnology Co., Ltd., Shanghai, China. The sequences of oligonucleotides are listed in Table S1 (Supplementary information). All DNA sequences were prepared by dissolving in a working buffer, Tris–HCl buffer (66 mM, pH 8.15) containing 6.6 mM $MgCl_2$, separately heated to 90 °C for 5 min, and then allowed to gradually cool down to room temperature before use (annealing). Healthy human serum sample was obtained from Southwest University Hospital of Chongqing, China, and then diluted ten folds with the working buffer.

2.2. Apparatus

Throughout the experiment, ultrapure water system was employed to produce purified 18.2 MΩ cm water. The pH of the working buffer was adjusted under the assistance of a pH-3B pH meter (Shanghai, Leici Instrument Company, Ltd., China). The experiment temperature was kept with a SD-101-005 DB super digital thermostat bath (Sida Experimental Equipment Ltd., Chongqing, China). All fluorescence measurements were performed on an F-2700 fluorescence spectrophotometer (Hitachi, Japan) equipped with an excitation light source of a 150 W Xenon lamp (Ushio Inc., Japan). Gel electrophoresis assays were performed using a Bio-Rad PowerPac-300 Power Supply, Bio-Rad minisubcellGT Electrophoresis Cell and Bio-Rad Universal Hood 2 DOC Electrophoresis Imaging Cabinet, Chemi, XR+ (Bio-Rad, USA).

2.3. Oligonucleotide sensing procedure

Various concentrations of target DNA were incubated with HP_1 (0.3 μM) and HP_2 (0.3 μM) in 90 μL of working buffer for 10 min at room temperature. After that, 25 U Exo Ⅲ in 10 μL of buffer was added making a hydrolyzing concentration of 0.25 U/μL and the mixtures were further incubated for 25 min at 37 °C. This was followed by the addition of 395 μL of working buffer and 5 μL of SG dyeing solution. The final mixed solution with a total volume of 500 μL was kept in darkness at room temperature for 10 min, and then the fluorescence intensity of each solution was measured.

2.4. Fluorescence measurements

The fluorescence measurements were performed at room temperature. The emission spectra were recorded in the range of 505–600 nm, with an excitation wavelength of 495 nm. The photomultiplier tube (PMT) voltage of the fluorescence spectrophotometer was 400 V, and the slit width was selected to be 10 nm for excitation and emission.

2.5. Polyacrylamide gel electrophoresis

To check the feasibility of our strategy, we freshly prepared seven solutions. Solution 1 contained 25 μM target DNA; solutions 2 and 3 included 3 μM HP_1 and HP_2, respectively; solution 4 was a mixture of 3 μM HP_1 and 3 μM HP_2; solution 5 consisted of 3 μM HP_1, 3 μM HP_2 and 1 U/μL Exo Ⅲ; solution 6 was of 3 μM HP_1, 3 μM HP_2 and 0.5 μM target DNA; and solution 7 was that of solution 6 adding Exo Ⅲ by a concentration of 1 U/μL. All the solutions were left reaction at room temperature for 10 min when only oligonucleotides were added, and then, incubated at 37 °C for 25 min after Exo Ⅲ was added.

Polyacrylamide gel (20%) was used to perform the polyacrylamide gel electrophoresis (PAGE) analysis by loading the samples mixed with 6 × loading buffer into the gel on a volume ratio of 1: 5. Gel electrophoresis separation was driven by a constant voltage of 120 V for 80 min in 1 × TBE buffer. After that, the gel was visualized with ethidium bromide staining and imaged by a Gel DOC XR+ system.

2.6. Application to real samples

To assess whether the proposed strategy can be applied in real clinical samples, healthy human dilute serum was adopted as model matrix and recovery tests were performed using the proposed method. The serum samples were diluted 10 times with the