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Label-free and ultrasensitive colorimetric detection of DNA based on target-triggered quadratic amplification strategy

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ABSTRACT

Highly sensitive detection of DNA plays a crucial role in biomedical research and clinical diagnosis. Herein, we developed a simple, label-free, isothermal, and ultrasensitive colorimetric method for amplified detection of DNA on the basis of a new quadratic amplification strategy. With the presence of three ingeniously designed hairpin structures and Exonuclease III (Exo III), the target DNA can trigger two independent cycles of reactions: hairpin assembly reaction and Exo III cleavage reaction, which are designed to initiate target DNA recycling amplification and reporter DNA amplification, respectively. Therefore, the proposed method exhibits a high sensitivity toward target DNA with a detection limit of as low as 81 fM, and it can discriminate mismatched DNA from completely matched target DNA. Furthermore, this method could be used as a universal tool for the detection of various DNA sequences and might be further extended for the detection of aptamer-binding molecules.

1. Introduction

Detection of trace amounts of DNA is of great importance to mutation analysis, clinical diagnosis, and gene therapy (Dong et al., 2012; Liu et al., 2013a; Xue et al., 2014). In order to improve detection sensitivity, signal amplification strategies are often employed in the fabrication of DNA biosensors (Liu et al., 2013a). Traditional amplification methods remain widely used in the field of nucleic acid research, such as polymerase chain reaction (PCR) (Mullis and Faloona, 1987), ligase chain reaction (LCR) (Barany, 1991), strand displacement amplification (SDA) (Walker et al., 1992), and rolling circle amplification (RCA) (Liu et al., 1996). Although these methods can achieve high sensitivity for target assays, they usually involve multiple assay steps and require the addition of many exogenous reagents (Xue et al., 2014). Thus, it is highly desired to exploit a new strategy that is simple, low-cost, and sensitive. Very recently, target-catalyzed hairpin assembly as a promising enzyme-free signal amplification strategy has been developed for DNA detection (Li et al., 2011, 2012; Zheng et al., 2012; Huang et al., 2012). To date, some fluorescent (Li et al., 2012; Huang et al., 2012) and electrochemical (Liu et al., 2013a; Qian et al., 2014) DNA biosensors have been developed on the basis of target-catalyzed hairpin assembly strategy. However, few reports are available on the development of colorimetric biosensors for DNA analysis.

DNAzymes are a class of functional artificial nucleic acids, which are isolated from an in vitro selection process named systematic evolution of ligands by exponential enrichment (SELEX) (Willner et al., 2008). The high catalytic activities toward specific substrates and high thermal stability make DNAzymes ideal biocatalysts for developing biosensing platforms (Willner et al., 2008; Gao and Li, 2013; Zhao et al., 2013). G-quadruplex/hemin DNAzyme as one of DNAzymes has received extensive attention in recent years (Zhang et al., 2011). The G-quadruplex/hemin DNAzyme displays peroxidase-like activity, and can catalyze the oxidation of colorless 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{2-}$) to the green-colored $ABTS^{\bullet-}$ with the presence of H_2O_2 (Li et al., 2010) or enhance the chemiluminescence of the luminol-H_2O_2 system (Niazov et al., 2004). Thus, it has been employed to develop many colorimetric and chemiluminescence biosensors for the detection of proteins, DNA, and other biomolecules (Xiao et al., 2004; Li et al., 2007; Shlyahovsky et al., 2007). Exonuclease III (Exo III) is a sequence-independent enzyme which does not require a specific recognition site (Liu et al., 2013b). It can catalyze the stepwise removal of mononucleotides from 3′ terminus of double-stranded DNA with blunt or recessed 3′-termini, while it shows limited activity on 3′-overhang end of double-stranded DNA or single-stranded DNA (Liu et al., 2013a; Gao and Li, 2013). Therefore, Exo III provides a versatile platform for amplified detection of DNA (Hsieh et al., 2010; Freeman et al., 2011; Xuan et al., 2012).

In this work, a new quadratic amplification strategy for label-free DNA amplified detection was proposed. This amplification

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strategy is composed of two major components. The first component is a target-catalyzed hairpin assembly reaction, in which the amplification is achieved by the cycling use of the target DNA. The second component is an Exo III cleavage reaction, which is designed to amplify the reporter DNA. With the significant quadratic signal amplifications, the proposed method can detect target DNA with an extremely low detection limit of 81 fM and a large dynamic range over 6 orders of magnitude. Moreover, this method might be further extended for detecting a variety of DNA sequences and aptamer-binding molecules.

2. Experimental section

2.1. Materials and reagents

Oligonucleotides used in this work were PAGE-purified and synthesized by Genscript (Nanjing) Co., Ltd. (Jiangsu, China), and their sequences are listed in Table S1. The oligonucleotide stock solutions (100 μM) were prepared in 20 mM Tris–HCl buffer (200 mM NaCl, 2 mM $MgCl_2$, and 20 mM KCl, pH=7.4). Each oligonucleotide was heated to 95 °C for 5 min, and then slowly cooled down to room temperature before use. Exonuclease III (Exo III) and hemin were purchased from Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). 4-(2-Hydroxyethyl)piperazine-L-ethanesulfonic acid sodium salt (HEPES), 2,2-azino-bis(3-ethylbenzothiozoline-6-sulfonic acid) diammonium salt (ABTS), H_2O_2, and [tris(hydroxymethy-l)aminomethane] (Tris) were purchased from Aladdin Reagents (Shanghai) Co., Ltd. (Shanghai, China). Other chemicals were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) and used without further purification. A hemin stock solution (1 mM) was prepared by dissolving 6.5 mg of hemin in 10 mL of dimethyl sulfoxide (DMSO) and stored in the dark at −20 °C. Double-distilled water, purified using a Milli-Q purification system (Billerica, MA, USA) with resistivity of 18.2 MΩ cm, was used for all experiments.

2.2. Procedure for DNA assay

The experiments were carried out in 80 μL of solution containing 20 μL of H1 (4 μM), 20 μL of H2 (4 μM), 20 μL of H3 (4 μM), 10 μL of Exo III (2.5 U/μL), and 10 μL of target DNA (varying concentrations), followed by incubating at 37 °C for 90 min. Then 10 μL of hemin (10 μM) and 90 μL of HEPES buffer (25 mM HEPES, 200 mM NaCl, 20 mM KCl, and 1% DMSO, pH=7.4) were added into the above solution and incubated for 60 min at room temperature. Finally, 10 μL of ABTS (120 mM) and 10 μL of H_2O_2 (40 mM) were added to the mixture to give the final concentrations of 6 mM and 2 mM, respectively. After incubation for 5 min, the resulting samples were used for the absorbance measurements and photographed.

2.3. Absorbance measurements

The absorption spectra of the samples were measured using a SpectraMax M5e Multi-Mode Microplate Reader (Molecular Devices, CA, USA) in the wavelength range of 390–490 nm with a step of 2 nm. Photographs of the solutions were taken using a canon EOS 600D camera.

3. Results and discussion

3.1. Design of hairpin structures for target-triggered quadratic amplification

The principle of target-catalyzed hairpin assembly was introduced in the seminal work by Pierce and co-works (Yin et al., 2008). Inspired by their work, we designed a simple target-triggered quadratic amplification circuit based on the ingenious combination of target-catalyzed hairpin assembly and Exo III-assisted signal amplification. Fig. 1 depicts the design process of hairpin structures for target-triggered quadratic amplification. The sequence of hairpin structures are described in terms of numbered domains, each of which represents a short fragment (5–10 nucleotides (nt)) of DNA sequence. Complementarity between numbered domains is denoted by an asterisk. The length of the target DNA is chosen based on kinetic and thermodynamic considerations. By using melting temperature (T_m) (calculated using OligoAnalyzer 3.1 software from IDT) to estimate the stability of DNA hybridization in solution, we found this colorimetric sensing system was more sensitive to short DNA with an optimum sequence length of 24–32 nt. We eventually chose 24 nt as the length of target DNA in this study. Once the domains 1, 2, 3 and 4 in target DNA were fixed, the domains in hairpin H1 were partially fixed, leaving domains 5, 6 and 7 to be designed. In order to avoid alternative foldings, OligoAnalyzer 3.1 was used to design the sequence of domains 5, 6 and 7 in H1, which in turn defined the sequence of hairpins H2 and H3. The left undefined domain 8 in H2 and domains 9 and 10 in H3 were also designed using OligoAnalyzer 3.1. One important application of this work is that for any unstructured short single-stranded DNA (ssDNA), three hairpin structures can be designed which do not initially interact between any two hairpins but can result in two recycling amplification cycles to achieve quadratic signal amplification in the presence of a ssDNA input that provides a high sensitivity for detecting trace amounts of DNA.

3.2. Principle of target-triggered quadratic amplification strategy for DNA detection

Scheme 1 depicts the new colorimetric biosensing strategy for ultrasensitive DNA detection. In this biosensing system, we designed three unlabeled hairpin structures termed H1, H2 and H3. Hairpin H1 contains two domains identified as I and II according to their different functions. Region I (red) is the target DNA recognition domain, and region II (skyblue) is partially hybridized at the stem region with region I. Hairpin H2 is designed to partially complementary to a segment (region I) of H1. Hairpin H3 contains two domains identified II′ and III. Region II′ (skyblue) is complementary to region II of H1, and region III (blue) is the G-quadruplex sequence. In hairpin H3, the G-quadruplex sequence (region III) is caged by partially hybridizing with region II′ to form a hairpin structure. As a result, the G-quadruplex sequence is prohibited to associate with hemin to form peroxidase-mimicking DNAzyme. These three hairpins (H1, H2, and H3) can coexist in solution until a target is introduced. Upon the addition of the target DNA (T), it interact with region I of hairpin H1, leading to an opening of H1. This opening of H1 results in the release of the single-stranded regions I and II. Subsequently, the released region I can serve as a toehold to hybridize with hairpin H2 to form a H1–T–H2 complex. This complex is inherently unstable, and T dissociates from the complex, leaving a stable H1–H2 duplex with protruding 3′-termini. The dissociated T can bind with a new hairpin H1 to initiate the target DNA recycling amplification cycle (Cycle I). It should be noted that the H1–H2 duplex with protruding 3′-termini cannot be digested by Exo III which specially cleaves DNA duplex with blunt or recessed 3′-termini. Moreover, the released region II in H1–H2 duplex can serve as another toehold to hybridize with hairpin H3 to form a H1–H2–H3 complex. Once H1–H2–H3 forms, it can be recognized by Exo III which stepwise hydrolyzes the mononucleotides of H3 from the blunt 3′-terminus in the direction of 3′- to −5′ and then releases the H1–H2 duplex and the G-quadruplex sequence. Importantly, the