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Highly sensitive fluorescence detection of target DNA by coupling exonuclease-assisted cascade target recycling and DNAzyme amplification

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ABSTRACT

Because of the intrinsic importance of nucleic acid as bio-targets, the simple and sensitive detection of nucleic acid is very essential for biological studies and medical diagnostics. Herein, a simple, isothermal and highly sensitive fluorescence detection of target DNA was developed with the combination of exonuclease III (Exo III)-assisted cascade target recycling and DNAzyme amplification. A hairpin DNA probe was designed, which contained the 3′-protruding DNA fragment as target recognition unit, the caged DNA fragment in the stem region as target analogue, and the caged 8–17 DNAzyme sequence in the loop region as signal response unit. Upon sensing of target DNA, the 3′-strand of hairpin DNA probe could be stepwise removed by Exo III, accompanied by the releasing of target DNA and autonomous generation of new target analogues for the successive hybridization and cleavage process. Simultaneously, the 8–17 DNAzyme unit could be exponentially released from this hairpin DNA probe and activated for the cyclic cleavage toward the ribonucleotide-containing molecular beacon substrate, inducing a remarkable fluorescence signal amplification for target detection. A low detection limit of 20 fM with an excellent selectivity toward target DNA could be achieved. The developed cascade amplification strategy may be further extended for the detection of a wide spectrum of analytes including protein and biological small molecules by combining DNA aptamer technology.

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1. Introduction

In order to profile low abundance of target DNA to serve the early-stage clinical diagnosis and medical treatment for some major diseases for example cancers, the development of signal amplification strategies to fabricate ultrasensitive DNA biosensor has recently received much efforts (Sassolas et al., 2008; Navani and Li, 2006; Sawyers, 2008; Turner, 2013; Wang et al., 2013a, 2013b). The target recycling strategy via the use of various nucleases for example exonuclease and endonuclease for signal amplification is especially intriguing (Lei and Ju, 2012; Zuo et al., 2010; Qiu et al., 2013; Gao et al., 2013). Among the different nucleases used for signal amplification, exonuclease III (Exo III) is employed more widely since it is a sequence-independent enzyme. It can only catalyze the stepwise removal of mononucleotides from 3′-terminus of double-stranded DNA in the case of substrate with a blunt or recessed 3′-terminus, and shows limited activity on single-stranded DNA or duplex DNAs with a protruding 3′-terminus (Liu et al., 2012; Zhao et al., 2011 ; Lee et al., 2005; Luo et al., 2013). Thus, Exo III provides a more versatile platform for amplified detection of DNA. Although great advances have been made toward the DNA detection by routine nuclease-based signal amplification strategy, it should be noted that the further improvement of the analytical performances, particularly sensitivity and selectivity, is still in high demand to satisfy the development needs of biological research, clinic diagnostics.

To further upgrade the detection sensitivity to make it to rival or even exceed that of polymerase chain reaction (PCR), the development of DNA nanomachine has drawn more and more concerns owing to its striking improvement for the detection performance toward target analytes (Weizmann et al., 2008; Wang et al., 2012). It holds a great promise to substitute PCR due to its easy operation, isothermal reaction, and high sensitivity (Nie et al.,

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2014; Yan et al., 2014). For example, the autonomous replication–scission–displacement strategy by means of nicking endonuclease and polymerase has been well developed for the amplified detection toward different biomolecules (Weizmann et al., 2006; Wang and Zhang, 2012; Wang et al., 2013a, 2013b; Jia et al., 2010; Zeng et al., 2013; Zhang et al., 2012). With the ingenious design of DNA recognition template or probe, the target analogues could be exponentially produced accompanying with the DNA replication–scission–displacement process and used for remarkable signal amplification. Recently, the quadratic enzymatic amplification strategy has been proposed for ultrasensitive miRNA and cytokines detection by using concurrent amplification cycles involving polymerase extension, nicking endonuclease cleavage and exonuclease-mediated target recycling (Duan et al., 2013; Zhou et al., 2014). However, the employment of multiple nucleases brings about the assay cost and also may increase the risk of false-positive signal output toward target detection. Furthermore, the autonomous DNA machine sensing system based on the use of sole exonuclease, endonuclease or DNAzyme has also been developed for the amplified detection of target analytes (Bi et al., 2012; Liu et al., 2013; Wang et al., 2011; Liu et al., 2014), yet it is usually confronted with the requirement of relatively complex probe or procedure design. Also, compared with some other detection methods for example electrochemistry, colorimetry, etc., fluorescence method is more simple and convenient. In this context, the development of simple, isothermal and autonomous amplification system for ultrasensitive fluorescence target detection is highly desirable for the applications in biomolecule diagnostics.

DNAzymes have been widely applied as signal amplifiers for enzyme-free and highly sensitive detection of various targets, such as metal ions, DNA, miRNAs, enzyme activity, small molecules, and so on (Liu and Lu, 2007; Tian et al., 2012; Zhang et al., 2010; Kim et al., 2007). They are a variety of catalytic DNA sequences selected by SELEX that show high catalytic hydrolytic cleavage activities toward specific substrates, while they are more stable than enzymes, and can be denatured and renatured many times without losing their catalytic activities toward substrates. As a typical example of DNAzymes, 8–17 DNAzyme is a DNA metalloenzyme catalyzing RNA transesterification in the presence of divalent metal ions, and has been used the most extensively in the applications mentioned above (Tang et al., 2013; Wang et al., 2013a, 2013b; Li et al., 2014).

Herein, a simple, isothermal and highly sensitive nucleic acid detection strategy was developed by the coupling of Exo III-assisted cascade target recycling and DNAzyme amplification. A hairpin DNA probe was designed, which contained the 3′-protruding DNA fragment for target recognition and the caged DNA fragment in the stem region as secondary target analogue. Also, the sequence of 8–17 DNAzyme was caged in the loop region and its activity was suppressed. Followed with the target-triggered and Exo III-assisted cascade cleavage recycling process, the 8–17 DNAzyme unit could be exponentially released from this hairpin DNA probe. Then, the cyclic cleavage of the activated 8–17 DNAzyme toward the ribonucleotide-containing molecular beacon substrate induced a remarkable amplified fluorescence signal for target detection. Owing to the distinct advantages for example simplicity in probe design and biosensor fabrication, cost-effective and high sensitivity, it thus holds a huge potential for the development of ultrasensitive biosensing platform for biomolecular detection in bioanalysis and disease diagnostics.

2. Experimental

2.1. Materials and chemicals

Exonuclease III were purchased from New England Biolabs Ltd. (Ipswich, MA, USA) and used without further purification. The DNA oligonucleotides were synthesized and purified by Takara Biotechnology Co., Ltd. (Dalian, China), and their sequences were listed in Table S1. Human serum samples were kindly provided by the Qingdao Center Hospital. All other chemicals were obtained from Shanghai Chemical Reagents (Shanghai, China) and used without further purification. Milli-Q water (resistance > 18 MΩ cm) was used in all experiments.

2.2. Procedures for DNA detection and assay optimization

Before DNA detection, all DNA samples were pretreated with the following procedure: heated to 90 °C and incubated for 5 min, and then cooled to 37 °C and incubated for 1 h. The obtained DNA solutions were stored at 4 °C for further use. The DNA hybridization and Exo III cleavage reaction was carried out in 100 μL of reaction mixtures containing 100 nM HP1 or HP2, 200 units Exo III, 10 mM tris–HCl buffer (pH 7.8, 10 mM $MgCl_2$, 50 mM NaCl) and varying concentrations of the target DNA. The reaction mixture was incubated at 37 °C for 30 min, followed by inactivation of Exo III at 70 °C for 20 min. Then, 100 μL of 25 mM HEPES buffer (pH 7.0, 200 mM NaCl) containing 200 nM MB and 100 μM Pb^{2+} were added into the above reaction mixture. After thoroughly mixing and incubation at 37 °C for 30 min, the fluorescence responses were recorded. The Exo III concentration optimization was conducted by using a series of Exo III concentrations (0.2, 0.5, 1, 1.5, 2, 2.5, and 3 unit/μL). The molar ratio of the MB to the HP1 was optimized by using different ratios (0.5, 1, 1.5, 2. 2.5, and 3).

2.3. Instruments

All fluorescence measurements were carried out on a F-4500 spectrometer with a scan rate at 1200 nm/min. The excitation wavelength was set at 480 nm. The slits for excitation and emission were set at 5 nm/5 nm.

3. Results and discussion

3.1. Design of strategy

Herein, a simple and isothermal Exo III-assisted cascade target recycling and DNAzyme amplification strategy was developed for the achievement of ultrasensitive detection of DNA, which was schematically illustrated in Fig. 1. A hairpin DNA probe (HP1) was designed, which consisted of two sequential domains **a** at 3′-termini, the loop region **b** and the domain **a*** at 5′-termini. In order to facilitate the binding of domain **a*** with the adjacent but not the distal domain **a**, two extra base-pairs were introduced in the stem region of HP1 to increase the hybridization stability. Thus, the HP1 shows a stabilized stem-loop structure with the protruding DNA fragment (domain **a**) at the 3′-termini, which can be recognized by target DNA. The base sequence for the domain **a*** in the stem region was the same with partial sequence of target DNA. Thus, once the domain **a*** was released from the stem region of HP1, it could be used as a target analogue to further hybridize with the 3′-protuding domain **a** of another HP1. The sequence of 8–17 DNAzyme was caged in the loop domain **b** and its activity was suppressed. The HP1 cannot be digested by Exo III, which specifically cleaves duplex DNA from blunt or recessed 3′-termini (Liu et al., 2012; Zhao et al., 2011 ; Lee et al., 2005). When the HP1

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