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Linear molecular beacons for highly sensitive bioanalysis based on cyclic Exo III enzymatic amplification

Chaoyong James Yang*, Liang Cui, Jiahao Huang, Ling Yan, Xiaoyan Lin, Chunming Wang, Wei Yun Zhang, Huaizhi Kang

State Key Laboratory of Physical Chemistry of Solid Surfaces, Key Laboratory for Chemical Biology of Fujian Province, Key Laboratory of Analytical Science, Department of Chemical Biology, College of Chemistry and Chemical Engineering, Xiamen University, Xiamen 361005, PR China

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ABSTRACT

Sensitive analysis or monitoring of biomolecules and small molecules is very important for many biological researches, clinical diagnosis and forensic investigations. As a sequence-independent exonuclease, Exonuclease III (Exo III) has been widely used for amplified detection of proteins and nucleic acids where displacing probes or molecular beacons are used as the signaling probes. However, displacing probes suffer slow hybridization rate and high background signal and molecular beacons are difficult to design and prone to undesired nonspecific interactions. Herein, we report a new type of probes called linear molecular beacons (LMBs) for use in Exo III amplification assays to improve hybridization kinetics and reduce background noises. LMBs are linear oligonucleotide probes with a fluorophore and quencher attached to 3′ terminal and penultimate nucleotides, respectively. Compared to conventional molecular beacons and displacing probes, LMBs are easy to design and synthesize. More importantly, LMBs have a much lower background noise and allow faster reaction rates. Using LMBs in cyclic Exo III amplification assay, ultrasensitive nucleic acid detection methods were developed with a detection limit of less than 120 fM, which is 2 orders of magnitude lower than that of conventional molecular beacons or displacing probes-based Exo III amplification assays. Furthermore, LMBs can be extended as universal probes for detection of non-nucleic acid molecules such as cocaine with high sensitivity. These results demonstrate that the combination of Exo III amplification and LMB signaling provides a general method for ultrasensitive and selective detection of a wide range of targets.

1. Introduction

The ability to detect molecular biomarkers with high sensitivity and selectivity is highly desired in various biological applications, such as rapid detection of pathogenic species and high-throughput screening of therapeutic and preventive agents (Kolpashchikov, 2010; Liu et al., 2009; Marti et al., 2007; Wang et al., 2009). Among available methods, nuclease-assisted signal amplification strategy has been employed in a variety of bioanalytical strategies. For example, with polymerase and nicking endonuclease, Fok I endonuclease or RNase H, exponential amplification reaction (EXPAR) (Kiesling et al., 2007; Tan et al., 2005; Van Ness et al., 2003; Weizmann et al., 2006a), DNA-based machine (Beyer and Simmel, 2006; Li et al., 2008a; Weizmann et al., 2006b; Weizmann et al., 2008), strand displacement amplification (SDA) (Connolly and Trau, 2010; Guo et al., 2009; He et al., 2010), isothermal chain amplification system (ICA) (Bekkaoui et al., 1996; Goodrich et al., 2004a; Jung et al., 2010; Kim and Chung, 2010; Kim et al., 2007a) and other amplification system (Li et al., 2008b; Zhou et al., 2010) have been developed for the detection of nucleic acids, proteins, small molecules and metal ions. Despite their high sensitivity and selectivity, restriction endonuclease-dependent amplification approaches generally require a specific enzymatic recognition sequence in the target sequence which significantly limits the number of sequences these methods can detect, and polymerase-dependent and RNase H-dependent amplification methods are time consuming and suffer from tedious manipulation (Lee et al., 2005; Weizmann et al., 2006a).

Exo III catalyzes stepwise removal of mononucleotides from blunt or recessed 3′-hydroxyl termini of duplex DNA, while it is not active on single-stranded DNA or 3′-protruding termini of double-stranded DNA. Different from restriction enzymes, Exo III does not require any specific enzymatic recognition sequence to function. With this property, various Exo III-assisted amplification methods have been developed for the detection of proteins or nucleic acids (Lee et al., 2005, 2006; Ou et al., 2010; Ren et al., 2004; Wang et al., 2005a; Xu et al., 2008).

* Corresponding author. Tel.: +86 592 218 7601; fax: +86 592 218 9959.
E-mail address: cyyang@xmu.edu.cn (C. Yang).

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Recently, we had proposed a cyclic enzymatic amplification method (CEAM) (Cui et al., 2010) based on Exo III and displacing probes. Displacing probes (Li et al., 2002; Cheng et al., 2004), developed by Li et al. based on the principle of displacement hybridization, consists of two complementary oligonucleotides of different lengths labelled with fluorophore and quencher respectively. In comparison with molecular beacons or other probes, these probes have the advantages of easier to design, synthesize, purify and thus are much cheaper and more applicable. In our design, the displacing probe is non-fluorescent when unbound and cannot be digested by Exo III until displacement hybridizing with a target sequence, leading to the release of a free un-quenched fluorophore. Because a single target sequence can initiate and cycle the release and digestion processes of numerous fluorophore strands from the displacing probes, a remarkable signal amplification is achieved. With this method, DNA can be detected in the picomolar range. More importantly, such a method does not require a restricted enzymatic recognition sequence in the target DNA, thus it has much better flexibility in choosing target sequences and offers wider applicability. At last, the method can be carried out at constant temperature with just one-step manipulation, avoiding the complex handling procedures generally required by other methods. Based on this method, we further developed a universal colorimetric platform for DNA detection with high sensitivity and excellent selectivity without any complicated design and instrument (Cui et al., 2011). However, because of their intrinsic property, displacing probes suffer from several drawbacks including high fluorescence background and slow hybridization kinetics. New probes which afford faster reaction yet high sensitivity and selectivity are thus in greatly needed.

Herein, we reported a new type of probes called linear molecular beacons (LMBs) as one type of signaling probe to use in the Exo III aided cyclic amplification detection method. LMBs are linear oligonucleotide probes with a fluorophore and quencher attached to 3′ terminal and penultimate nucleotides respectively. Compared to conventional molecular beacons, LMBs do not require any intramolecular structure and thus is very easy to design. Because both quencher and fluorophore are attached to the same strand with adjacent positions, efficient quenching can be obtained with very low probe background signal. Using our LMBs, rapid and ultrasensitive nucleic acid detection method was developed with detection limit of less than 120 fM, which is 2 orders of magnitude lower than that of conventional molecular beacons or displacing probes-based Exo III amplification assays, and 5 orders of magnitude lower than traditional molecular beacon assays without any amplification involved. Furthermore, LMBs were also used as probes to explore the detection of non-nucleic acids molecules such as cocaine. These results demonstrate that the combination of Exo III amplification and LMB signaling provides a general method for ultrasensitive and selective detection of a wide range of targets.

2. Experimental

2.1. Chemicals and reagents

Exo III was purchased from Takara Biotechnology Co. Ltd. (Dalian, China) and used without further purification. All DNA sequences (see Table S1 of the Supplementary Materials) were synthesized on a PolyGen DNA synthesizer and the reagents were purchased from Glen Research (Sterling, VA, USA). A 3′-Fluorescein CPG column, Thymidine C-5 Dabcyl were used for the synthesis of LMBs. The complete DNA sequences were then deprotected in concentrated ammonia at 65 °C overnight and purified by HPLC. An Agilent 1100 HPLC with a Promosil C18 reversed phase column (5 μm, 250 mm × 4.6 mm) was applied. A solution of 0.1 M triethylamine acetate (TEAA) was used as HPLC buffer A, and HPLC-grade acetonitrile was used as buffer B. The collected product was then vacuum-dried, desalted with a NAP-5 column, and stored at −20 °C for future use. Absorbance was measured on an Agilent 8453 UV/Vis spectrometer and used to calculate concentration. MALDI-TOF-MS (Microflex, Bruker Daltonics) was used to confirm the successful synthesis of LMBs (see Fig. S1 of the Supplementary Materials).

2.2. Fluorescence measurements

Fluorescence measurements were carried out on a RF-5301-PC Fluorescence Spectrophotometer (Shimadzu, Japan). Excitation and emission wavelengths were set at 490 and 517 nm, respectively, with 5 nm bandwidth. The emission spectra were obtained by exciting samples at 490 nm and scanning the emission from 500 to 650 nm. All experiments were conducted in 20 mM Tris–HCl (pH 8.0) buffer containing 5 mM $MgCl_2$ and 450 mM NaCl. The amplified detection of target was performed in 400 μL solution consisting of 100 nM displacing probes or LMBs, 5 U of Exo III and various concentrations of DNA target at room temperature for 0.5 h.

2.3. Cocaine assay

The cocaine assay followed literature procedures with minor modifications (He et al., 2010). Upon testing various conditions, the following procedure was used to study the concentration-dependent changes in fluorescence experiments: 40 μL of cocaine solution of a specific concentration were mixed with 2 μL of Cocaine Aptamer Fragment1 solution (1 μM) for 10 min. After adding 2 μL of 100 μM LMBs, the mixture was incubated for 60 min at room temperature. The resulting solution and 0.5 μL of 2 U/μL Exo III were subsequently added into the reaction buffer, followed by incubation in 37 °C for 60 min. Then the fluorescence emission spectra were recorded.

3. Results and discussion

3.1. Design of linear molecular beacons for Exo III-assisted signal amplification

The principle of signal amplification based on Exo III has been described in details previously (Cui et al., 2010, 2011). As shown in Fig. 1, the reporter probe is able to hybridize with target DNA to form a duplex with a recessed 3′ terminus that can be digested by Exo III. Because Exo III only cleaves the 3′ recessed probe strand from the duplex, the target sequence will then be released to bind another probe and initiate a new cleavage process. Through such a cyclic hybridization–hydrolysis process, a single target DNA strand can induce the cleavage of a large number of probes. When the probes are designed as molecular beacons or displacing probes, each cycle of cleavage can free an un-quenched fluorophore so that the fluorescence intensity of the solution will increase with the progress of cycling reaction.

When molecular beacons or displacing probes are used, the Exo III assisted amplification method can detect as low as pM of target sequence. This sensitivity is not as high as those observed in the case of some other nucleases. The major factors leading to unsatisfied sensitivity include high probe background signal (Yang et al., 2005a) and unfavorable hybridization kinetics. Take displacing probes for example, the longer positive strand (F strand) is labeled with a fluorophore at the 5′-end and the shorter negative strand (Q-strand) is labeled with a quencher at the 3′-termini, so that the fluorophore and the quencher groups are in close contact in the duplex form (Cheng et al., 2004). However, a high background signal exist because F-strand cannot completely associated with the Q-strand due to hybridization equilibrium. For molecular beacon