Biosensors and Bioelectronics 68 (2015) 365–370

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

A novel molecular beacon-based method for isothermal detection of sequence-specific DNA via T7 RNA polymerase-aided target regeneration

Bin-Cheng Yin, Shan Wu, Jin-Liang Ma, Bang-Ce Ye*

Lab of Biosystem and Microanalysis, State Key Laboratory of Bioreactor Engineering, East China University of Science & Technology, Shanghai 200237, China

ARTICLE INFO

Article history:
Received 7 November 2014
Received in revised form
7 January 2015
Accepted 8 January 2015
Available online 9 January 2015

Keywords:
Molecular beacon
Nucleic acid detection
Isothermal detction
Signal amplification

ABSTRACT

Developing molecular beacon (MB)-based method for DNA detection has been of great interest to many researchers because of its intrinsic advantages of simplicity, rapidity, and specificity. In this work, we have developed a novel MB-based method for isothermal detection of sequence-specific DNA via T7 RNA polymerase-aided target regeneration strategy. The proposed method involves three primary processes of target-mediated ligation by T4 DNA ligase, transcription reaction by T7 RNA polymerase, and MB switch for signal output. Upon the hybridization with DNA target, a rationally designed MB and a pair of primers encoded with T7 promoter sequence were ligated via the formation of a phosphodiester bond by T4 DNA ligase. The resultant joint fragment acted as template to initiate T7 RNA polymerase-mediated transcription reaction. Correspondingly, a great amount of RNA strands complementary to MB and partial primers were transcribed to initiate new cyclic reactions of MB switch, ligation, and transcription. With such signal amplification strategy of the regeneration of target-like RNA fragments, our proposed assay achieved a detection limit as low as ~10 pM, which was ~3 orders of magnitude lower than the traditional MB-based method with a recognition mechanism in 1:1 stoichiometric ratio between MB and target molecule.

1. Introduction

So far, sequence-specific and sensitive detection of DNA still attracts substantial scientific interests in a variety of application fields, including medical diagnostics (Santiago et al., 1997; Schafer and Hawkins, 1998; Favis et al., 2000), pathogen identification (Jin et al., 2009), environmental and food safety monitoring (Lermo et al., 2007; Pafundo et al., 2010), and biodefense application (Sergeev et al., 2004; Sulaiman et al., 2007). Since some DNA targets of interest may be present in small amounts, thus sensitive methods to detect a unique DNA target from high-diversity environment are highly in demand. Sequence-based amplification of specific DNA target is an effective way to increase small amounts of a specific sequence to the detectable levels. Various amplified DNA assays to sensitively detect unique target sequences have been developed (Zuo et al., 2010; Connolly and Trau, 2011; Wu et al., 2011; Yu et al., 2014). These methods are mainly divided into two broad categories based on reaction condition: thermal cycling and isothermal amplification.

The polymerase chain reaction (PCR), considered as golden standard for DNA amplification since introduced in 1985 (Saiki et al., 1985), is the most widely used thermal-cycling protocol. It has significantly impacted the genetics and molecular biology. Via thermal-cycling protocol in raising and lowering temperature of the reaction mixture, the reaction proceeds exponentially to amplify the amount of DNA target by repeatedly denaturing template DNA, annealing primers at specific sites in the denatured template, and extending the primers by thermostable DNA polymerase. Although PCR-based methods have been widely used by researchers, the requirement of an expensive thermal cycler to precisely control the reaction temperature would limit the application in point-of-care settings or resource-limited locations. To avoid this problem, a variety of isothermal amplification methods, such as rolling circle amplfication (RCA) (Fire and Xu, 1995; Lizardi et al., 1998), strand displacement amplification (SDA) (Walker et al., 1992a, 1992b), isothermal exponential amplification reaction (EXPAR) (Jia et al., 2010; Tan et al., 2008), helicase-dependent amplification (HDA) (Vincent et al., 2004), nucleic acid sequence-based amplification (NASBA) (Deiman et al., 2002), loop-mediated amplification (LMA) (Notomi et al., 2000), and cross priming amplification (CPA) (Xu et al., 2012), have been developed as thermal cycler-free alternatives to the PCR-based methods. Undoubtedly,

* Corresponding author. Fax: +86 021 64252094.
E-mail address: bcye@ecust.edu.cn (B.-C. Ye).

http://dx.doi.org/10.1016/j.bios.2015.01.020
0956-5663/

these isothermal methods have made significant progress toward DNA detection, especially for the "on the spot" testing. These methods have their own strengths and limitations. For example, RCA relies on the casecade enzymatic process to generate very long single-stranded DNA molecules with tandem repeats; but it is tedious and time-consuming (over 8 h) to finish the ligation of the padlock probes and branched rolling-circle amplification reaction. In the case of HDA, the amplification scheme proceeds the same as PCR via simultaneous and continuous DNA unwinding and amplification, essentially. The HDA allows faster rate on exponential amplification than that of PCR with the joint participation of UvrD helicase and its accessory protein MutL protein, single-strand binding protein, and exo$^-$ Klenow fragment. However, this method is problematic owing to the involvement of complicated reaction components of multiple specialized and expensive enzymes, which not only greatly restricts the possible application and also raises the detection cost. NASBA is a transcription-based amplification method, specifically designed for RNA detection. It eliminates heat denaturation process via a set of transcription and reverse transcription reactions to produce a great amount of single-stranded RNA amplicons by the joint action of three enzymes of avian myeloblastosis virus reverse transcriptase, RNase H and T7 DNA dependent RNA polymerase. Similar to HDA, requirement of multiple enzymes makes the entire reaction complicated. Specifically, the condition optimization procedures for three enzymes coexistence in one tube seems to be laborious and difficult. SDA and EXPAR rely on endonuclease to initiate the isothermal displacement of one DNA strand by cleaving the nicking site, and polymerase to generate the new nickable recognition site in a continuous cycle-after-cycle format. However, SDA and EXPAR may suffer the repeatability problem stemming from random operation between endonuclease and polymerase, and limited specificity due to nonspecific amplification. In constrast to the aboved enzyme-assisted isothermal methods, both CPA and LAMP methods offer enzyme-free amplification way for isothermal DNA detection by using a strand-displacement DNA polymerase, however the complexity in primer design and multiple primers employment would restrict their scope of applications and thus decrease their generality. From a view of enzyme-catalyzed amplification assays with a high efficiency, it is still constantly desirable to develop new isothermal DNA detection methods, avoiding the complexity design and multiple enzymes employment (no more than two enzymes used).

Among the above mentioned methods, the introduction of a primer encoded with T7 promoter sequence to initiate T7 RNA polymerase-mediated transcription reaction in NASBA is a simple and efficient way to proceed the self-amplification at constant temperature. T7 RNA polymerase is a DNA-dependent RNA polymerase, to catalyze the synthesis of RNA fragment in a 5′–3′ direction on either single-stranded DNA or double-stranded DNA downstream from its promoter. Inspired by this efficient catalytic property of T7 RNA polymerase, here we have developed a novel molecular beacon (MB)-based method for isothermal detection of specific DNA. Among various fluorescence-based DNA probes, MB is the most effective probe for DNA detection as it has intrinsic advantages of simplicity, rapidity, and specificity; and can be used directly without separation. In traditional MB-based strategy, the inherent limitation of target-to-signal ratio in 1:1, in which one target only causes one quenched fluorescent probe to fluoresce, would induce low sensitivity. To overcome this limitation, we have combined MB and transcription reaction to achieve the one-to-multiple amplification effect. It is designed such that, a MB probe with a 3′-overhang end, which is complementary to half-sequence of DNA target, is designed to link with 5′-phosphate modified primers encoded with intact T7 promoter sequence in the presence of DNA target. With this elaborate design, the produced jointed fragment not only acts as a template for T7 RNA polymerase to generate a great amount of RNA fragments, but also undergoes conformational change to open its hairpin structure in MB portion with the transcription reaction proceeds. The transcribed RNA fragment, containing both DNA target sequence and the complementary sequence of MB probe, would trigger a new round of ligation reaction between MB and primers and subsequent transcription reaction, resulting in one-to-multiple amplification effect. By combining MB as signal output, DNA ligase and T7 RNA polymerase as mechanical activators, our proposed method could quantitatively detect a sequence-specific DNA target as low as 10 pM, which was ~3 orders of magnitude lower than those of the traditional MB-based method.

2. Materials and methods

2.1. Reagents and materials

Oligonucleotides and MB probes were custom-synthesized by Sangon Biological Engineering Technology & Services Co., Ltd.

Table 1
The sequence information of DNA oligonucleotides and MB probes used in this work.

Name	Sequence* (5′→3′)
MB1	FAM-ACCTT*CTATAG***CAAACACCATTGTCACACTCCACCTCAGC***CTATAG*AAGGT-DABCYL
MB2	FAM-*ACTATAGG***TATACAACCTACTACCTCAC***CCTATAGT*-DABCYL
MB3	FAM-ACCGCCGG**ACAAGGAGAGTCAA**CCGGCGGT-(DABCYL)- AGAGA*CCCTATAGTGAGTCGTATTA*
MB4	FAM-CCGCCGG**ACAAGGAGGCTGGAGTCAA**CCGGCGG-DABCYL
C-MB1	ACCTTCTATAGGCTGAGGTGGAGTGTGACAATGGTGTTTGCTATAGAAGGT
C-MB2	ACTATAGGGTGAGGTAGTAGGTTGTATACCTATAGT
C-MB3	TAATACGACTCACTATAGGGTCTCTACCGCCGGTTGACTCTCCTTGTCCGGCGGT
C-MB4	CCGCCGGTTGACTCCAGCCTCCTTGTCCGGCGG
Promoter	*TAATACGACTCACTATAGGG*
MB5	DABCYL-ACCGCCGG**ACAAGTGGAGTCAA**CCGGCGGT-(FAM)-GAAACTATACAA
C-MB5	TTGTATAGTTTCACCGCCGGTTGACTCCACTTGTCCGGCGGT
P1	Pi-CCTACTACCTCAGTAGGAAC*CCCTATAGTGAGTCGTATTAGTGATC*
P2	*GATCACTAATACGACTCACTATAGGG*
T1	TGAGGTAGTAGGTTGTATAGTT
T2	TGAGGTAGTAG<u>C</u>TTGTATAGTT
T3	TGAGGTA<u>C</u>TAGGTTG<u>A</u>ATAGTT
T4	TGAG<u>C</u>TAGTAG<u>C</u>TTG<u>A</u>ATAGTT
T5	**TGAGGTAGTAGGTTG<u>A</u>ATAGTT**

* The bases in bold represent the loop-structure in MB probes. The bases in italics represent T7 promoter sequence. The underlined bases in T2, T3, T4, and T5 sequences represent the mutation sites in tested DNA targets compared to target T1.