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ORIGINAL PAPER

Colorimetric detection of genetically modified organisms based on exonuclease III-assisted target recycling and hemin/G-quadruplex DNAzyme amplification

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Abstract
An isothermal colorimetric method is described for amplified detection of the CaMV 35S promoter sequence in genetically modified organism (GMO). It is based on (a) target DNA-triggered unlabeled molecular beacon (UMB) termini binding, and (b) exonuclease III (Exo III)-assisted target recycling, and (c) hemin/G-quadruplex (DNAzyme) based signal amplification. The specific binding of target to the G-quadruplex sequence-locked UMB triggers the digestion of Exo III. This, in turn, releases an active G-quadruplex segment and target DNA for successive hybridization and cleavage. The Exo III impellent recycling of targets produces numerous G-quadruplex sequences. These further associate with hemin to form DNAzymes and hence will catalyze H_2O_2-mediated oxidation of the chromogenic enzyme substrate $ABTS^{2-}$ causing the formation of a green colored product. This finding enables a sensitive colorimetric determination of GMO DNA (at an analytical wavelength of 420 nm) at concentrations as low as 0.23 nM. By taking advantage of isothermal incubation, this method does not require sophisticated equipment or complicated syntheses. Analyses can be performed within 90 min. The method also discriminates single base mismatches. In our perception, it has a wide scope in that it may be applied to the detection of many other GMOs.

Keywords GMO · CaMV 35S · DNA detection · Isothermal amplification · Molecular beacon · Oligonucleotide · Absorption intensity · Microplate photometer · Biosensor

Introduction

A genetically modified organism (GMO) is a living organism whose genetic material has been modified by introducing an exogenous desired DNA fragment into the genome. GMOs can improve crop yield, reduce production cost, and prevent environmental damage by reducing the amount of herbicides. However, public attention over the potential risks of GMOs to human health has also been growing in the past few years [1–3]. Hence, developing efficient methods that can accurately detect the presence of GM contents is essential to reduce these undesired risks. Currently, the most commonly recommended method is the Taqman-based quantitative polymerase chain reaction (qPCR) due to its relatively high levels of precision and accuracy [4]. Nevertheless, the results of qPCR are easily affected by many factors according to the amplification curve, including the PCR inhibitor, the experience of the technicians and the matrix effect of the PCR tubes [5]. Moreover, the requirement of expensive and sophisticated equipments makes it impractical for non-wealthy areas and for field testing. As an alternative, numerous new methods, including colorimetric [6], electrochemical [7], quartz crystal microbalance [8], and surface plasmon resonance (SPR) [9] methodologies have also been developed. Among these strategies, label-free colorimetric methods have attracted particular attention for its low-cost, simplicity, speediness, and even only utilizing naked eyes [10, 11]. However, most of these methods are of unsatisfactory sensitivity. In order to solve the problem of lack of sensitivity, several isothermal amplification strategies, such as strand displacement

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reaction (SDR) [12], hybridization chain reaction (HCR) [13], rolling circle amplification (RCA) [14], and nicking endonuclease signal amplification (NESA) [15], have been applied to achieve signal amplification. These isothermal amplification techniques do not require special conditions for thermal cycling and are highly compatible to biosensor systems. Especially, an enzyme-aided target recycling strategy based on exonuclease III (Exo III) is presented for simple and sensitive detection of nucleic acid. Unlike nicking endonucleases, Exo III does not require a specific recognition site and is able to selective catalyze the stepwise removal of mononucleotides from the blunt or the recessed 3′-termini of double-stranded DNA (dsDNA), while its activity on 3′-overhang end of dsDNA or single-stranded DNA (ssDNA) is limited [16–18]. As far as we know, there is no report about Exo III-assisted signal amplification strategy combines with colorimetric method for developing GMO assays, although this amplification strategy is very suitable for constructing universal detection platform.

DNAzymes are a class of functional nucleic acids, which can be isolated using an in vitro selection method called systematic evolution of ligands by exponential enrichment (SELEX) [19, 20]. G-quadruplex/hemin DNAzyme as one of DNAzymes exhibits high peroxidase-like activity and can effectively catalyze the H_2O_2-mediated oxidation of 2, 2′-azionbis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^{2-}$) into the colored free-radical anion $ABTS^{\cdot-}$, generating a blue-green colorimetric signal, which can be monitored simply by the bare eye or a spectrophotometer [21]. A series of classic strategies has been constructed for the formation of DNAzyme by using hairpin probe. When target DNA binding with stem opened the hairpin DNA, and then the G-quadruplex/hemin complex can be obtained in the presence of hemin [22]. Owing to the competitive advantage of DNAzymes over traditional protein enzymes including high chemical stability, ease synthesis and low price, nowadays DNAzymes have been extensively applied as a catalytic label for amplified colorimetric detection of biomolecules.

Herein, we first developed a colorimetric method for GMO detection by combining with DNAzyme-signal amplification and Exo III assisted-target recycling. In brief, the target CaMV 35S promoter sequence specific binding to the molecular beacon triggers the Exo III digestion reaction to release active G-quadruplex sequence and free target, which initiates a new recycling, and thus produces cascades of DNAzyme in the presence of hemin. The color change resulting from DNAzyme-catalyzed H_2O_2-medated $ABTS^{2-}$ oxidation herein is served as a signal output and can be instantly recognized by naked eye and/or photometer. Under optimal conditions, the detection sensitivity is greatly improved, and the detection limit (LOD) is obtained as low as a few femtomoles in 10 μL. Moreover, this biosensor is capable of discriminating single oligonucleotide mismatch of the DNA sequences, showing a promising platform for GMO analysis.

Experimental

Materials and reagents

$ABTS^{2-}$, Triton X-100, dimethyl sulfoxide (DMSO), and 4-(2-Hydroxyethyl) piperazine-L-ethanesulfonic acid sodium salt (HEPES) were purchased from Sangon Biological Engineering Technology & Co., Ltd. (Shanghai, China, http://www.sangon.com/). Exo III was obtained from Takara Biotechnology Co., Ltd. (Dalian, China, http://www.takara.com.cn/). Hemin, H_2O_2, KCl, and NaCl were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China, http://www.sinoreagent.com/). Hemin stock solution (5 mM) was prepared in DMSO and stored in the dark at −20 °C. Hemin, $ABTS^{2-}$ and H_2O_2 working solutions were freshly prepared in a HEPES buffer (pH 7.4) containing 25 mM HEPES, 200 mM NaCl, 20 mM KCl, 0.05% Triton X-100, and 1% DMSO before used. Other reagents were all of analytical grade and used without further purification. Ultrapure water with resistivity of 18.2 MΩ cm was used for all experiments.

The oligonucleotides were synthesized by Sangon Biological Engineering Technology & Co., Ltd. (Shanghai, China, http://www.sangon.com/) and purified using high-performance liquid chromatography. The base sequences are listed as follows:

UMB: 5’ TCA ACG GGT TGG GCG GGA TGG GTT TCA ACC CGT TGA AGA TGC CTC TGC C 3’
CaMV 35S: 5’ GGC AGA GGC ATC TTC AAC GAT GGC 3’
Single-base mismatched DNA: 5’ GGC AGA GGC ATC ATC AAC GAT GGC 3′
Two-base mismatched DNA: 5’ GGC ACA GGC ATC ATC AAC GAT GGC 3’
Non-complementary DNA: 5′ GAA CTC GTA CCA TAG CAG CAC GTA 3’

All oligonucleotides were dissolved in tris-ethylenediaminetetraacetic acid (TE) buffer (pH 8.0, 10 mM Tris-HCl, 1 mM EDTA) and stored at −20 °C, which were diluted in water prior to use.

Procedure for DNA assay

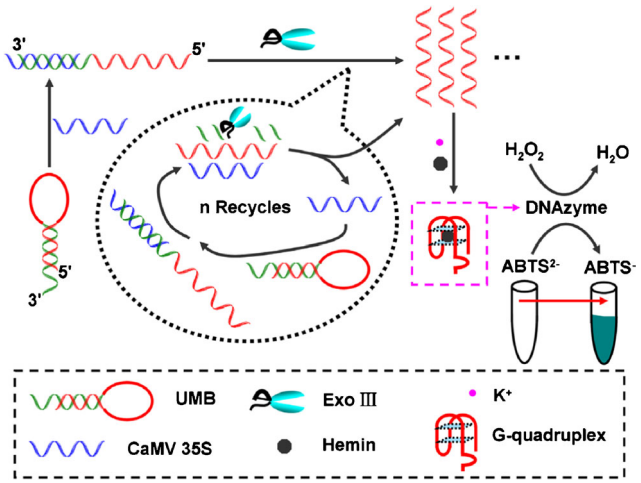
In order to facilitate the molecular beacons to fold into stable stem-loop structures, the UMB solution was heated at 90 °C for 5 min, followed by cooling down to room temperature for at least 2 h before use [23]. The detailed procedure for DNA detection was as follows. First, the Exo III-assisted target recycling amplification reaction was performed by mixing a 5 μL reaction buffer containing 200 mM Tris-HCl (pH 8.0), 20 mM Mg_2Cl, 120 mM sodium citrate, 1.2 M NaCl, 4 mM

DTT, 3 μL of UMB (50 μM), 1 μL of Exo III (50 $U \cdot \mu L^{-1}$), and 10 μL of target DNA (various concentrations), followed by incubating at 37 °C for 30 min. Then 4 μL of hemin (50 μM) and 112 μL HEPES buffer (25 mM HEPES, 200 mM NaCl, 20 mM KCl, 0.05% Triton X-100, and 1% DMSO, pH 7.4) were added into the above solution and incubated for 60 min at room temperature. Then 60 μL $ABTS^{2-}$ (20 mM) and 4 μL H_2O_2 (100 mM) were added to the mixture to give the final concentrations of 6 mM and 2 mM, respectively. After that, the resulting solution was dropped into 96-well microplate and incubated at room temperature for 5 min. Finally, the photographs were taken or the signal respones were collected at 420 nm by a Multiskan FC Microplate Photometer (Thermo Scientific, Wilmington, DE, USA, https://www.thermofisher.com/).

Results and discussion

Principle of the strategy for GMO detection

Scheme 1 illustrates the working principle of Exo III and DNAzyme dual-signal amplification approach for sensitive and visual GMO CaMV 35S promoter sequence detection. Since the hairpin structure probe shows improved ability to distinguish mismatches, one kind of unlabeled stem-loop DNA MB (UMB) was designed as target recognition probe. In the absence of target DNA, the G-quadruplex sequence-locked UMB is resistant to catalytic digestion by Exo III due to the 3′ protruding end of its stem sequence. The introduction of target DNA can hybridize with the 3′ protruding terminus of UMB and open its stem-loop structure to form dsDNA, as well as form the 3′ recessed end recognition sequence for Exo III. Then, Exo III can stepwise hydrolyze the mononucleotides from the 3'-OH terminus of the hybridized dsDNA, resulting in the releases of the target DNA and the G-quadruplex containing intermediate sequence. Eventually, each target DNA can initiate many recycling cycles to trigger the digestion of many UMBs, generating numerous active G-quadruplex structures. Upon the addition of hemin, these liberated G-quadruplexes can form massive peroxidase-mimicking DNAzymes that can catalyze H_2O_2-mediated oxidation of colorless $ABTS^{2-}$ to green-colored $ABTS^{\cdot-}$. Due to the dual-amplification nature of the strategy, the presence of low concentrations of target DNA can potentially result in significantly intense color change for colorimetric detection.



Scheme 1 Schematic principle of the dual-signal amplification strategy for highly sensitive detection of GMO

Feasibility of target-triggered amplification strategy

To clarify the feasibility of the designed signal amplification strategy, a comparing experiment was carried out and the result is shown in Fig. 1. The absorption intensity of the solution is relatively low in the absence of target GMO DNA. While in the presence of target, a dramatically significant increase in absorption intensity can be observed. Such increase is basically due to the hybridization of target DNA with UMB, which opens UMB and leads to the exposure of unlocked G-quadruplex sequences for subsequent formation of peroxidase-mimicking DNAzymes. These DNAzymes catalyze H_2O_2-medated $ABTS^{2-}$ oxidation, causing an increase in absorption intensity. More interestingly, the addition of the Exo III can lead to the recycling and reuse of target GMO DNA, resulting in the formation of more G-quadruplex/hemin DNAzymes and further increase in absorption intensity. These results strongly indicate that the detection sensitivity can be dramatically increased by using Exo III-assisted target recycling signal amplification.

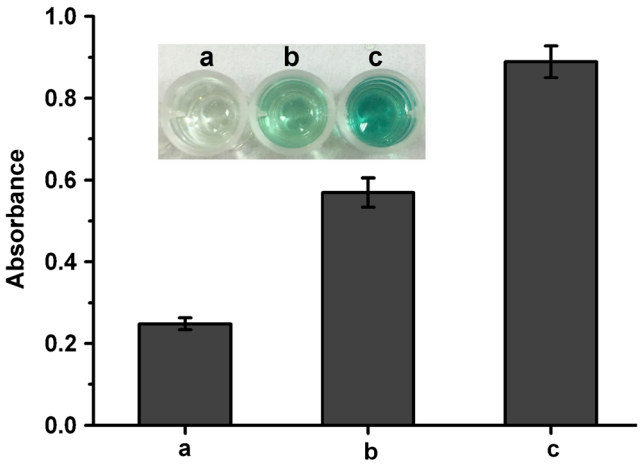


Fig. 1 The comparing experiments of the designed method performed without target (**a**), 500 nM target without Exo III (**b**), and 500 nM target with 50 U Exo III (**c**), individually. Signal responses were collected at 420 nm. Error bar is the standard deviation of three independent measurements

Optimization of experimental conditions

The following parameters were optimized: (a) concentration of Exo III; (b) reaction temperature; (c) reaction time. The respective data and figures are given in the Electronic Supporting Material. The following experimental conditions were found to give best results: (a) concentration of Exo III: 50 $U \cdot \mu L^{-1}$; (b) reaction temperature: 37 °C; (c) reaction time: 30 min.

Analysis specificity of the method

The specificity of the method for GMO assay was further evaluated by using different kinds of DNA sequences, i.e., completely target DNA, single-based mismatched DNA, two-based mismatched DNA, and non-complementary DNA at the same concentration of 100 nM. As shown in Fig. 2, single nucleotide mismatch can be clearly distinguished with the method. When the method is applied to detect the non-complementary sequence, the absorption intensity is as small as the background. These results demonstrate that this method displays excellent selectivity and held promising potential for single nucleotide polymorphism (SNP) analysis.

Analytical performance of the assay

Under the optimal experimental conditions, the absorption intensities increase with the increasing target concentrations (Fig. 3b). This indicates that the amount of active G-quadruplexes is highly dependent on the concentration of trigger target. Even 0.5 nM target triggers remarkable color change that is easily distinguished by naked eyes (Fig. 3a (b vs. a)). The absorption intensity is proportional to the logarithm value of target DNA concentration over a 3-decade range from 0.5 nM to 500 nM (inset of Fig. 3b). The regression equation is $Y = a + b\log_{10}C$ with a correlation coefficient of 0.9963, where Y and C represent the absorption intensity and the DNA concentration, respectively. The LOD was calculated to be 0.23 nM in a 3σ rule. Considering the required sample volume of 10 μL per analysis, the latter corresponds to the smallest detectable absolute amount of 2.3 fmol. The sensitivity of the method has improved more than 1 order of magnitude in contrast to previously reported colorimetric method [6] and is similar to that obtained with other assays (Table 1). These results clearly demonstrate that the strategy is efficient for sensitive colorimetric detection of DNA. To estimate the reproducibility of the assay, the imprecision of five different assays were examined. The coefficients of variation (CVs) were 5.7% at 1 nM and 3.2% at 100 nM of target DNA, respectively, indication remarkable precision and reproducibility.

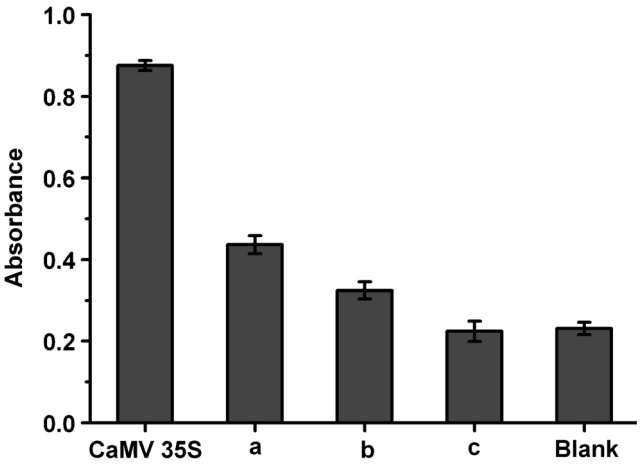


Fig. 2 Histograms of absorption intensity for 500 nM: complementary target DNA (CaMV 35S), single-base mismatched DNA (**a**), two-base mismatched DNA (**b**), non-complementary DNA (**c**), and the absence of DNA (blank). Error bars represent the standard deviations of three independent measurements

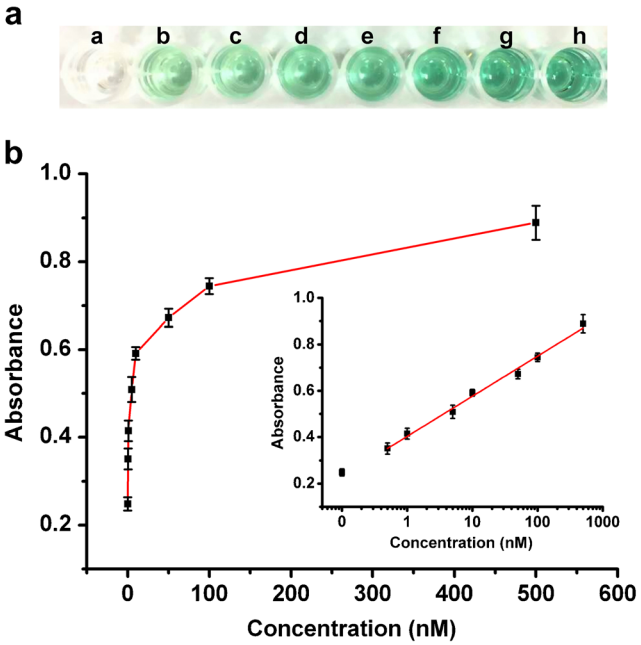


Fig. 3 **a** Photograph for amplification detection of target DNA at 0, 0.5, 1, 5, 10, 50, 100 and 500 nM (from a to h), **b** The corresponding of absorption intensity on target DNA concentration. Inset shows the linear relationship between the absorption intensity and the log of concentrations of target DNA. Signal responses were collected at 420 nm. The experiment was performed under optimal conditions. Error bars represent the standard deviations of three independent measurements

Table 1 Comparison between the proposed assay and other reported methods for the GMOs detection

Analytical technique	Detection limit	Linear range	Reference
Colorimetric method	5 nM	50–500 nM	[6]
SPR spectroscopy	1 nM	1–100 nM	[24]
Fluorescent biosensor	2 nM	5–9 nM	[25]
Electrochemical biosensor	1 nM	1–10 nM	[26]
DVD-based biochip	50–900 fg	–	[27]
Colorimetric method	0.23 nM	0.5–500 nM	This work

Real sample assay

Spiked CaMV 35S promoter sequences with different concentration in tap water were testing to evaluate the application feasibility of our study in real sample condition. As shown in Table S1, good recovery percentages were obtained that indicates non-significant interference from the tap water components on the strategy. Hence, these results illustrates that the colormetric method held potential for GMOs analysis in real samples.

Conclusions

We developed an isothermal colorimetric method for amplified detection of CaMV 35S promoter sequence in GMO. With the introduction of Exo III assisted-target recycling and hemin/G-quadruplex DNAzyme amplification, the method shows a linear dynamic range from 0.5 to 500 nM with a limit of detection of 0.23 nM. The strategy also exhibited excellent specificity to discriminate single base mismatched sequences well. Although we anticipate this sensitive and specific method can be extended to detection of various other GMOs by changing the UMB sequences, a further improvement can be made to enable high-throughput detection. In addition, preliminary application of this method in the spiked tap water samples shows a good recovery percentage, and the application of the method in biological samples will be studied in the future.

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Compliance with ethical standards The author(s) declare that they have no competing interests.

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