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High sensitive and label-free colorimetric DNA detection based on nicking endonuclease-assisted activation of DNAzymes

Juan Li, Qiu-Hong Yao, Hua-E. Fu, Xiao-Long Zhang, Huang-Hao Yang*

The Key Lab of Analysis and Detection Technology for Food Safety of the MOE, Fujian Provincial Key Laboratory of Analysis and Detection Technology for Food Safety, College of Chemistry and Chemical Engineering, Fuzhou University, Fuzhou 350002, PR China

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ABSTRACT

Horseradish peroxidase mimicking DNAzyme (HRP-DNAzyme) attracts growing interest as an amplifying label for biorecognition and biosensing events, especially for DNA detection. However, in the traditional designs, one target molecule can only generate one HRP-DNAzyme, which limits the signal enhancement and thus its sensitivity. In this article, we propose an amplified and label-free colorimetric DNA detection strategy based on nicking endonuclease (NEase)-assisted activation of HRP-DNAzymes (NEAA-DNAzymes). This new strategy relies on the hairpin-DNAzyme probe and NEase-assisted target recycling. In the hairpin-DNAzyme probe, the HRP-DNAzyme sequence is protected in a "caged" inactive structure, whereas the loop region includes the target complementary sequence. Upon hybridization with target, the beacon is opened, resulting in the activation of the HRP-DNAzyme. Meanwhile, upon formation of the duplex, the NEase recognizes a specific nucleotide sequence and cleaves the hairpin-DNAzyme probe into two fragments. After nicking, the fragments of the hairpin-DNAzyme probe spontaneously dissociate from the target DNA. Amplification is accomplished by another hairpin-DNAzyme probe hybridizing to the released intact target to continue the strand-scission cycle, which results in activation of numerous DNAzymes. The activated HRP-DNAzymes generate colorimetric or chemiluminescence readout signals, thus providing the amplified detection of DNA. The detection limit of the colorimetric method is 10 pmol/L, which are three orders of magnitude lower than that without NEase. In addition, the detection limit of the chemiluminescence method is 0.2 pmol/L. Meanwhile, this strategy also exhibits high discrimination ability even against single-base mismatch.

1. Introduction

Deoxyribozymes or DNA enzymes (DNAzymes) are catalytic nucleic acids that mimic the functions of enzymes [1]. They are selected via in vitro selection or SELEX (systemic evolution of ligands by exponential enrichment) techniques [2]. DNAzymes have been applied to various biological and chemical reactions, such as DNA cleavage [3], DNA ligation [4], N-glycosylation [5] and phosphorylation [6]. Several recent review articles summarized different applications of DNAzymes for sensing and nanotechnology applications [7]. One interesting example of DNAzymes that reveals peroxidase-like activity includes a supramolecular complex between hemin and a single-stranded guanine-rich nucleic acid [8]. This DNAzyme is called horseradish peroxidase-mimicking DNAzyme (HRP-DNAzyme), which catalyzes the H_2O_2-mediated oxidation of 2,2′-azino-bis(3-ethylbenzothiozoline)-6-sulfonic acid ($ABTS^{2-}$) to produce the colored product ($ABTS^{\bullet-}$) [9], or stimulates the generation of chemiluminescence in the presence of H_2O_2 and luminol [10]. The HPR-DNAzyme has attracted growing interest as an amplifying label for biorecognition and biosensing events. The easy synthetic preparation of DNAzymes and the reduced nonspecific adsorption of DNAzymes make catalytic nucleic acids attractive reporter units. More importantly, they are more stable and robust than protein enzymes. Indeed, numerous recent studies used HRP-DNAzyme as an amplifying unit for biosensing events, such as telomerase activity [10,11], small molecules [12], metal ions [13], and methyltransferase activity [14].

DNA detection plays a critical role in biomedical research, gene therapy and clinical diagnostics [15]. Extensive efforts have been made to develop many sensitive methods for DNA detection based on modern biotechnology and nanotechnology, including the use of HRP-DNAzyme [11,16–19]. For example, Willner and co-workers [11(a)] reported that the caged HRP-DNAzymes in the stem region were used for the amplified detection of DNA, reaching a detection limit of 200 nmol/L. The HRP-DNAzyme was also used as a label for the chemiluminescence detection of DNA with a detection limit of 1 nmol/L [10]. However, in these methods, each target generated a

* Corresponding author. Tel.: +86 591 22866135; fax: +86 591 22866135.
E-mail address: hhyang@fzu.edu.cn (H.-H. Yang).

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Table 1
Sequences of the used oligonucleotides (in 5′ to 3′ direction).

Type	Sequence
P1[a]	GGG TAG GGC GGG TTG GG TAGAT***GAGT C***CGTC↓CTGCT CCC AA CCC
T1[b]	AGC AGG ACG GAC TCA TC
T2[c]	AGC AGG ACG GAG TCA TC
T3[c]	AGC AGG AGG GAC TCA TC
T4[c]	AGG AGG ACG GAC TCA TC
T5[d]	GAT GAA GAA AGA GAG AC

[a] Hairpin-DNAzyme probes: the underlined bold letters are DNAzyme sequence. The italic bold letters are the recognition sequence of Nt.BstNBI, and the arrow indicates the nicking position.
[b] Perfectly complementary target.
[c] Single-base-mismatched target (the mismatched base is underlined).
[d] Non-complementary target.

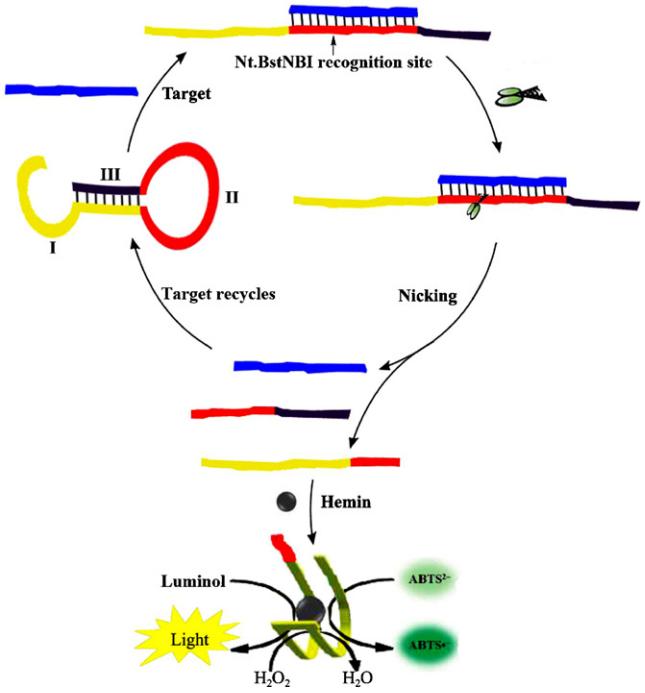
single HRP-DNAzyme. This 1:1 ratio limits signal enhancement and thus the sensitivity of the assay. In addition, it should be noted that the detection limit of the assay using DNAzyme as a catalytic label is about 2 orders of magnitude higher as compared to the analogous analysis using HRP as a biocatalyst. Recently, some ways have been proposed to overcome this problem. One way was to introduce Au nanoparticles (AuNPs) as biobarcodes. AuNPs were functionalized with many copies of DNAzymes to generate a strong output signal [11(b)]. The HRP-DNAzyme could also be very efficiently produced by polymerase chain reaction (PCR) [16] or rolling circle amplification (RCA) [17]. Furthermore, Willner and co-workers designed an ingenious DNA machine to strand displacement of the synthesized DNAzyme for the amplified analysis of DNA [18]. However, these amplified methods needed complicated modification or polymerase replication process. Consequently, it is still highly desirable to develop simple and sensitive strategies to improve the sensitivity of DNA assays based on HRP-DNAzyme as a catalytic label.

In this paper, we take advantage of NEase to create a new amplified DNA detection strategy using the HRP-DNAzyme as a label. NEase is a special family of restriction endonuclease and occurs either naturally or via gene engineering. NEases can recognize a specific sequence along a double-strand DNA, however, they hydrolyze only one specific strand instead of both strands, leaving a nick in the DNA [20]. Until now, over 200 NEases have been studied and many of them are available commercially. Our new strategy relies on the hairpin-DNAzyme probes and NEase-assisted target recycling. By employing this strategy, the detection limit of the colorimetric method is 10 pmol/L, which are three orders of magnitude lower than that without NEase. Furthermore, due to the high selectivity of NEases, this strategy also exhibits high discrimination ability even against single-base mismatch.

2. Experimental

2.1. Materials

Hemin was purchased from Sigma and was used without any further purification. A hemin stock solution was prepared in dimethyl sulfoxide (DMSO) and stored in the dark at −20 °C. The DNA oligonucleotides were synthesized by Shanghai Sangon Biotechnology Co. Table 1 shows the sequences of the used oligonucleotides. The NEase (Nt.BstNBI) and NEBuffer 3 (50 mmol/L Tris–HCl, 10 mmol/L $MgCl_2$, 100 mmol/L NaCl, 1 mmol/L dithiothreitol, pH 7.9) were obtained from New England BioLabs. 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and luminol were purchased from Sigma. All the other reagents were analytical grade.



Scheme 1. Schematic representation of analysis of DNA based on NEase-assisted activation of DNAzymes

2.2. Nicking assay

Each DNA was heated to 90 °C for 5 min, and slowly cooled down to room temperature. The hairpin-DNAzyme probe and target DNA were incubated at 55 °C with Nt.BstNBI in NEBuffer 3 buffer. Typically, the incubation was performed for 0–90 min with 0–80 U of NEase. After incubation, the resulting mixtures were heated at 80 °C for 20 min to deactivate the NEase. Then the mixtures were used for colorimetric and chemiluminescence assay.

2.3. Colorimetric measurements

Experiments were performed in 25 mmol/L HEPES buffer with 200 mmol/L NaCl and 20 mmol/L KCl pH 7.4. For the cuvette assay, hemin and ABTS were added to a final concentration of 375 nmol/L and 2 mmol/L, respectively. The peroxidase-mimicking reaction was started by addition of H_2O_2 (final concentration of 2 mmol/L). The color development was followed at 414 nm with a Lambda 750 UV/vis spectrometer (PerkinElmer).

2.4. Chemiluminescence measurements

Chemiluminescence experiments were performed using a BPCL-Weak Luminescence Analyzer (Institute of Biophysics, Chinese Academy of Science, Beijing, China). Measurements were made in a cuvette that included a buffer solution consisting of 25 mmol/L HEPES buffer with 200 mmol/L NaCl and 20 mmol/L KCl, pH 9.0 and 1 nmol/L hemin, 0.4 mmol/L luminol, and 20 mmol/L H_2O_2.

3. Results and discussion

3.1. Principle of DNA detection based on NEase-assisted activation of DNAzymes (NEAA-DNAzymes)

The detailed concept of NEAA-DNAzymes is illustrated in Scheme 1. This new strategy relies on the hairpin-DNAzyme probes and NEase-assisted target recycling. The hairpin-DNAzyme probe

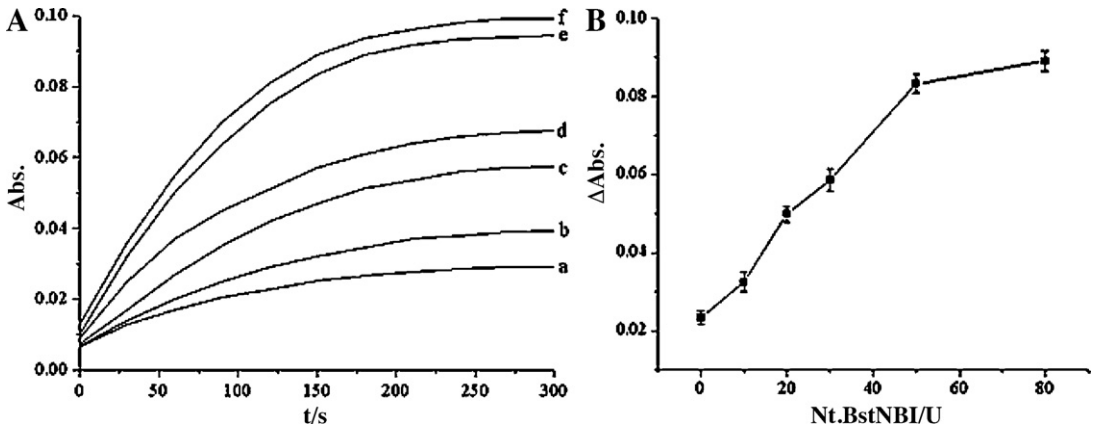


Fig. 1. (A) The time-dependent absorbance changes upon analyzing different concentrations of Nt.BstNBI at the fixed concentrations of P1 (200 nmol/L) and T1 (100 pmol/L). The curves (a–f) were obtained with different concentrations of Nt.BstNBI: 0, 10, 20, 30, 50 and 80 U, respectively. (B) The absorbance change (ΔAbs) in 5 min was plotted as a function of the Nt.BstNBI concentration. Scale bars indicate the standard deviation in quadruplicate experiments.

(termed as P1 in the following text) contains three domains termed as I, II, III according to their different functions. Region I is the HRP-DNAzyme sequence. In the hairpin probe, the DNAzyme sequence (region I) is partially caged in the duplex structure of the stem by hybridization with region III. As a result, the G-riched DNAzyme segment is prohibited to combine with the hemin molecule and this formation of the HRP-DNAzyme is inactive. Region II is a single-stranded loop of the beacon, which is complementary to the target sequence (termed as T1) and simultaneously contains a NEase recognition sequence. Upon hybridization with target, the beacon is opened, resulting in the activation of the HRP-DNAzyme. Meanwhile, upon formation of the duplex, the NEase recognizes specific nucleotide sequence and cleaves the P1 into two fragments. After nicking, the affinity of target DNA and the P1 is reduced and target DNA dissociates from the duplex structure. The released intact target DNA is able to hybridize with another P1 to continue the strand-scission cycle. In this way, one target DNA is able to open multiple P1, resulting in activation of numerous DNAzymes. Upon completion of the strand-scission cycle, the activated HRP-DNAzymes generate colorimetric or chemiluminescence readout signals, thus providing the amplified detection of DNA. Therefore, the proposed NEAA-DNAzymes strategy integrates two steps of signal amplification. In the first step, each target can go through many cycles, resulting in activation of numerous DNAzyme units. In the second amplification step, the activated DNAzyme units act as a catalyst to produce numerous readout signals.

3.2. Optimization of nicking reaction

To demonstrate the utility of the proposed NEAA-DNAzymes strategy, we designed a hairpin-DNAzyme probe (P1). The NEase used in this case is Nt.BstNBI, which recognizes a simple asymmetric sequence, 5′-GAGTC-3′, and cleaves only one DNA strand, 4 bases away from the 3′ end of its recognition site. Since the yield of nicked products is largely dependent on the concentrations of NEase and nicking time, various incubation times and the concentrations of NEase were investigated firstly.

Fig. 1A shows the time-dependent absorbance changes in the presence of the 200 nmol/L P1 and 100 pmol/L T1 upon analyzing different concentrations of NEase (0–80 U). As the concentration of NEase increased, the absorbance changes (ΔAbs) were intensified, implying enhanced cleavage of the hairpin-DNAzyme probes and generation of more HRP-DNAzymes. As shown in Fig. 1B, ΔAbs at 414 nm linearly increased with increasing the concentrations of NEase from 0 to 50 U and increases slowly after about 50 U.

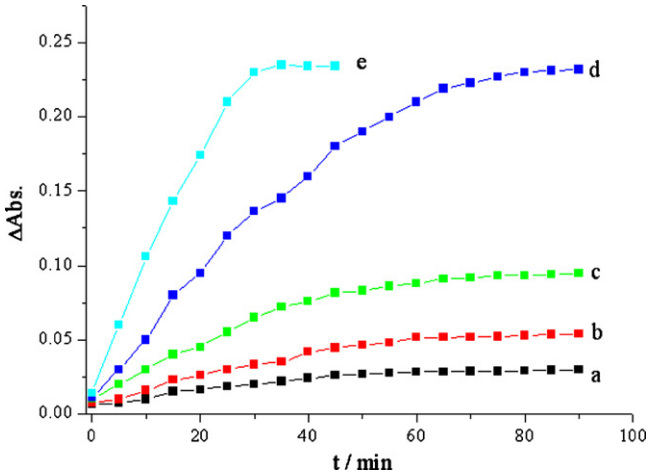


Fig. 2. The absorbance change (ΔAbs) in 5 min was plotted as a function of nicking time. The curves were obtained with different concentrations of T1, a–e: 0, 10, 100, 500 and 2000 pmol/L, respectively.

Therefore, 50 U is selected for nicking reaction in this work unless otherwise specified.

Next, in order to study the nicking process, the absorbance changes at 414 nm versus different nicking time were recorded (Fig. 2). With the increase of nicking time, the absorbance changes gradually increase. As anticipated, nicking reaction was extremely rapid. The absorbance reached a plateau after about 30 min in the presence of 2 nmol/L T1 and 50 U of NEase (Fig. 2, curve e). By decreasing the target concentration to 500 pmol/L, the reaction rate slowed down and the absorbance reached a plateau after about 90 min (Fig. 2, curve d). For achievement of the balance between short detection time and high signal intensity, 90 min was selected for the nicking reaction in this work.

3.3. Determination of detection limit

In the proposed NEAA-DNAzymes strategy, signal amplification brought about significant increase in the sensitivity of DNA detection. To investigate the signal amplification ability of this strategy, we compared the detection sensitivity of the strategy with NEase to that without NEase.

Under the optimization conditions, the time-dependent absorbance changes upon analyzing different concentrations of T1 without NEase are shown in Fig. 3A. The absorbance values gradually increased as the concentration of T1 was increased. This is

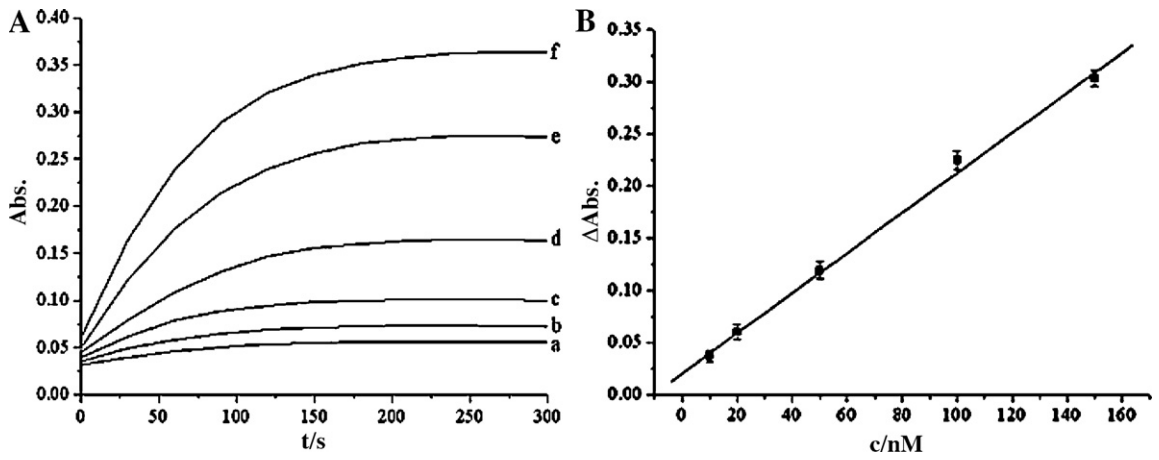


Fig. 3. (A) The time-dependent absorbance changes upon analyzing different concentrations of T1 at the fixed concentrations of P1 (200 nmol/L) without NEase, a–f: 0, 10, 20, 50, 100 and 150 nmol/L, respectively. (B) Calibration curve corresponding to the analysis of different concentrations of T1. Scale bars indicate the standard deviation in quadruplicate experiments.

consistent with the fact that the more the hairpin is opened, the higher the amounts of DNAzymes are activated. The response of the system in the absence of T1 is shown in Fig. 3A (curve a). The resulting absorbance was almost similar to that generated by free hemin and might be considered as the background signal. The calibration curve for analyzing T1 is shown in Fig. 3B, indicating that the detection limit is 10 nmol/L without NEase, which is similar to the previously reported results using the HRP-DNAzyme as a label [21].

Under the same conditions, Fig. 4A shows the time-dependent absorbance changes upon analyzing different concentrations of T1 with NEase. One may realize that small time-dependent absorbance changes were observed even in the absence of T1 (curve a). This oxidation absorbance changes were due to the inefficient hemin-catalyzed oxidation of $ABTS^{2-}$ by H_2O_2 and were observed only with hemin [12(a)]. This increased the background signal. Thus, the absorbance changes depicted in curve a, may be considered as the background signal of the system. Nevertheless, the signal was significantly above the background even in the presence of 10 pmol/L T1. The absorbance changes gradually increased as the concentration of T1 was increased. The calibration curve for analyzing T1 is shown in Fig. 4B, indicating that the detection limit is 10 pmol/L with NEase, which is three orders of magnitude lower than that without NEase and similar to the rolling circle amplification method using HRP-DNAzyme as a label [17(b)].

Moreover, the HRP-DNAzyme can catalyze the generation of chemiluminescence in the presence of luminol-H_2O_2. The NEAA-DNAzymes strategy was also characterized using the chemiluminescence (CL) method. The CL analysis (Fig. 5A) shows the changes of CL intensities upon analyzing different concentrations of T1. As the T1 concentration increased above 0.2 pmol/L, the CL signals became higher than the background. The generated CL intensity increased with increasing T1 concentrations, which could be attributed to increased contents of the activated DNAzyme. The CL intensity had a linear relationship with the logarithm of T1 concentration in the range of 0.2–10 pmol/L (Fig. 5B). The assay allowed for the detection of T1 at concentration as low as 0.2 pmol/L.

3.4. The specificity of the NEAA-DNAzymes strategy

The specificity of this strategy was investigated by using P1 to hybridize with various DNA sequences (complementary DNA T1, single-base-mismatch DNA T2–T4 and non-complementary DNA T5 mentioned in Table 1). To quantitatively evaluate the discrimination ability of our sensor, we defined the discrimination factor (DF) as (Abs2–Abs0)/(Abs1–Abs0), where Abs1, Abs2 and Abs0 are the value of absorbance changes of complementary target (T1), mismatched DNA (T2–T5) and the background (without target), respectively. The results were presented in Fig. 6. It can be seen

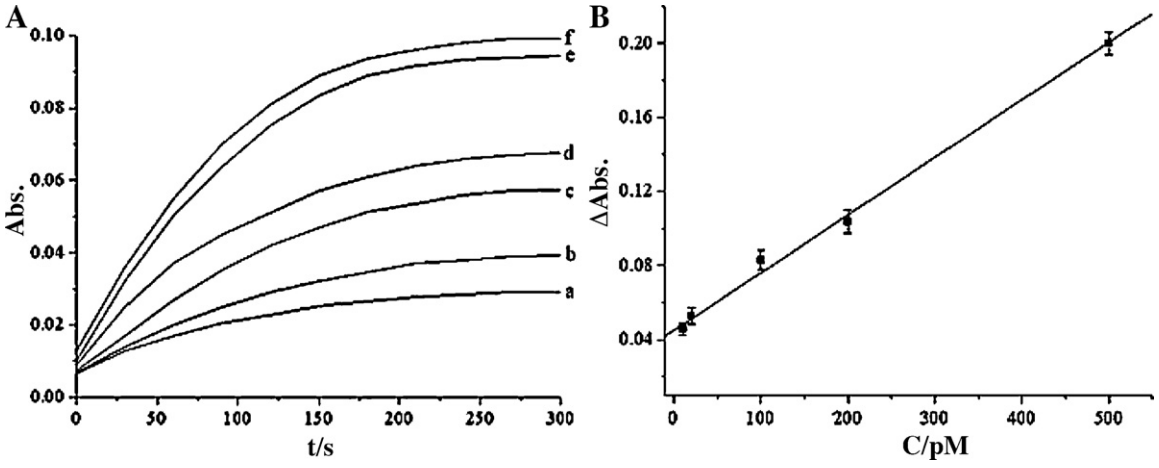


Fig. 4. (A) The time-dependent absorbance changes upon analyzing different concentrations of T1 at the fixed concentrations of P1 (200 nmol/L) with NEase, a–f: 0, 10, 20, 100, 200 and 500 pmol/L, respectively. (B) Calibration curve corresponding to the analysis of different concentrations of T1 with NEase. Scale bars indicate the standard deviation in quadruplicate experiments.

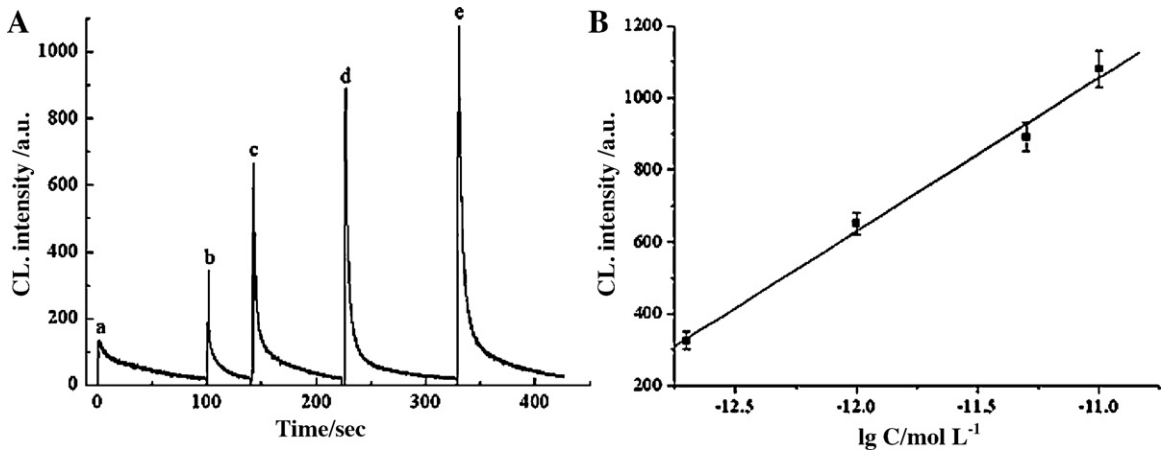


Fig. 5. (A) Chemiluminescence intensities upon analyzing T1 upon analyzing different concentrations of T1 at the fixed concentrations of P1 (10 nmol/L) with NEase, a–e: 0, 0.2, 1, 2 and 10 pmol/L, respectively. (B) Calibration curve corresponding to the analysis of different concentrations of T1. Scale bars indicate the standard deviation in quadruplicate experiments.

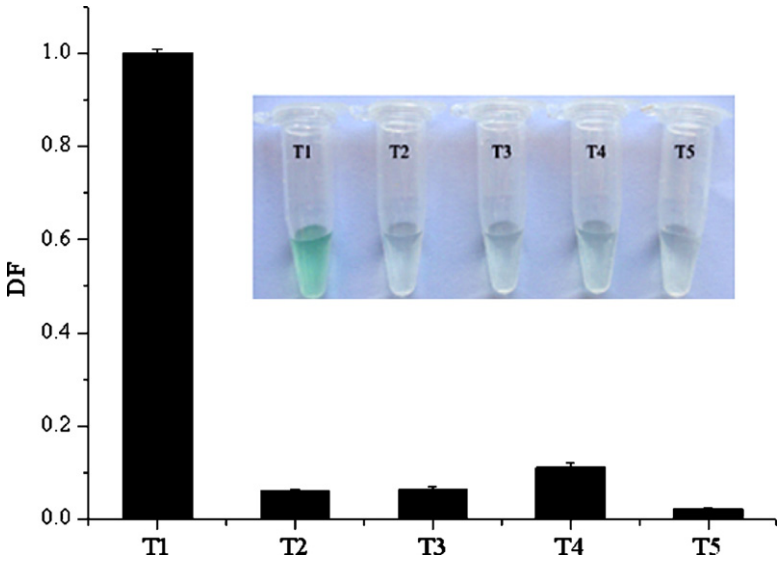


Fig. 6. Detection of single base mismatch. The concentrations of T1, T2, T3, T4 and T5 were 200 pmol/L. Inset: photograph showing colorimetric responses of the NEAA-DNAzymes strategy towards the single base mismatch. Each column is the average of four measurements, and the error bar is the standard deviation.

that DF is highly dependent on the position of mutation. Although T2, T3 and T4 all have only a single mismatched base, DF decreased obviously as compared with that for T1. DF varied due to one or both of the following effects of mutation [20]. Firstly, it directly disturbed the enzyme binding and/or nicking sites. Secondly, it lowered the stability of the target–probe hybrid. Therefore, the proposed NEAA-DNAzymes strategy had excellent specificity. Mutation of T2 occurs at the centre of the enzyme binding site, resulting in the dramatic drop in DF. Surprisingly, although mutation T3 is at neither binding site nor nicking site, its effect is as strong as that of T2, probably because it can influence both binding and nicking. Mutation of T4 is the furthest from the binding site, and is also out of the nicking site. So it has the least influence on DF among these three single-base-mismatch DNA. Yet it still causes significant drop in the DF, probably due to its influence on the stability of the duplex. In addition, as expected, the minimum DF can be observed for T5, since no successful hybridization occurs due to the sequence mismatch between the P1 and the non-complementary sequence. Furthermore, the result of this assay can be clearly observable with the naked eyes (Fig. 6 inset). These facts clearly indicated that our strategy had excellent discrimination ability for the detection of a single-base mismatch.

4. Conclusions

In conclusion, a sensitive and label-free colorimetric DNA strategy based on NEAA-DNAzymes is reported in this study. This strategy integrates two steps of signal amplification. In the first step, NEase-assisted target recycling makes each target go through many cycles, resulting in activation of numerous DNAzyme units. In the second amplification step, the activated DNAzyme units act as a catalyst to produce numerous readout signals. The sensitivity of the NEAA-DNAzymes strategy was three orders of magnitude higher than that without NEase. Meanwhile, due to the high selectivity of NEases, this strategy also exhibits high discrimination ability even against single-base mismatch. Furthermore, the single-base mismatch can be clearly visualized with the naked eye. It must be mentioned that the NEAA-DNAzymes strategy can only be applied to the DNA sequence containing the recognition site of the NEase. However, more and more NEases have been obtained via gene engineering and parts of them are available commercially. Therefore, through selecting endonuclease and designing signaling probe according the target sequence, we believe that our strategy could offer a universal approach for DNA detection using HRP-DNAzyme as a label.

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