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PAPER IN FOREFRONT

A simple enzyme-assisted cascade amplification strategy for ultrasensitive and label-free detection of DNA

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Abstract

A simple fluorescence biosensor is developed based on the enzyme-assisted cascade amplification strategy. The amplification system consists of a hairpin-structure DNA (H-DNA) and exonuclease III. The target DNA can hybridize with the H-DNA and initiate exonuclease III-assisted target recycling amplification to generate abundant G-rich DNA (G-DNA). One region of G-DNA is designed to possess the same sequence as target DNA. Thus, the G-DNA can also hybridize with H-DNA and initiate the digestion of H-DNA. The cascade strategy in this amplification system causes the concentration of G-DNA to grow exponentially. The fluorescence intensity of *N*-methylmesoporphyrin IX (NMM) is highly enhanced due to the formation of G-quadruplex configuration. Under optimal conditions, the cascade system could achieve an admirable sensitivity with a detection limit of 52 fM for HIV DNA, and guarantees a satisfactory specificity. Moreover, the cascade system could be implemented for other target DNA detections by substituting the recognition region of the H-DNA. In this way, a detection limit of 65 fM for HBV DNA could be achieved by the cascade system. The target DNA analysis in a real serum sample further indicates that this biosensor has potential for future application in clinical diagnosis.

Keywords Biosensor · Cascade · Label-free · Hairpin DNA · Exonuclease III

Introduction

Signal amplification strategies have become an important research topic due to the demand of various domains, including environmental risk [1], food safety [2], clinical diagnostics [3, 4], and bioanalysis [5, 6]. Nucleic acid amplification techniques are very valuable and highly coveted due to their robustness, versatility, and simplicity [7–9]. Up to now, many nucleic acid amplification techniques have been developed to improve the sensitivity and selectivity [10–12], e.g., polymerase chain reaction [13] and rolling circle amplification [14, 15]. These techniques have an adequate sensitivity, but the requirement of expensive instrumentation and sophisticated operation greatly limits the applications in point-of-care testing [16–18]. Therefore, it is still a challenge to construct a simple and universal amplification strategy with high sensitivity and adequate selectivity.

Hairpin DNA is a single-stranded oligonucleotide with a stem-loop structure under appropriate conditions [19–22]. Benefiting from the stimuli-induced conformation switch, hairpin DNA has been widely employed for the construction of a biosensor and amplification device [23, 24]. Yuan's group reported a 3-D DNA nanomachine for mucin 1 protein detection. The recognition element is hairpin-structure DNA. In the presence of the target, it will induce the hairpin DNA switch to a new conformation for initiating the biosensor [25]. Hairpin self-assembly amplification strategy is an isothermal amplification technique and is one of the most promising candidates [26]. Up to now, various biosensors have been reported based on hairpin self-assembly [27–29]. Zhang et al. constructed a

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Kun Wang and Fu-Heng Zhai contributed equally to this work.

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fast-assembled biosensor based on catalytic hairpin assembly for colorimetric detection of oligonucleotides [30]. Bi's group reported a cross-catalytic hairpin assembly system for DNA detection, which utilized four different hairpin DNAs for constructing a biosensing platform based on toehold-mediated strand displacement [31, 32]. However, the mixed hairpin DNAs may lead to a high background signal, and influence the analytical performance [33, 34]. In order to reduce the background signal and improve the performance of the biosensor, a simple and universal cascade amplification strategy is constructed based on a hairpin DNA and exonuclease III (EXO III), which provides a new direction for developing nucleic acid amplification techniques [35–37].

Cascade is an important method for an exponential amplification strategy [38–40]. A cascade system can cause the exponential growth kinetics, which can improve the sensitivity and reduce the reaction time [41–44]. Chen's group utilized two hairpin DNAs and EXO III to construct an EXO III-assisted cascade signal amplification strategy for DNA detection [45]. Although the cascade amplification strategy achieved high sensitivity, the separate reaction and the EXO III inactivation may lead to a complicated and time-consuming operation. Li's group reported a cascade amplification strategy based on two DNAs and EXO III for chemiluminescence detection of DNA [46]. However, this method utilized the DNAzyme to catalyze the luminol–H_2O_2 reaction to generate the signal, which may limit the applications in serum detection. Inspired by those strategies, we proposed a cascade amplification strategy (CAS) based on a single hairpin DNA and EXO III. To achieve an exponential DNA amplification, the CAS takes advantage of EXO III-assisted target recycling amplification to create an autonomous cascade mechanism, overcoming the high background of hairpin-based amplification strategy. The EXO III has a high exodeoxyribonuclease activity for duplex DNA with an overhanging 3′ end, which provides selective enzymatic digestion of H-DNA upon hybridizing with target DNA. Taking HIV DNA detection as a trial system, when the HIV DNA hybridizes with the H-DNA, the H-DNA/HIV DNA duplex is formed with a blunt 3′ end of H-DNA. Then, the EXO III digests the H-DNA from the 3′ terminus, releasing the G-rich DNA (G-DNA). The G-DNA is well-designed, which also can hybridize with H-DNA and initiate the digestion of H-DNA. The proposed CAS is composed by the two recycle reactions. By virtue of the novel strategy, the biosensor achieves a satisfactory detection limit of 52 fM for HIV DNA. The CAS can discriminate the mismatched target DNA even with one-base difference. Moreover, the CAS can be implemented for other target DNA detections by substituting the recognition region of the H-DNA, e.g., HBV DNA detection. The target DNA analysis in a real serum sample further indicates that the present biosensor has potential for future application in clinical diagnosis.

Materials and methods

Materials and reagents

DNAs were purchased from Sangon Biotechnology Co., Ltd. (Shanghai, China). The sequences of DNAs are shown in Table S1 in the Electronic Supplementary Material (ESM). DNA was prepared by dissolving it in 50 mM Tris-HCl buffer (containing 50 mM $MgCl_2$, 100 mM KCl) at pH 7.0 and stored at − 20 °C. The H-DNA was heated to 90 °C for 10 min, then slowly cooled down to room temperature before use, in order to make it completely form a hairpin structure. *N*-Methylmesoporphyrin IX (NMM) was purchased from Porphyrin Products (Logan, UT, USA). The DNA marker was purchased from Thermo Fisher Scientific (USA). Other chemicals were purchased from Aladdin Reagent (Shanghai, China). All reagents used were of analytical reagent grade unless specified. Milli-Q ultrapure water of 18 MΩ cm was used throughout the study.

Procedure of the cascade amplification system

The working solution containing 500 nM H-DNA and 60 U EXO III was mixed with different concentrations of target DNA, and reacted at 37 °C for 90 min. Then, 1.2 μM NMM was introduced to incubate for another 30 min. Finally, the fluorescence of the solution was measured by a Hitachi F-7000 fluorescence spectrometer. The emission spectra were recorded from 555 to 750 nm at an excitation wavelength of 399 nm. Human serum samples were obtained from the Hospital of Northeast University (Shenyang, China).The detection in human serum samples was performed with the same procedure. The human serum samples were 50-fold diluted with Tris-HCl buffer prior to detection. Then, different concentrations of target DNA were spiked in the diluted serum sample, and detected with the same procedure.

Polyacrylamide gel electrophoresis

Polyacrylamide gel (10%) was prepared with Tris-acetic acid-EDTA buffer (40 mM Tris, 20 mM acetic acid, 20 mM EDTA, 125 mM magnesium acetate pH 7.5). Each sample was prepared in Tris-acetic acid-EDTA buffer, and the concentration of DNA was 2 μM. Ten microliters of each sample was mixed with 1 μL of native tracking dye buffer before loading into the gel. Then, gel electrophoresis ran under a constant voltage of 200 V for ca. 3 h. Subsequently, the gel was stained in ethidium bromide solution for 10 min. Finally, a fluorescence imaging system (KODAK Gel Logic 200 Imaging System, USA) was used to record the photograph of the gel.

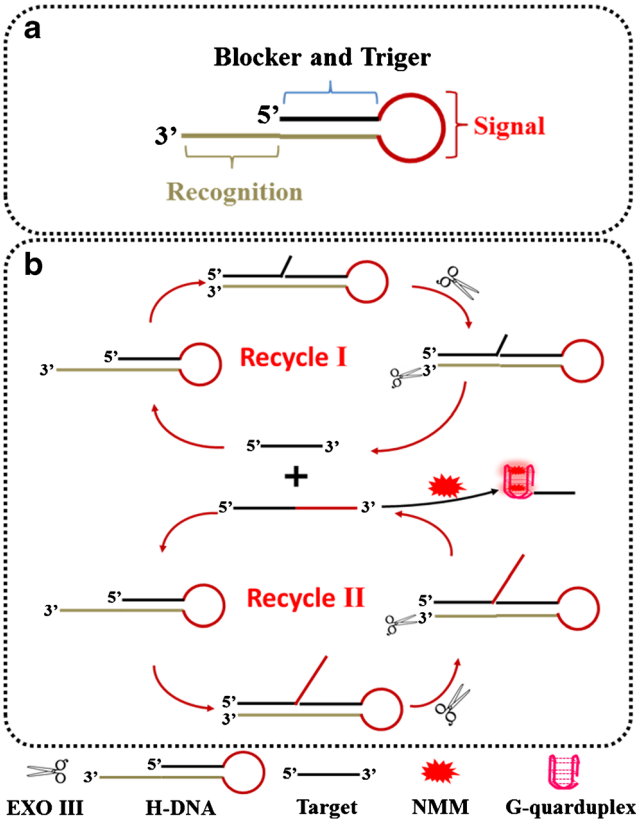

Fig. 1 Schematic illustration of a label-free and cascade amplification strategy for DNA detection. The amplification strategy is developed by exploiting H-DNA and exonuclease III (EXO III), where the fluorescence of *N*-methylmesoporphyrin IX (NMM) is used as signal

Results and discussion

Principle of the label-free and cascade amplification strategy for DNA detection

In this amplification system, a single hairpin DNA and EXO III are utilized to construct the sensing platform, as shown in Fig. 1. In the absence of target DNA, the spontaneous recycle reaction is hindered, since the EXO III hardly shows any activity toward 3′-overhang ends of DNA. Upon the introduction of target DNA, target DNA first hybridizes with the single-strand domain of H-DNA to form a double-strand stem. Then, the EXO III can degrade the H-DNA from the 3′-blunt ends to produce the stretches of single-strand DNA (G-DNA). It is noteworthy that the G-DNA contains two regions. One region is the G-quadruplex sequence, which can form a G-quadruplex structure and enhance the fluorescence of NMM. The other region is designed to possess the same sequence as target DNA. Therefore, the G-DNA can initiate the EXO III-assisted target recycling reaction and degrade the H-DNA to generate G-DNA. The cascade amplification strategy guarantees the sensitivity of the biosensor. Furthermore, the label-free signal avoids the expensive labeling procedure of the reporter probe, which holds potential for future application in resource-limited settings.

To demonstrate the feasibility of the CAS, the fluorescence of different components is monitored by the same procedure for target detection. As shown in Fig. 2A, a minimal fluorescence intensity of NMM is observed for the solution containing H-DNA and NMM (curve a). It is because the G-quadruplex sequence is blocked in the hairpin structure, and unable to enhance the fluorescence of NMM. When the EXO III is introduced, the solution still exhibits a low fluorescence intensity (curve b). It is because the EXO III hardly shows any activity toward 3′-overhang ends of DNA. Upon the addition of target DNA, a remarkable fluorescence intensity of NMM is observed (curve c), indicating that the G-quadruplex structure is formed and the recycle reaction is performed as expected. These results demonstrate that the CAS is feasible for DNA detection.

The DNA interactions in the CAS are validated by polyacrylamide gel electrophoresis analysis. As shown in Fig. 2B, the bands in lane a and lane b correspond to HIV DNA and H-DNA, respectively. When the H-DNA and HIV DNA are mixed, a new band shows in lane c, indicating that the HIV DNA can hybridize with the H-DNA to form the HIV DNA/

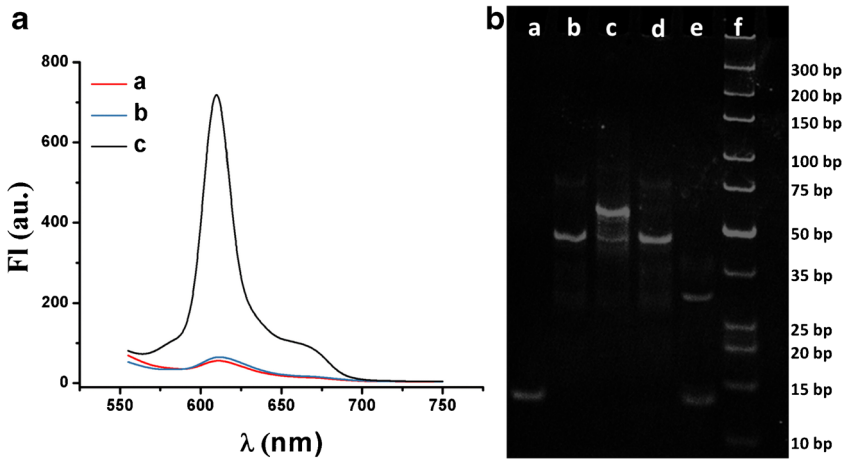

Fig. 2 (**A**) The florescence responses in the presence of various components, including H-DNA/NMM (a), H-DNA/NMM/EXO III (b), and H-DNA/NMM/EXO III/HIV DNA (c). (**B**) Polyacrylamide gel electrophoresis (PAGE) analysis of CAS: HIV DNA (a), H-DNA (b), HIV DNA/H-DNA (c), H-DNA/EXO III (d), HIV DNA/H-DNA/EXO III (e), and DNA marker (f)

H-DNA duplex. In lane d, the band of H-DNA is apparent without other new bands, because the EXO III fails to degrade the H-DNA in the absence of HIV DNA. When HIV DNA is added in the system, the band of H-DNA almost disappears and a new band shows in lane e, indicating that the EXO III degrades the H-DNA to generate the G-DNA. The results herein demonstrate the feasibility of the CAS for target DNA detection.

Optimization of experimental conditions for the biosensor

The amount of NMM plays an important role in the biosensor. If the concentration of NMM is too low, the fluorescence intensity would be low even when all the H-DNAs are cleaved by EXO III. On the other hand, if the concentration of NMM is too high, the excess NMM would result in a high background signal. Hence, the concentration of NMM is investigated to fix the best signal-to-background ratio. As shown in Fig. S1 (see ESM), the fluorescence intensity increases with the increase of NMM concentration from 0.2 to 1.2 μM, and then moderately increases. Therefore, 1.2 μM NMM is chosen to give the best signal-to-background ratio. In order to improve the performance of the biosensor, the dosage of EXO III and the incubation time for CAS are also investigated. As shown in Fig. S2A (see ESM), the fluorescence intensity increases with the increase of EXO III concentration up to 60 U, and then reaches a plateau. Similarly, the fluorescence signal increases with the increase of incubation time, until the time reaches 90 min (ESM Fig. S2B). Therefore, the optimum conditions for the CAS are 80 U EXO III and 90 min for incubation.

Analytical performance of the CAS

To investigate the analytical performance of the CAS, the sensitivity and the detection range are evaluated by monitoring the fluorescence change of NMM. As shown in Fig. 3, the fluorescence of NMM gradually increases with the increase of HIV DNA concentration from 0.1 to 2 pM. The fluorescence intensity at 610 nm is proportional to the HIV DNA concentration. A linear relationship between fluorescence intensity and HIV DNA concentration is obtained from 0.1 to 1 pM. There is a regression equation of $FI = 515C_{HIV} + 61$ with a correlation coefficient of $R = 0.995$, where FI represents the fluorescence intensity at 610 nm. According to 3σ/slope, the limit of detection (LOD) is calculated to be 52 fM. In order to evaluate the performance of the CAS, several other detection methods are also selected for comparison. Obviously, the CAS still shows high sensitivity, simple composition, and comparable reaction time with respect to previously reported methods (ESM Table S2). This favorite sensitivity is contributed by the cascade strategy, which causes the concentrations of trigger-DNA and signal-DNA to grow exponentially. The CAS provides a new direction for developing the DNA amplification strategy.

Selectivity is also significant for real clinical application. Hence, various mutants are introduced into the system to evaluate the specificity of the FAS. The influence of the mismatch location has been investigated by testing three kinds of single-mismatch sequences (the sequences of mutants are listed in the ESM Table S1). As shown in Fig. 4A, when the mismatch is located at the end of the sequence, it can be easier distinguished than that located at the middle of the sequence. The sequence specificity of this method probably comes from two reasons. One is the difference in the melting temperature between fully matched and mismatched

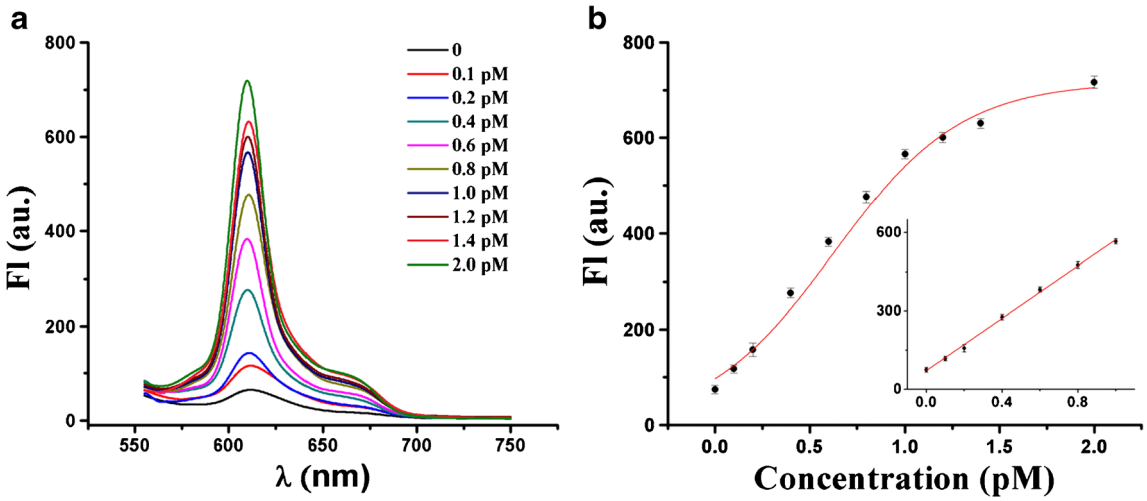

Fig. 3 (**A**) The fluorescence responses corresponding to various concentrations of HIV DNA ranging from 0 to 2 pM. (**B**) The fluorescence intensity in the presence of various concentrations of HIV DNA. Inset: calibration curve of fluorescence intensity versus HIV DNA concentration

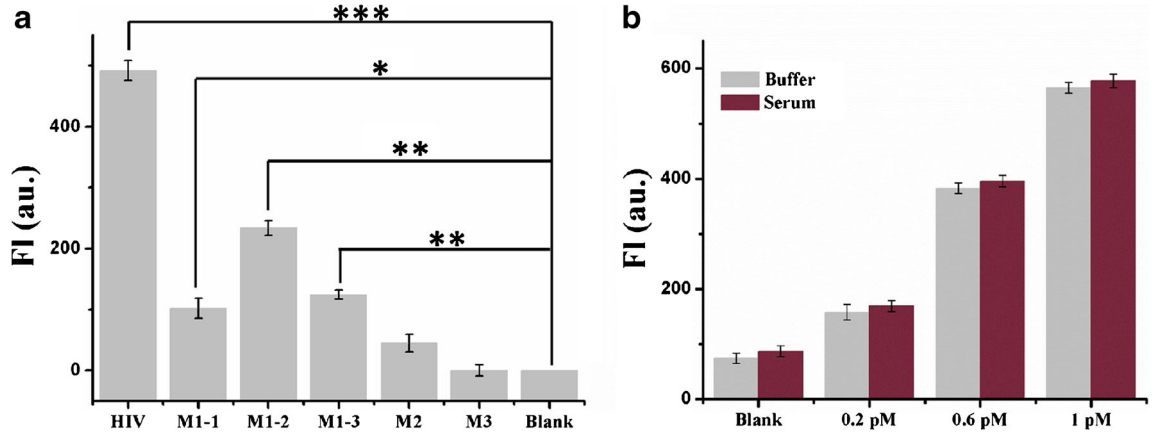

Fig. 4 (**A**) The fluorescence intensity in the presence of 1 pM HIV DNA, 1 pM single-base-mismatched DNA (M1-1, M1-2, M1-3), 1 pM two-base-mismatched DNA (M2), and 1 pM three-base-mismatched DNA (M3). (**B**) Results obtained from the buffer solution and the diluted serum samples spiked with different concentrations of HIV DNA. Blank represents the control sample containing the same reaction mixture without target or mutants. Statistical analysis illustrated by asterisks with tracing lines represents significant difference between the control and treatment groups. One asterisk, $P < 0.001$; two asterisks, $P < 0.00001$; three asterisks, $P < 0.000001$; one-way ANOVA

targets; the other is the specific catalytic activity of EXO III to double-stranded DNA hybrid. The mismatch "gap" causes the resulting DNA hybrid to be feeble, which weakens the coupling between the EXO III binding and cleavage, and, as a result, slows down the cleavage rate and produces obviously different fluorescence signals. Simultaneously, the signal change caused by two-base mismatch is even smaller. The fluorescence in the presence of three-base mismatch is very close to that of the blank. The excellent selectivity is contributed by the selectivity of EXO III. In order to investigate the potential application for gene detection in the complicated biosample, the CAS is performed in the diluted human serum sample. The HIV DNA is spiked in the diluted human serum sample, and detected with the same procedure of HIV DNA detection in the buffer. As shown in Fig. 4B, the signal generated by HIV DNA in human serum is similar to that in the buffer. The favorite performance of this method in serum probably comes from two reasons. Firstly, the stable performance of EXO III in diluted serum samples causes the result. Secondly, the simple content of this method makes the strategy have strong flexibility in the complicated biosample. Hence, the proposed CAS has potential for application in clinical diagnosis.

Adaptability of CAS for other DNA targets

Due to the simple components in CAS, it is possible to design CAS for other targets by substituting the recognition region of the H-DNA. In order to investigate the adaptability of CAS, we apply the CAS for the detection of HBV DNA. The recognition region of the H-DNA is substituted with the complementary sequence of HBV DNA. Then, the HBV DNA can be detected with the same procedure of HIV DNA detection. As shown in Fig. 5A, the fluorescence intensity is intensified with

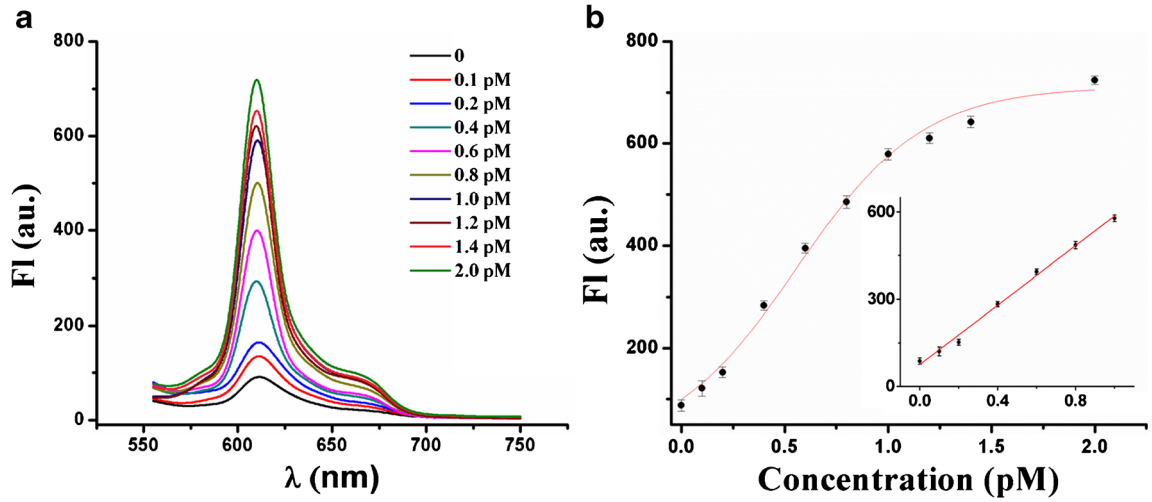

Fig. 5 (**A**) The fluorescence spectra of NMM in the presence of different concentrations of HBV DNA. (**B**) The peak fluorescence intensity as a function of HBV DNA concentration. Inset: calibration curve of peak fluorescence intensity versus HBV DNA concentration

the increase of HBV DNA concentration, which is similar to the result of HIV DNA detection. According to 3σ/slope, the LOD is calculated to be 65 fM (Fig. 5B). The same design can be employed for other target detections. A target-dependent structure-switching method has been presented in our previous work [47], which makes the FAS hold the potential to expend the application in other targets with aptamer.

Conclusions

A simple and label-free cascade amplification strategy is developed by combining hairpin DNA with EXO III. The novel DNA amplification strategy provides an admirable sensitivity with a LOD of 52 fM for HIV DNA detection, which is significantly higher than that of previously reported methods. The CAS can distinguish single-base mismatched mutants from target DNA, and exhibits a favorable performance for DNA detection in the human serum sample. Moreover, the CAS can be employed for HBV DNA detection, which holds potential application in other target detections. We believe that this simple but powerful DNA amplification strategy would further diversify the bioanalysis utility.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals This study was approved by the ethical review committee of The Hospital of Northeast University (Shenyang, China). Written informed consent was given by the volunteer from whom the human serum sample was obtained.

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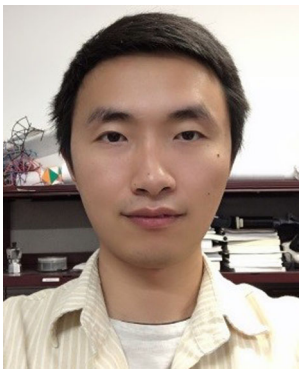

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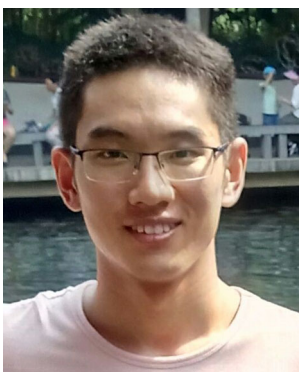

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