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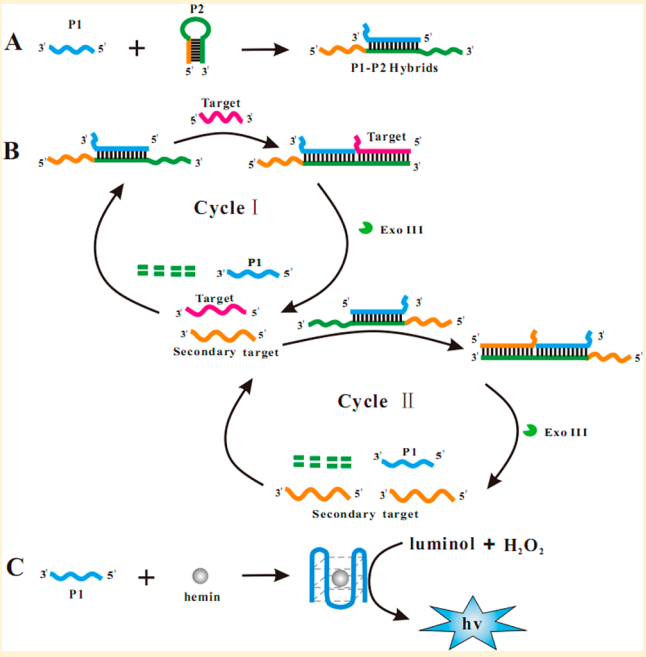
Exonuclease III-Assisted Cascade Signal Amplification Strategy for Label-Free and Ultrasensitive Chemiluminescence Detection of DNA

Yuan Gao and Baoxin Li*

Key Laboratory of Analytical Chemistry for Life Science of Shaanxi Province, School of Chemistry & Chemical Engineering, Shaanxi Normal University, Xi'an 710062, China

Supporting Information

ABSTRACT: Detection of ultralow concentrations of specific nucleic acid sequences is a central challenge in the early diagnosis of genetic diseases and biodefense applications. Herein, we report a simple and homogeneous chemiluminescence (CL) method for ultrasensitive DNA detection. It is based on the exonuclease III (Exo III)-assisted cascade signal amplification and the catalytic effect of G-quadruplex–hemin DNAzyme on the luminol–H_2O_2 CL system. A quadruplex-forming DNA probe hybridizes a hairpin DNA probe to construct a duplex DNA probe as recognition element. Upon sensing of target DNA, the recognition of target DNA and the duplex DNA probe triggers the Exo III cleavage process, accompanied by releasing target DNA and generating a new secondary target DNA fragment. The released target DNA and the secondary target DNA are recycled. Simultaneously, numerous quadruplex-forming sequences are liberated and bind hemin to yield G-quadruplex–hemin DNAzyme, which subsequently catalyze the luminol–H_2O_2 reaction to produce strong CL emission. This method exhibited a high sensitivity toward target DNA with a detection limit of 8 fM, which was about 100 times lower than that of the reported DNAzyme-based colorimetric system for DNA detection with Exo III-assisted cascade signal amplification. This method provides a simple, isothermal, and low-cost approach for sensitive detection of DNA and holds a great potential for early diagnosis in gene-related diseases.



Detection of nucleic acids is a central research field in modern bioanalytical science because of its broad applications in molecular diagnostics, forensic investigation, genetic therapy, and biomedical development.[1,2] The disease-related nucleic acids are often found only in trace amounts in biological extracts, and seeking a simple and convenient method for probing ultralow concentrations of specific single-stranded DNA (ssDNA) has risen to become a great challenge in recent years.[3] Over the past decades, polymerase chain reaction (PCR) has evolved as the most frequently used technique for detecting low levels of DNA targets.[4] Despite the attractive sensitivity of PCR, the requirements of precise temperature control, a complex sets of primers, and professional handling procedures with high costs limit the wide application of this technique.[5] In order to overcome this problem, the nuclease-assisted signal amplification strategy, in which one target molecule leads to many cycles of target-dependent nuclease digestion of reporter molecules, has been developed for sensitive DNA detection.[6−8] The target recycling strategy can indirectly amplify the amounts of target DNA, which holds great promise as a substitute for PCR due to its easy operation and isothermal reaction.[6] Among the signal amplification methods, nicking enzyme signal amplification was first proposed and attracted considerable interest.[9] Unfortunately, because nicking enzyme is sequence-specific and requires the nucleic acid substrate to contain certain sequence for recognition, the application is limited to the number of DNA sequences that can be recognized by nicking enzyme.[10,11]

Recently, an exonuclease III (Exo III)-assisted target recycling method has been proposed for amplified DNA detection.[3,8,11−14] Unlike nicking endonucleases, Exo III does not require a specific recognition site and can catalyze the stepwise removal of mononucleotides from the 3′-hydroxyl termini of double-stranded DNA. It is not active on 3′-overhang ends of double-stranded DNA or ssDNA. Thus, Exo

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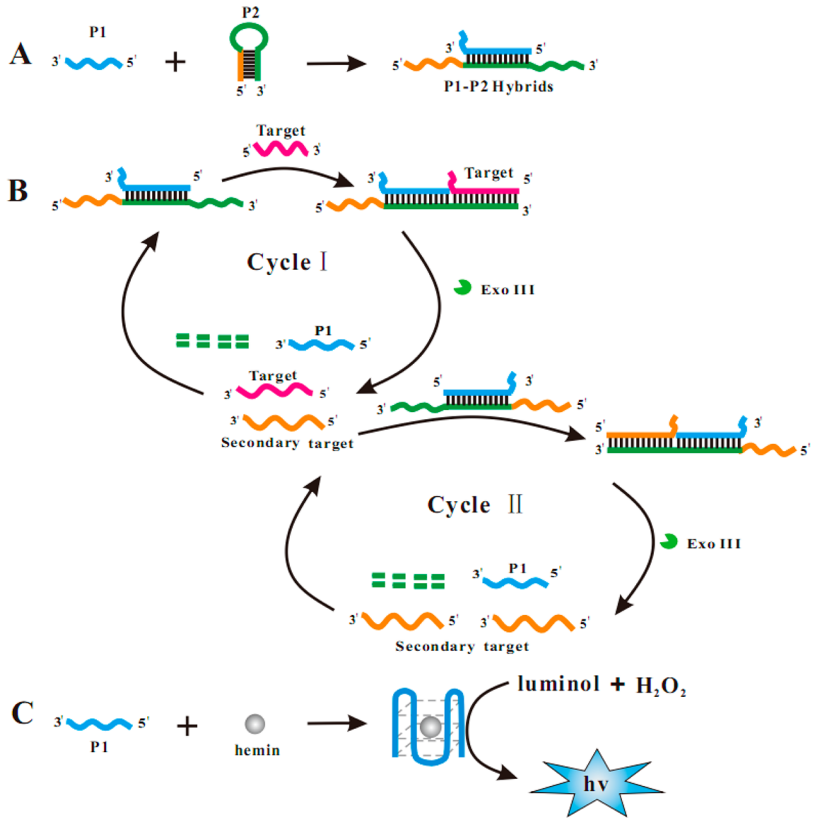
Scheme 1. Schematic Illustration of the Cascade Signal Amplification Strategy for DNA Detection: (A) Formation of the Catalytically Inactive Hybrid Probe (P1−P2) through Hybridization of a Quadruplex-Forming DNA Probe (P1) and a Hairpin DNA Probe (P2), (B) Exo III-Assisted Cascade Recycling Amplification Process, and (C) Binding of G-Rich DNA with Hemin To Yield DNAzyme, Catalyzing the Oxidation of Luminol by H_2O_2 To Produce CL



III provides a more versatile platform for amplification detection of DNA. However, most of the reported works using Exo III-assisted amplification were involved in labeling procedures (such as tagging fluorophore for optical detection[11−15] and tagging ferrocene or other redox-active labels for electrochemical assays[16−18]) and other modifications to immobilize the probe on carriers.[19−21] Therefore, a strategy for label-free and immobilization-free detection of DNA sequences that not only reduces the cost of the assay but also simplifies the operation is in great demand.

Herein, we develop an Exo III-assisted cascade signal amplification strategy for label-free and ultrasensitive chemiluminescence (CL) detection of DNA (Scheme 1). Very recently, cascade amplification by catalytic DNAzyme has been introduced in bioanalysis and biosensing.[16,19,22−28] The cascade amplification has been demonstrated to dramatically amplify the signal output. In the reported sensing platform based on cascade amplification by catalytic DNAzyme, 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS)−H_2O_2 chromogenic reaction has predominantly been used as signal readout, and the analytes can be detected by absorbance methods.[24−28] Although colorimetric detection is low-cost and easy to operate, the colorimetric method presents some inherent shortcomings (low sensitivity and narrow linear range). In addition, ABTS can turn green after oxidation, but the green product is not stable and can quickly decay to a colorless product in aqueous media within 10 min.[29] So, the ABTS−H_2O_2 chromogenic system is not an ideal signal readout for G-quadruplex DNAzyme. In this study, we employ DNAzyme-driven CL for signal readout in the cascade signal amplification strategy. Taking advantage of the amplification efficiency of the cascade signal amplification strategy and the intrinsically high sensitivity of CL detection, the proposed method provides an ultrasensitive platform for DNA detection with a wide dynamic range from 0.01 to 1.0 pM and an extremely low detection limit of 8 fM. Moreover, CL-based readouts permit a dynamic range and the use of simple instrumentation.

EXPERIMENTAL SECTION

Chemicals and Materials. All oligonucleotides were HPLC-purified and synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). The sequences of oligonucleotides are listed in Table 1. The oligonucleotide stock solutions (20 μM) were prepared in Tris−HCl buffer (140 mM NaCl, 20 mM $MgCl_2$, 20 mM KCl, pH 7.4) and diluted to the desired concentration with the

Table 1. Sequences of the Used Oligonucleotides

oligonucleotides	sequence (5′-to-3′)
probe 1 (P1)	CAC TGG GTT GGG CGG GAT GGG TTT T
probe 2 (P2)	GGA AGA CTC ATG TTA TCC CGC CCA ACC CAG TGG AGT CTT CC
probe 3 (P3)	TTA TCC CGC CCA ACC CAG TGG AGT CTT CC
T	GGA AGA CTC TTG T
T1	GGA ATA CTC TTG T
T2	GTA AGA CGC TTG T
Tn	ATG GAT GAA TTG T

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same Tris−HCl buffer. Each oligonucleotide was heated to 90 °C for 10 min and slowly cooled to room temperature before use to unwind the single-stranded oligonucleotide. Exo III was purchased from Sangon Biotechnology Co., Ltd. (Shanghai, China). Luminol and hemin were obtained from Sigma (St. Louis, MO). Luminol stock solution (25 mM) was prepared by dissolving 4.43 g of luminol in 20 mL of NaOH (0.10 M) and then diluting to 1 L with water. Luminol solution was stored in the dark for 1 week prior to use. Hemin stock solution (25 mM) was prepared by dissolving hemin in a water−dimethyl sulfoxide (DMSO) mixture (volume ratio, H_2O/DMSO = 90/10) and stored in the dark at −20 °C. Hydrogen peroxide (H_2O_2, 30%) was obtained from Xi'an Chemical Reagent Plant (Xi'an, China). The working H_2O_2 solutions were prepared fresh daily from 30% (w/w) H_2O_2. Unless otherwise indicated, all reagents and solvents were purchased in their highest available purity and used without further purification. Ultrapure water (18 MΩ cm^{-1}) was obtained through a Millipore Milli-Q water purification system (Billerica, MA), and was used in all experiments.

Apparatus. The CL intensity was measured and recorded with a model IFFM-D chemiluminescence analyzer (Xi'an Ruimai Electronic Sci. Tech. Co. Ltd., Xi'an, China). The circular dichroism (CD) spectra were performed on a Chirascan CD spectrophotometer (Applied Photophysics, Leatherhead, UK), of which the lamp was always kept under a stable stream of dry purified nitrogen (99.99%) during experiments. An HH-1 electric-heated thermostatic water bath (Beijing Kewen Instrumental Factory, Beijing, China) was used to control the reaction temperature at 0.1 °C intervals.

Exo III-Aided Cascade Target Recycling Amplification Reaction. The detailed procedure for Exo III-assisted cascaded recycling amplification was as follows. First, the DNA duplex P1−P2 hybrid probe was prepared by mixing 50 μL of P1 (0.5 μM) and 50 μL of P2 (0.5 μM) in 20 mM Tris−HCl buffer (pH 7.4, 70 mM NaCl, 10 mM $MgCl_2$, 20 mM KCl). The P1−P2 hybrid was heated to 90 °C for 10 min and allowed to cool to room temperature for at least 30 min. Second, the P1−P2 hybrid was incubated with 10 μL of Exo III (2.0 U/μL) and different concentrations of target DNA for 110 min at 25 °C. Finally, the 50 μL hemin (0.8 μM) solution was added into the above obtained solution, keeping the mixtures at room temperature for 60 min to form the G-quadruplex−hemin DNAzyme.

Chemiluminescence Measurement. The CL measurements was performed at room temperature in the Tris−HCl buffer (pH 9.0) by mixing 100 μL of the above prepared G-quadruplex−hemin DNAzyme solutions and 100 μL of H_2O_2 (1.0 mM) solution. Then, 20 μL of the resulting solution was decanted to the quartz cuvette (used as CL reactor), and the CL reaction was triggered by injecting 100 μL of luminol (0.5 mM). The CL signal was measured and recorded with the IFFL-D chemiluminescence analyzer. The target DNA concentration was quantified by the CL peak intensity.

RESULTS AND DISCUSSION

Probe Design and Analytical Principle. The Exo III-assisted cascade signal amplification strategy for label-free and homogeneous CL detection of DNA is demonstrated in Scheme 1. A quadruplex-forming DNA probe (denoted as P1) and a hairpin DNA probe (denoted as P2) were designed. P2 possess a loop region, which is complementary to P1. The stem region at the 3′-terminus (the green part in Scheme 1A) is complementary to the target DNA, and the stem region at the 5′-terminus (the yellow part) is complementary to the sequence of the green stem region. There is no special requirement for the stem region of P2 at the 3′-terminus. Thus, the scheme is general and can analyze the target with a random sequence. At first, the P1−P2 hybrid probe is prepared in the solution by hybridizing P1 and P2 (Scheme 1A). In the P1−P2 hybrid probe, P1 (DNAzyme sequence) is partially caged in the duplex structure. As a result, the G-rich segment (blue strand) is prohibited to bind hemin, and the DNAzyme sequence is inactive in the absence of target DNA. Moreover, the P1−P2 hybrid duplex probe is ingeniously designed with the protruding 3′-termini in order to avoid the digestion of the P1−P2 hybrid probe by Exo III, which specifically cleaves duplex DNA from blunt or recessed 3′-termini.[8] When the P1−P2 hybrid probe is challenged with target DNA, the recognition of target DNA with the dangling ssDNA at the 3′-end of P2 by a template-enhanced hybridization process leads to the formation of DNA duplex with a blunt 3′-terminus. In this case, Exo III can preferentially bond to the duplex region and catalyze the stepwise removal of mononucleotides from the blunt 3′-terminus of P2 in the 3′-to-5′ direction. After the duplex is fully consumed, the target DNA and P1 (DNAzyme sequence) are released. Then the release target DNA can bind to another 3′-protruding terminus of the P1−P2 hybrid probe to trigger a new cleavage process, which constitutes cycle I in Scheme 1B. At the same time, the yellow fragment of P2 is also released during each cleavage process, and the yellow fragment of P2 can be used as a secondary target DNA. The secondary target can hybridize P1−P2 hybrid probe to form DNA duplex with a blunt 3′-terminus, and then Exo III can catalyze the cleavage the duplex DNA, releasing again the yellow fragment of P2 as the secondary target DNA for the next cleavage process and P1 (DNAzyme sequence). This constitutes cycle II in Scheme 1B. On the basis of the spontaneous cleavage of cycles I and II, one target DNA can produce a larger number of free DNAzymes in a way of exponential amplification. Upon completion of the Exo III-assisted recycling, the released DNAzyme sequence can bind hemin with the help of K^+ to yield G-quadruplex−hemin DNAzyme (as shown in Scheme 1C), which can catalyze the luminol−H_2O_2 reaction to produce strong CL emission.[30−33] The number of G-quadruplex−hemin DNAzyme is positively related to the CL intensity, and thus, the Exo III-assisted target recycling leads to significant amplification of the readout signal.

To verify the feasibility of the Exo III-assisted cascade signal amplification strategy for label-free and ultrasensitive CL detection of DNA in Scheme 1, the target-induced CL signals in the presence and the absence of Exo III were recorded by using a CL analyzer. Typically, the CL system of luminol−H_2O_2 in the presence of catalyst is a flash-type reaction, in which the luminescence signal rises very sharply at the start of the reaction and decays quickly within seconds. Therefore, we obtained sharp, peak-shaped, time-dependent luminescence profiles in CL measurements. As shown in Figure 1, P1 probe can bind hemin to form highly active DNAzyme (curve b), whereas hemin itself yields very low catalytic activity in this CL reaction (curve a). An obvious signal enhancement is observed upon the addition of 0.5 pM target DNA and 20 U Exo III (curve e). In contrast, the CL intensity of 0.5 pM target DNA in the absence of Exo III (curve d) is the same as the background (curve c). In this system, hemin itself can catalyze the luminol−H_2O_2 CL reaction in the presence of P1−P2

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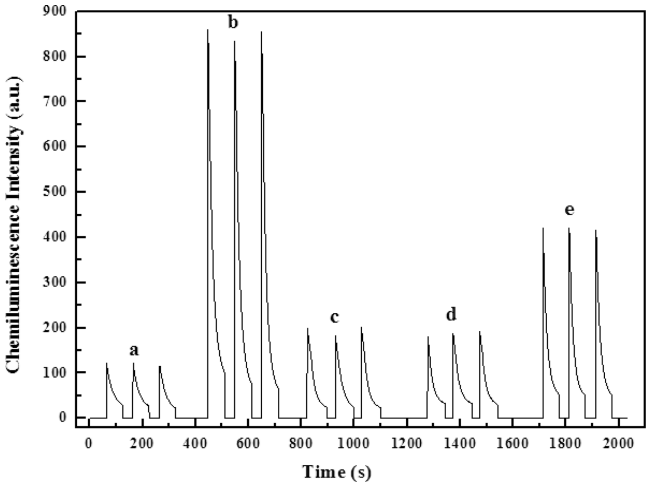
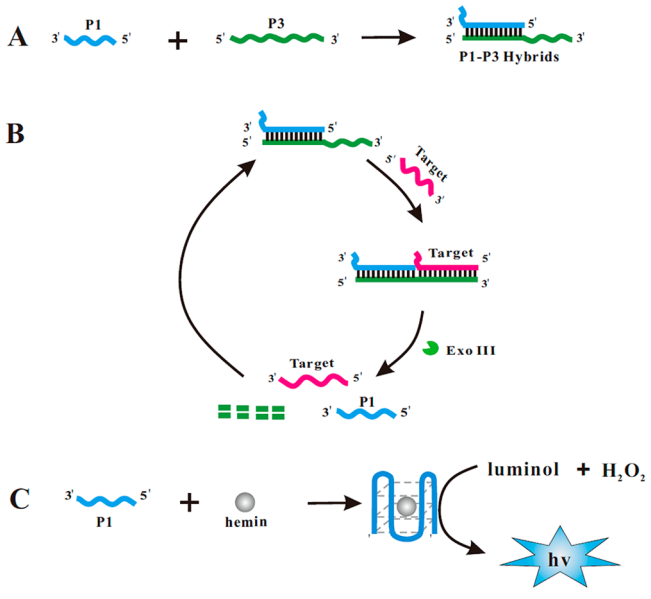


Figure 1. Measurement results of the CL biosensing system under different conditions: (a) hemin, (b) P1 + hemin, (c) P1−P2 + hemin, (d) P1−P2 + target + hemin, and (e) P1−P2 + target + Exo III + hemin. Experimental conditions: 0.5 μM P1, 0.5 μM P2, 0.5 pM target, 20 U Exo III, 0.8 μM hemin, 0.5 mM luminol, and 1.0 mM H_2O_2.

hybrid probe, which results in the background. Meanwhile, the control experiments showed that Exo III itself did not affect the CL of luminol−H_2O_2. This strongly indicated that only target DNA can trigger the cleavage process by Exo III to release the DNAzyme sequence for the amplified CL response.

In order to check the amplification efficiency of the Exo III-assisted cascade signal amplification strategy, the common amplification approach with one cycling process was designed for comparison. As shown in Scheme 2, P3 probe does not contain the yellow fragment of P2. After the cleavage of the duplex DNA by Exo III, the secondary target DNA cannot be released. So, there is only one cycling amplification process. The CL responses of 0.1 and 1.0 pM target DNA were measured in the cascade signal amplification strategy and the common amplification strategy, respectively. As shown in Figure 2, for the same concentration of target, the CL signals in

Scheme 2. Schematic Illustration of the Common Signal Amplification Strategy for DNA Detection: (A) Formation of the Catalytically Inactive Hybrid Probe (P1−P3) through Hybridization of a Quadruplex-Forming DNA Probe (P1) and a Linear DNA Probe (P3), (B) Exo III-Assisted Target Recycling Amplification Process, and (C) Binding of G-Rich DNA with Hemin To Yield DNAzyme, Catalyzing the Oxidation of Luminol by H_2O_2 To Produce CL



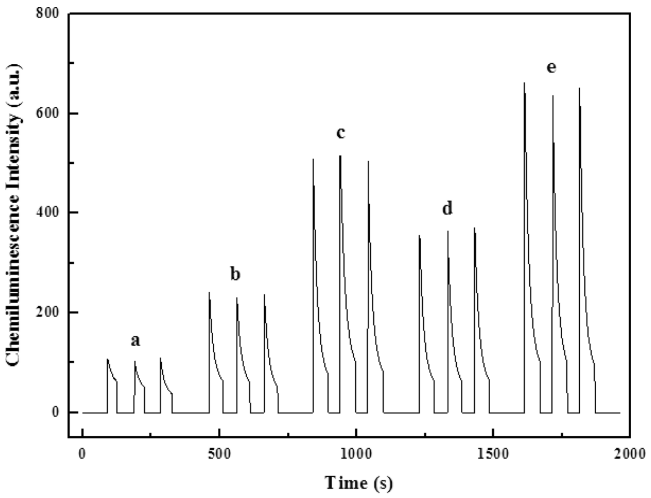


Figure 2. Measurement results of the CL biosensing system under different conditions: (a) hemin, (b) P1−P3 + target (0.1 pM) + Exo III + hemin, (c) P1−P3 + target (1.0 pM) + Exo III + hemin, (d) P1−P2 + target (0.1 pM) + Exo III + hemin, and (e) P1−P2 + target (1.0 pM) + Exo III + hemin. Experimental conditions: 0.5 μM P1, 0.5 μM P2, 0.5 μM P3, 20 U Exo III, 0.8 μM hemin, 0.5 mM luminol, and 1.0 mM H_2O_2.

the cascade signal amplification strategy were significantly higher than that in the common amplification approach. These results clearly demonstrated that our proposed cascade signal amplification strategy performed with higher amplification efficiency. So, the Exo III-assisted cascade signal amplification strategy can offer great potential for detection of target DNA at ultralow concentration.

CD Characterization. The viability of the proposed strategy was further investigated by CD spectra. As shown in Figure 3, the CD spectrum of P1 probe displayed a negative peak at ca. 240 nm and a positive peak at ca. 260 nm (curve a), which is characteristic of typical parallel G-quadruplex DNA conformation.[34,35] After hybridizing with P2, the peak at ca. 260 nm disappeared and a new peak at ca. 280 nm appeared (curve b), which is characteristic of double-stranded DNA. This suggested the formation of P1−P2 hybrid. After addition of target DNA into P1−P2 hybrid probe, the CD spectrum of the system (curve c) did not change. Upon adding Exo III to the system, the peak at 280 nm disappeared and the peak at 260 nm appeared (curve d). The reason is that Exo III can stepwise remove mononucleotides from 3′-hydroxyl termini of double-stranded DNA, and the P1 probe is released, thus forming the parallel G-quadruplex DNA conformation in the system. These results further validate the developed strategy.

Optimization of Experimental Conditions. In this assay, the amount of P1−P2 hybrid probe plays an important role in the performance of the system. In this system, P1 hybridized P2 with the same concentrations to form P1−P2 hybrid probe. The effect of P1−P2 hybrid concentration was studied through the CL response toward the detection of 1.0 pM target DNA. The results showed that 0.5 μM P1−P2 hybrid probe could

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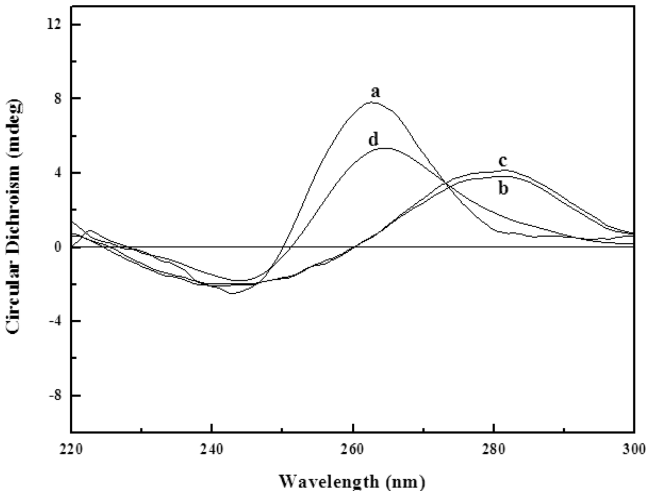

Figure 3. CD spectra of different samples: (a) P1, (b) P1−P2, (c) P1−P2 + target, and (d) P1−P2 + target + Exo III. Experimental conditions: 5.0 μM P1, 5.0 μM P2, 5.0 nM target, and 20 U Exo III.

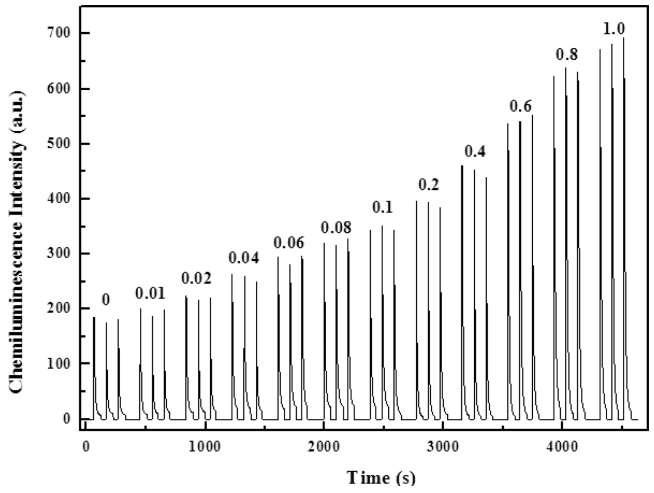

Figure 4. Representative recorder outputs of the CL biosensing system in the presence of different concentrations of target DNA under the optimized experiment conditions.

achieve the best signal-to-background ratio. In this proposed assay, the sensitivity can be dramatically increased by using the Exo III-assisted cascade signal amplification strategy. So, the amount of Exo III is the key factor in this system. The experimental results showed that the sensitivity for detecting target DNA increased with increasing Exo III concentration until a plateau was reached at 20 U. Taking into account the consumption of enzyme, 20 U Exo III in the final solution was used. The concentration of Exo III in the final solution was then set to 20 U for subsequent experiments. The incubation time of Exo III and DNA was also optimized. When the assay was conducted at room temperature (ca. 25 °C), the CL signal increased with increasing incubation time. The continuous increase of CL signal indicated that the designed target recycling was indeed taking place. Taking into account the analysis time, the incubation time was set at 110 min. DNA hybridization and Exo III enzyme reaction are usually slow, so the analysis time is long. Of course, the reaction temperature would rise from room temperature (25 °C) to 37 °C, and the analysis time could be shortened.

Furthermore, the response of the developed CL method for DNA was also strongly influenced by the CL reaction conditions (such as luminol concentration, H_2O_2 concentration, hemin concentration, and media pH). Different CL conditions were investigated by fixing 0.5 μM P1−P2 hybrid probe and 1.0 pM target DNA. The experimental results (Figure S1, Supporting Information) showed that the optimal concentrations of luminol, H_2O_2, and hemin were 0.5 and 1.0 mM and 0.8 μM, respectively. The optimal pH of CL reaction media was 9.0.

Performance of the CL Biosensing for DNA. Under the optimized conditions, experiments were carried out by adding target DNA with different concentrations into the system to examine whether the CL change could be used for DNA quantification. As shown in Figure 4, the CL intensity increases with the increasing target DNA concentration, to reveal a linear relationship in the target DNA concentration range from 0.01 to 1.0 pM (Figure 5). The limit of detection (taken to be 3 times the standard deviation in the blank solution) was 8 fM, which was about 100 times lower than that of the reported DNAzyme-based colorimetric system for DNA detection with Exo III-assisted cascade signal amplification.[24−28] Furthermore,

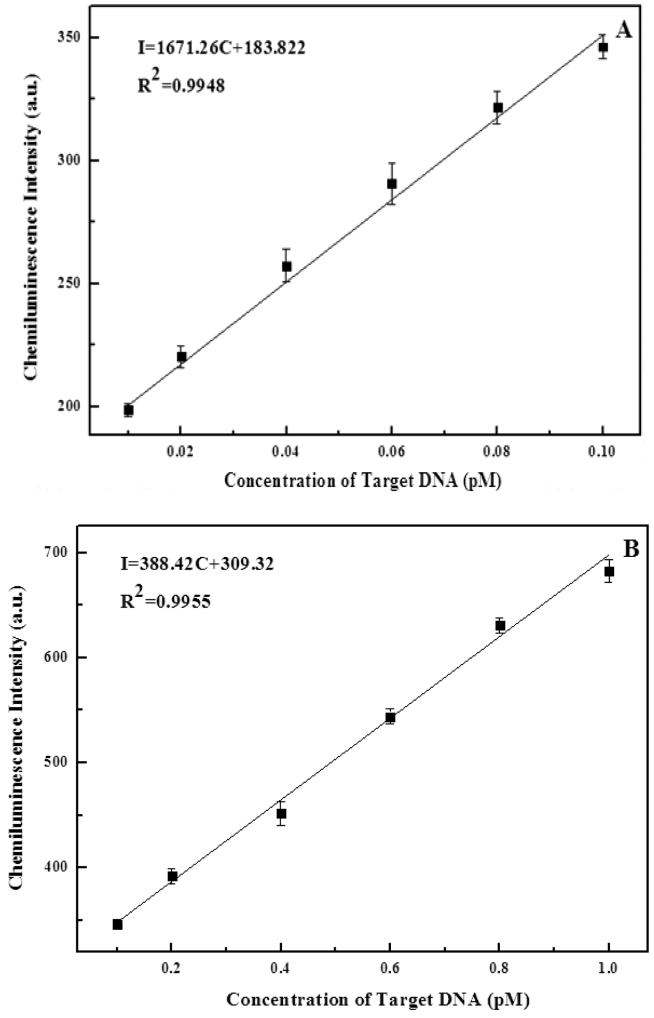

Figure 5. Linear relationship between the CL intensity and target DNA concentration from 0.01 to 0.1 pM (A) and 0.1 to 1.0 pM (B). Error bars represent the standard deviations of three independent measurements.

for comparison, we also measured the CL response of the common amplification strategy with one cycling process

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(Scheme 2). The results showed that the target DNA could be detected quantitatively in the range from 0.1 to 1.0 pM (Figure S2, Supporting Information), and the detection limit was 0.05 pM, which was much higher than that obtained in the Exo III-assisted cascade signal amplification. Thus, such high sensitivity of this proposed method can be attributed to two factors: (1) the high amplification efficiency of the cascade signal amplification strategy and (2) the intrinsically high sensitivity of CL detection.

The specificity of this proposed method for detecting DNA was investigated by testing the CL response to four kinds of DNA sequences, including complementary target DNA (T), a single-base mismatched DNA (T1), two base mismatched DNA (T2), and the noncomplementary DNA (Tn) at the same concentration of 1.0 pM. Figure 6 shows the CL intensity

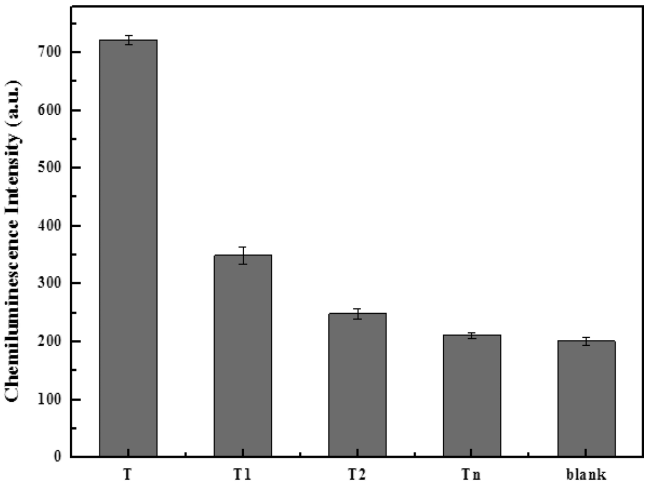

Figure 6. Specificity of the assay for DNA detection by hybridizing probe DNA with different target DNA: completely complementary target DNA (T), single-base mismatched DNA (T1), two-base mismatched DNA (T2), noncomplementary DNA (Tn), and the absence of DNA (blank), respectively.

changes with target DNA and other mismatched DNA strands. The relative CL intensities for T1 and T2 were about 28.8% and 9.6% of that for perfect target at the same concentration, respectively. The noncomplementary DNA (Tn) showed almost the same response with the blank solution. Thus, this method exhibited a good performance to discriminate perfect complementary target and the base mismatched targets and held great potential for single nucleotide polymorphism analysis.

CONCLUSION

A simple G-quadruplex DNAzyme-based CL platform for ultrasensitive DNA detection has been demonstrated in this study. In the presence of Exo III, target DNA can trigger the cascade recycling processes to release massive G-quadruplex sequences for production of numerous HRP-mimicking DNAzyme to achieve signal amplification. Due to the significant signal amplification and the intrinsically high sensitivity of CL detection, as low as 8 fM target DNA can be detected, which is far more sensitive than most of the DNA assays reported so far, and holds a great potential for early diagnosis in gene-related diseases. In this strategy, the DNA probe does not need labeling or modifying, and the assay occurs in the liquid phase and is homogeneous, which avoids the separation and washing steps. The method does not require sophisticated experimental techniques and instruments. As a result, it is simple, cheap, and easy to operate. The features make the proposed assay particularly useful for point-of-care application in the developing world. Furthermore, the current strategy may be extended for the detection of aptamer-binding molecules. Thus, it opens a promising avenue to develop the Exo III-assisted cascade signal amplification strategy for the fabrication of an ultrasensitive CL method. Of course, it is necessary to bear in mind that this method did not achieved the 5−10 copy sensitivity level offered by PCR-based techniques.[36]

ASSOCIATED CONTENT

Supporting Information

Figures S1 and S2, as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Fax: +86-29-81530727. E-mail: libaoxin@snnu.edu.cn.

Notes

The authors declare no competing financial interest.

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