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Ultrasensitive electrochemical biosensor for specific detection of DNA based on molecular beacon mediated circular strand displacement polymerization and hyperbranched rolling circle amplification

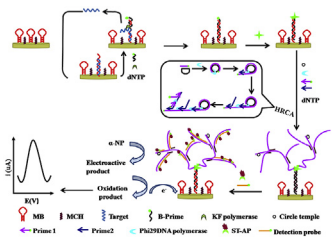
Xiaolu Li, Jing Guo, Qian Zhai, Jing Xia, Gang Yi*

Key Laboratory of Clinical Laboratory Diagnostics (Ministry of Education), College of Laboratory Medicine, Chongqing Medical University, Chongqing 400016, China

HIGHLIGHTS

- Signal amplification contains circular strand displacement polymerization and hyperbranched rolling circle amplification.
- Molecular beacon (MB) were used as capture probe to improve the specificity.
- The developed method showed high sensitivity and specificity for detection of DNA.

GRAPHICAL ABSTRACT



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ABSTRACT

Using a cascade signal amplification strategy, an ultrasensitive electrochemical biosensor for specific detection of DNA based on molecular beacon (MB) mediated circular strand displacement polymerization (CSDP) and hyperbranched rolling circle amplification (HRCA) was proposed. The hybridization of MB probe to target DNA resulted in a conformational change of the MB and triggered the CSDP in the presence of bio-primer and Klenow fragment (KF exo$^-$), leading to multiple biotin-tagged DNA duplex. Furthermore, the HRCA was implemented to product amounts of double-stranded DNA (ds-DNA) fragments using phi29 DNA polymerase via biotin-streptavidin interaction. After the product of HRCA binded numerous biotinylated detection probes, an ultrasensitive electrochemical readout by further employing the streptavidin-alkaline phosphatase. The proposed biosensor exhibited excellent detection sensitivity and specificity with a log-linear response to target DNA from 0.01 fM to 10 pM as low as 8.9 aM. The proposed method allowed DNA detection with simplicity, rapidness, low cost and high specificity, which might have the potential for application in clinical molecular diagnostics and environmental monitoring.

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1. Introduction

Development of DNA sensors for specific detection of trace amounts of DNA has become imperative in clinical diagnosis, gene therapy, pathogen detection and environmental monitoring [1–4]. Over the past decades, different techniques have been developed for DNA detection, such as colorimetric biosensors [5], chemiluminescent biosensors [6], surface plasmon resonance biosensors [7], electrochemical biosensors [8], DNA microarrays [9] and so on. Among these protocols, the electrochemical biosensors have attracted particular attention in different fields owing to its small dimensions, easy operation, rapid response, low cost, high sensitivity and selectivity [10,11].

* Corresponding author.
E-mail address: yigang666@126.com (G. Yi).

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Recently, various signal amplification strategies have been established for sensitive detection of DNA, such as nanomaterials [12], hybridization chain reaction (HCR) [13], exonuclease III assisted signal amplification [14], strand displacement amplification [15], rolling circle amplification (RCA) [16] and catalytic hairpin assembly (CHA) [17]. Among them, rolling circle amplification (RCA), as an isothermal amplification technique, has been used in a variety of assays owing to its simplicity and high efficiency [18]. However, the sensitivity of RCA based biosensors is lower than that of PCR. To achieve ultrasensitive detection, hyperbranched rolling circle amplification (HRCA) has been developed derived from RCA, which is an isothermal and exponential amplification through a turn-by-turn cascade of primer extension and strand displacement [19,20]. For HRCA, a circular template and a pair of universal primers are used. The circular template is a long oligonucleotide about 100 bases in length, which can be ligated and circularized by the action of DNA ligase [21,22]. Rolling circle amplification (RCA) is then carried out by one universal primer, while the other universal primer with the same sequence of the circular template can identify and bind complementarily to the RCA product of the circular template. HRCA is performed by incubating with DNA polymerase, which resulting in the formation of a large amount of double-stranded DNA (ds-DNA). The HRCA could generate more than 10^9 copies of each circle within 1 h [23]. Moreover, the performance of HRCA is simple and cost-effective compared with PCR and less time consuming than culture, which has been widely applied for sensitive detection of viral RNA [24], single nucleotide polymorphisms [25], mutation [26,27] and proteins [28].

Molecular beacon (MB) mediated circular strand displacement polymerization (CSDP), as another isothermal DNA amplification method, can further improve the specificity and sensitivity of the biosensor. The hairpin structure of MB is beneficial for binding DNA target efficiently and is excellent specificity for discriminating from mismatched sequence. CSDP uses lengthening of an oligonucleotide primer to replace the target sequence based on the ability of DNA polymerase, and thus releases the target to initiate a new polymerization cycle [29–31]. Due to the DNA recognition event, the MB mediated CSDP process can transfer the conformational change of the MB into the cyclic nucleic acid strand displacement polymerization [32]. As an efficient and specific hybridization assay, it has become an essential step for nucleic acid strand detection and quantification in the process of designing DNA sensors.

In this study, we combined MB mediated CSDP and HRCA to develop a simple and ultrasensitive electrochemical biosensor for specific detection of DNA, in which target recycling and cascade signal amplification are successfully achieved. Compared with other fluorescent, colorimetric and electrochemical methods for the detection of DNA, our strategy exhibits superior specificity towards target DNA and has a lower detection limit. Due to these advantages of this method, it may provide a simple and powerful platform for DNA screening in bioanalysis, clinical diagnosis, pathogen detection, and environmental monitoring.

2. Materials and methods

2.1. Reagent

All oligonucleotides were synthesized and purified by Sangon (Shanghai, China). The sequences were showed in Table 1 and stored in Tris-HCl (10 mM, pH 8.0) containing 1 mM ethylenediaminetetraacetic acid. DNA hybridization buffer was phosphate buffered saline (PBS, 137 mM NaCl, 2.5 mM Mg^{2+}, 10 mM Na_2HPO_4, 2.0 mM KH_2PO_4, PH 7.4). 6-Mercapto-1-hexanol (MCH), streptavidin-alkaline phosphatase (ST-AP), α-naphthyl phosphate (α-NP), bovine serum albumin (BSA) and salmon sperm DNA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Klenow fragment (3′–5′ exo$^-$) (KF exo$^-$), 10 × NEB buffer 2 (1 × NEB buffer 2: 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9 at 25 °C), phi29 DNA Polymerase, 10 × phi29 buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 4 mM DTT, pH 7.5 at 25 °C) were obtained from New England Biolabs Inc. (USA). T4 DNA Ligase and 10 × T4 DNA Ligase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, pH 7.5 at 25 °C), deoxynucleotide solution mixture (dNTPs) were purchased from Sangon (Shanghai, China). Gold-view and DL20 DNA Marker were purchased from Takara (China). The washing buffer was PBS (0.1 M, pH 7.4) containing 0.05% (w/v) Tween-20. All other reagents were of analytical reagent grade, and Millipore-Q water (≥18 MΩ) was used in all experiments.

2.2. Apparatus

All electrochemical measurements were recorded with CHI660D electrochemical workstation (Chenhua Instruments, Shanghai, China). All experiments were carried out with a conventional three-electrode system composed of Ag/AgCl electrode as reference, and platinum wire as auxiliary a 3-mm diameter gold electrode as working electrode. The differential pulse voltammetry (DPV) measurement was performed from 0.0 to +0.5 V with a pulse period of 0.2 s, a pulse width of 0.05 s, and a pulse amplitude of 70 mV. Differential pulse voltammetry (DPV), electrochemical impedance spectroscopic (EIS) and square wave voltammetry (SWV) were carried out at room temperature.

2.3. Preparation of the MB-modified sensing electrode

The bare gold electrode was polished with 0.05 mm alumina slurries and ultrasonically treated in deionized water. The electrode was then soaked in piranha solution (H_2SO_4:H_2O_2 = 3:1) for 10 min followed by rinsing thoroughly with deionized water to eliminate other substances. Afterwards, the pretreated electrode was rinsed with ultrapure water and allowed to dry at room temperature. 10 μL 100 nM MB probe was dropped on the prepared electrode surface and incubated overnight at 4 °C. After washing with washing buffer, the obtained electrode was immersed into 10 μL of 1 mM MCH solution for 1 h to obtain well-aligned DNA monolayer and occupy the left bare sites [33]. Then the obtained electrode was immersed in salmon sperm DNA and 1% BSA for 30 min to avoid nonspecific adsorption of DNA and enzyme on the electrode surface. The electrochemical biosensor was rinsed with the washing buffer and used for following operation.

2.4. Preparation of circularization mixture

96 μL of ligation buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP) containing 100 nM circular template oligonucleotide was incubated at 37 °C for 30 min, followed by the addition of 4 μL of T4 DNA ligase and incubation at 37 °C for 1 h. Afterwards, T4 DNA ligase was inactivated by heating the reaction mixture at 65 °C for 10 min. At last, the circularization mixture was stored at 20 °C.

2.5. Target DNA detection protocol

The target DNA was diluted to the desired concentration with the DNA hybridization buffer. After the prepared electrochemical sensor was further washed with washing buffer, 10 μL target DNA was dropped on the sensor surface, incubated for 1 h at 37 °C and rinsed. Then, the MB mediated CSDP was implemented by adding 10 μL reaction mixture (2 U KF exo$^-$, 500 mM dNTPs, 100 nM B-

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Table 1
Sequence of synthesized oligonucleotides probes used in this work.

Name	Sequence (5′ to 3′)
SH-modified molecular beacon (MB)	SH-$(CH_2)_6$-ACCGCCGGACAAGGAGAGTCAACCGGCGGTAGAGACCCTATAGTGAGTCGTATTA
Target DNA	TTGACTCTCCTTGT
Biotion-modified primer (B-primer)	Biotin-$(CH_2)_6$-TAATACGACTCACTATAGGG
Circular template	P-TATGCCCAGCCCTGTAAGATGAAGATAGCGCAGAATGGTCGGATTCTCAACTCGTATCTGCCCTGACTTC
Primer 1	Biotin-$(CH_2)_6$-AAAAAAGAAGTCAGGGCAGA
Primer 2	AAAAAACGAGTTGTGAATCC
Detection probe	Biotin-$(CH_2)_6$-AAAAAAGCGCAGAATGGT
Single -base mismatch (SM)	TTGACTATCCTTGT
Double-base mismatch (DM)	TTGACTATACTTGT
Non-complement sequence (NC)	GCATTGGCAACCCA

prime, 1 × KF buffer) on the electrode surface, incubated at 37 °C 1.5 h and rinsed with the washing buffer. The electrode was then incubated with 10 μL of 100 nM streptavidin solution at 37 °C for 30 min. Subsequently, the HRCA-reaction was proceeded at 37 °C for 1 h by immersing the achieved electrode in 10 μL HRCA-reaction buffer, which containing 4 μL circularization mixture, 1 μM primer 1, 1 μM primer 2, 0.2 U phi29 DNA polymerase, and 0.6 mM dNTPs. Following washed with washing buffer, 10 μL of 1 μM biotinylated detection probe was dropped onto the biosensor surface and hybridized for 30 min at 37 °C. After rinsed with DEA buffer, 10 μL of 1 μg mL^{-1} ST-AP was dropped onto its surface and incubated at 37 °C for 30 min, and washed thoroughly with DEA buffer containing 0.05% Tween-20. Finally, the electrochemical DPV measurements were performed in DEA buffer containing 1 mg mL^{-1} of α-NP substrate.

2.6. Gel electrophoresis

The amplification products of CSDP and HRCA reaction were analyzed by 8% nondenaturating polyacrylamide gel electrophoresis (PAGE) in 1 × TBE buffer (90 mM Tris-boric acid, 2 mM EDTA, pH 8.0) at a 120 V constant voltage at room temperature for 20 min. Gel images were recorded by an imaging system (Bio-Rad Laboratories, USA).

3. Results and discussion

3.1. Design of the electrochemical sensor

As shown in (Fig. 1), the SH-modified molecular beacon probe (MB) was immobilized on the gold electrode though Au-thiol interaction. Upon binding to target DNA, MB undergoed a conformational change and leaded to stem separation. The opened stem then hybridized with the bio-primer to initiate polymerization of DNA strand with the aid of the dNTPs and KF exo-, which led to the release of the target DNA. Meanwhile, the released target DNA bound with another MB to trigger next polymerization cycle, resulting in the multiplication of the biotin-tagged DNA. Then the HRCA primers and circular templates anchored multiple biotin-tagged DNA duplex on the biosensor surface through strong biotin-streptavidin interaction. Afterwards, the HRCA reaction proceeded on the electrode surface. Briefly, the primer 1 hybridized with circular templates and extended isothermally at its 3′ end by phi29 DNA polymerase to generate multimeric single-stranded (ssDNA), which serves as the template for the primer 2 binding. Primer 2 was further extended by phi29 DNA polymerase to displace the downstream growing DNA strands. This displaced strand contains multiple binding sites for primer 1 hybridization again. As a result, a large number of double-stranded DNA (ds-DNA) fragments with different lengths had been produced on the electrode surface. Finally, the capture probes specifically hybridized with the HRCA product. The streptavidin-alkaline phosphatase (ST-AP) was labeled on the capture probe by the specific recognition of ST and biotin. AP catalysed the hydrolysis of the electroinactive α-naphthy phosphate to α-naphthol; this product is electroactive and has been detected by mea ns of differential pulse voltammetry, eventually produce an amplified electrochemical signal [34]. The cascade signal amplification strategies might provide a sensing platform for sensitive and specific detection of DNA.

3.2. Characterization of biosensor fabrication

Electrochemical impedance spectroscopy (EIS) and square wave voltammetry (SWV) measurements were employed to investigate the features of surface-modified electrodes [35]. In the terms of EIS, $[Fe(CN)_6]^{3-/4-}$ was utilized as the redox probe and the EIS curves were obtained in 0.5 mM $[Fe(CN)_6]^{3-/4-}$ containing 0.4 M KCl. The semicircle diameter was equal to electron-transfer resistance (Ret). As shown in Fig. 2A, bare Au electrode exhibited an almost straight line (curve a) reflecting the excellent electrochemical conductivity which indicated that the electro active ions transported easily on the electrode surface. Causing by the electrostatic repulsion between the negatively charged phosphate backbone of the DNA and the anionic $[Fe(CN)_6]^{3-/4-}$ ions, the Ret increased after the modification of the MB probe was self-assembled onto the bare electrode (curve b). Subsequent surface blocking with MCH led to an increase of Ret (curve c). Ret further increased after the target DNA was hybridized with MB (curve d), since an opened negatively charged interface electrostatically repelled the negatively charged $[Fe(CN)_6]^{3-/4-}$ redox probe. After the CSDP, the Ret also increased significantly (curve e). Following binding streptavidin, the Ret increased dramatically (curve f). When HRCA reaction was finished, the Ret was sharply increased (curve g), this can be explained by the fact that the HRCA products containing large amount of ds-DNA had been accumulated on the electrode surface. These results were in a good agreement with those obtained from SWV measurements (Fig. 2B), in which the peak currents varied upon the assembly and binding processes. All these experimental results demonstrated that the sensing interface has been successfully fabricated according to Fig. 1.

3.3. Signal amplification performance of designed biosensor

To verify the feasibility of the proposed method for the detection of the target DNA, DPV measurements were performed with and without the cascade signal amplification strategy. As shown in Fig. 3A, in the absence of target DNA, there was only a small DPV peak observed (curve a). The DPV signal corresponding to 10 pM target DNA showed remarkable increase with the introduction of CSDP (curve b), indicating the CSDP formed a lot of biotin-tagged DNA duplex for signal amplification. Following the HRCA reaction, the significant signal amplification had been received (curve c). To

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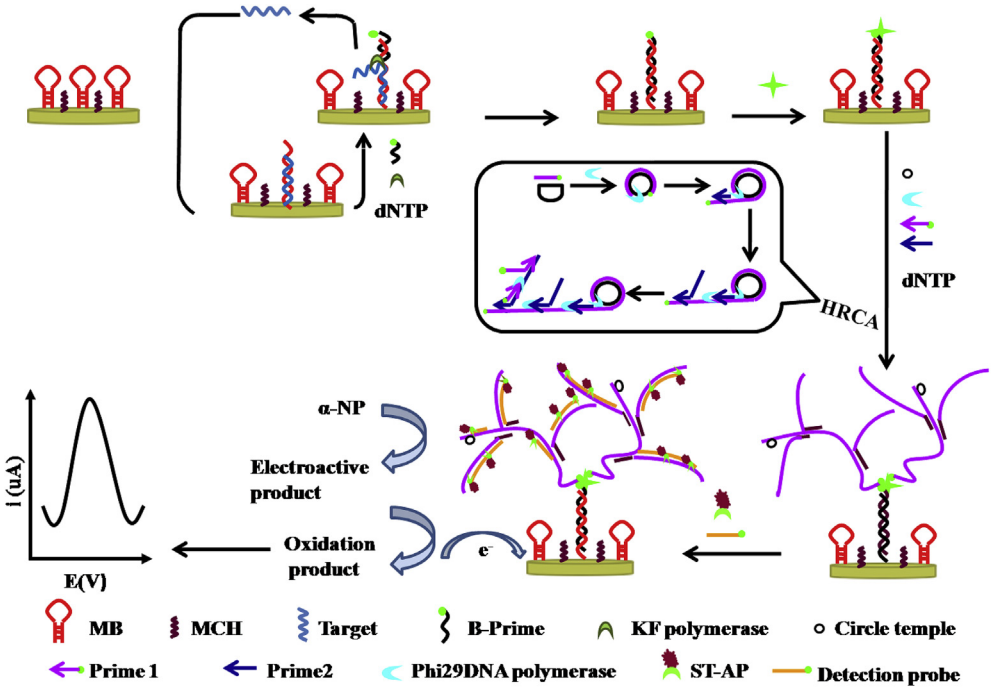

Fig. 1. Schematic illustration of the electrochemical DNA biosensor based on the cascade signal amplification of MB mediated CSD and HRCA.

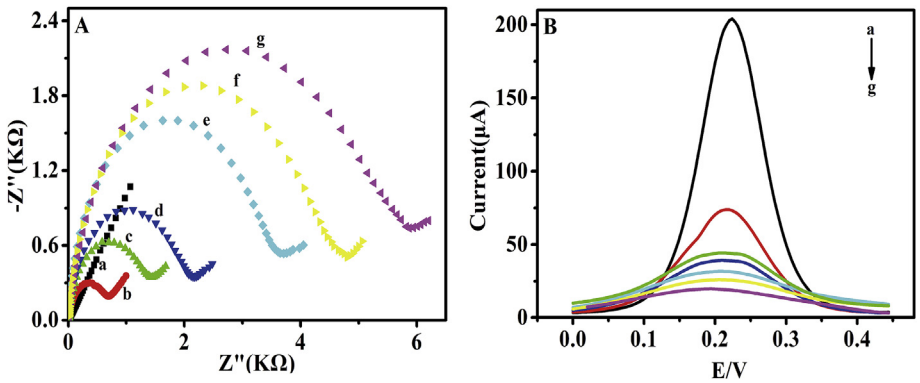

Fig. 2. EIS (A) and SWV (B) in 0.4 M KCl containing 0.5 mM $Fe(CN)_6^{3-/4-}$ at different stages: bare gold electrode (a), MB modified electrode (b), MCH and BSA immobilized on the electrode surface (c), MB modified electrode after hybridization with target DNA (d), CSD (e), streptavidin binding (f) and HRCA (g).

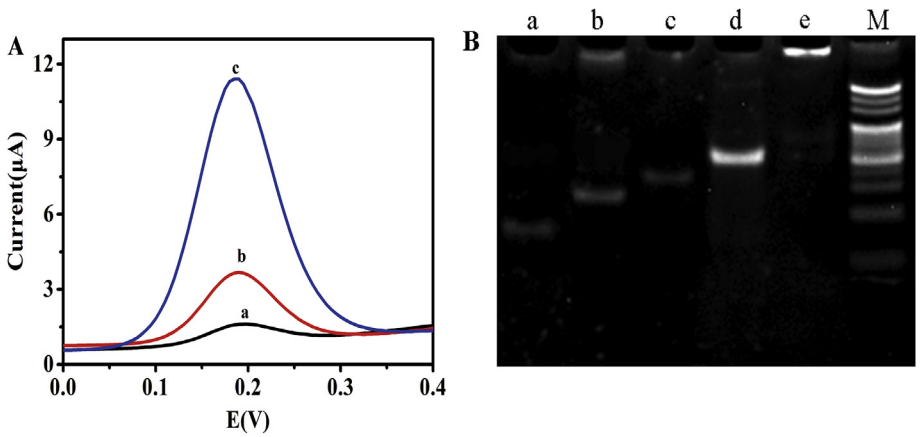

Fig. 3. (A) Comparison of DPV peak currents in the absence (blank) (a) and the presence of 10 Pm target with CSDP (without HRCA) (b) and CSDP-HRCA (c). (B) The native PAGE analysis: Line a: MB; Line b: product of MB hybridized with target; Line c: product of CSDP; Line d: circular template; Line d: product of HRCA; M: 20 bp marker.

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further confirmed the validity of the proposed method, a native polyacrylamide gel electrophoresis (PAGE) experiment was carried out. As shown in Fig. 3B, MB exhibited one band (lane a). After hybridization with the target DNA, the mobility of the hybrid was reduced, which corresponded to the formed double stranded DNA (lane b). A new product band with a slow migration speed was observed (lane c) when CSDP was performed in the presence of polymerase and dNTPs. Compared with the band of circular template (lane d), the high molecular weight products band showed a decrease of mobility significantly after HRCA reaction (lane e). These results showed that the cascade signal amplification strategy was successful.

3.4. Optimization of experimental conditions

In order to obtain the optimal sensing performance, the different experimental parameters were investigated. The CSDP process could be affected by a series of factors including the concentration of KF exo$^-$ and the reaction time. The peak current of response was plotted against the amount of the KF exo$^-$ in 10 μL CSDP mixture from 0.05 U to 0.3 U (Fig. 4A). The signal increased with the increasing amount of KF exo$^-$ until the enzyme amount exceeded 0.2 U, which was then chosen as advised. The time duration of the CSDP process was examined from 30 min to 150 min (Fig. 4B). After 90 min the signal reached a plateau, so 90 min was chosen as the duplication time.

T4 DNA ligase is important for ligation and circularization of the circular templates, so the concentration of T4 DNA ligase was optimized. As shown in Fig. 4C, the signal increased with the increasing amount of T4 DNA ligase and beyond 4 U, the signal did not exhibit further increasing. Therefore, 4 U was selected as the optimum concentration of T4 DNA ligase.

The phi29 DNA polymerase and reaction time are vital factors to affect the output of HRCA. Fig. 4D demonstrated the effect of the concentration of phi29 DNA polymerase in 10 μL HRCA solution on the DPV current response. The signal increased with increasing dosage from 0.05 U to 0.6 U, and beyond 0.2 U, the signal did not exhibit further increasing. Thus 0.2 U phi29 DNA polymerase was chosen as the optimal dosage during the following studies. Similarly, the reaction time of HRCA reaction was investigated too. As shown in Fig. 4E, with the increasing HRCA reaction time, the DPV current response was gradually enhanced, and finally reached a constant value after 60 min. Therefore, 60 min was chosen as the optimum reaction time for HRCA. dNTPs is another key factor for strand extension in HRCA reaction, so the concentration of dNTPs was optimized. With the increasing dNTPs concentration, the signal enhanced and finally reached a plateau after 0.6 mM (Fig. 4F). Thus, 0.6 mM of dNTPs was chosen for the subsequent experiments.

3.5. Sensitivity of designed biosensor

Under optimal conditions, the sensitivity and dynamic range of our proposed method were evaluated toward target DNA at different concentrations. As shown in Fig. 5A, the peak current increased with the increasing concentration of target DNA. Fig. 5B shows a linear relationship between the enhanced signal intensity of the system and the logarithm of target DNA concentration from 0.01 fM to10 pM with a correlation coefficient of 0.9985. The linear equation was $\Delta I/(\mu A) = 3.452 + 1.626\ \lg C\ (fM)$ (here, ΔI is the difference signal intensity in the presence and absence of target DNA, C is the concentration of target DNA). According to the signal which was equivalent to 3 times the standard deviation of the blanks, the detection limit (LOD) was 8.9 aM, which was comparable or even better than some of other reported methods (Table 2). Such an attractive detection limit of our sensing strategy can be primarily attributed to the mechanism of combining CSDP and HRCA strategies. Among the methods in Table 2, the assay time of almost references were between 4 h and 5 h which were similar with that of our work and reference [38] was longer than ours.

3.6. Specificity, reproducibility and stability of the assay

In addition, in order to investigate the specificity of the proposed biosensor for the detection of target DNA, four different synthetic oligonucleotides including perfect complementary target

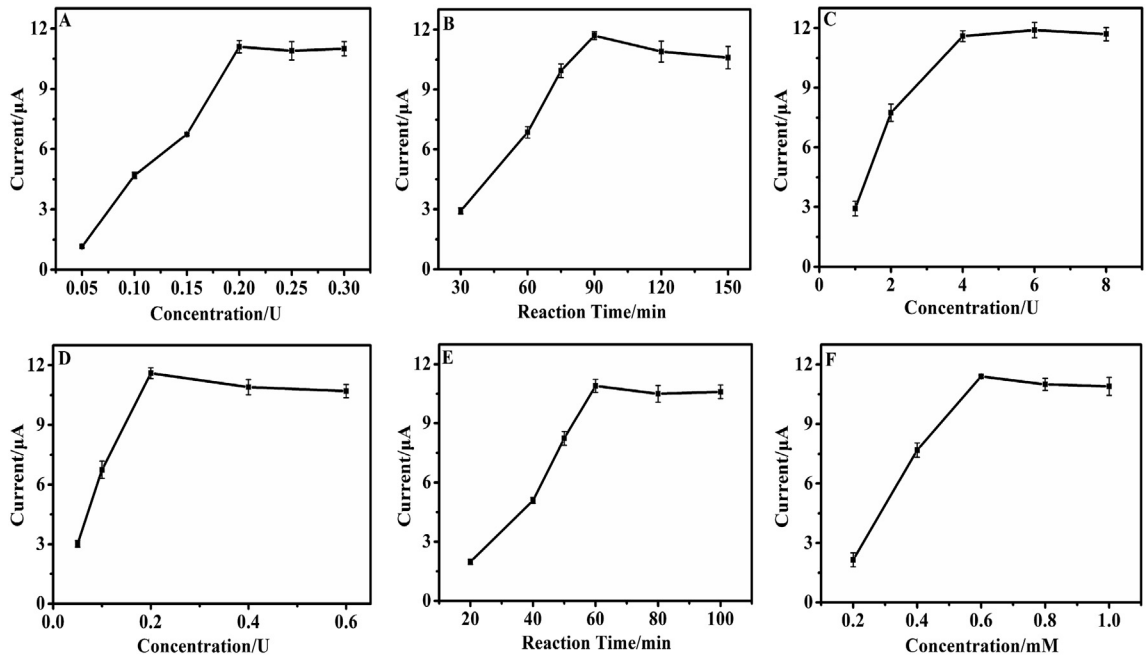

Fig. 4. Optimizations of experimental conditions: concentration of KF exo$^-$ (A) and reaction time in the CSD (B), concentration of T4 DNA Ligase (C), concentration of phi29 DNA polymerase (D), incubating time (E) and concentration of dNTPs (F) in HRCA. When one parameter changed, the others were under their optimal conditions.

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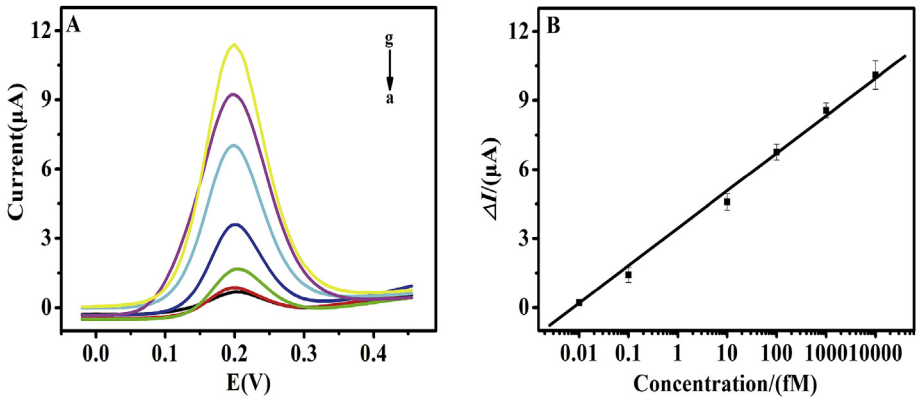

Fig. 5. (A) Typical DPV curves responding to 0, 0.01, 0.1, 10, 100, 1000 and 10,000 fM of target DNA (from a to g). (B) Plot of DPV peak current vs. logarithm of target DNA concentration from 0.01 fM to10 pM. The error bars represent the standard deviations in three repetitive experiments for each concentration.

Table 2
Comparison between the proposed assay and other reported methods for DNA detection.

Signal amplification method	Analytical technique	Linear range (mol L^{-1})	Detection limit (mol L^{-1})	Assay time (h)	References
HCR	Fluorescence	5×10^{-7}–2×10^{-13}	10^{-15}	2	[36]
CSRP	LSV	10^{-12}–10^{-16}	3×10^{-17}	4.5	[37]
RCA	Raman	10^{-7}–10^{-11}	10^{-11}	5	[38]
CSDPR/HCR	DPV	10^{-7}–10^{-14}	8×10^{-15}	6.2	[39]
Exonuclease	Impedance	10^{-8}–10^{-11}	4.2×10^{-11}	4.8	[40]
HRCA	DPV	8×10^{-11}–5×10^{-15}	1.6×10^{-15}	4.5	[41]
MB-Mediated CSDP/HRCA	DPV	10^{-11}–10^{-17}	8.9×10^{-18}	5	This work

DNA, single-base mismatched strand (SM), double-base mismatched strand (DM) and non-complementary strand (NC) were analyzed. As shown in Fig. 6A, the signal intensity for 10 pM of SM (curve b), 10 pM of DM (curve c) and 10 pM of NC (curve d) were similar to that of black (curve e). However, in the presence of target DNA, there was a remarkable increase in the DPV current (curve a) and showed a response 16.41 times as much as that of the single-base mismatched strand (Fig. 6B). These results indicated that the biosensor displayed excellent specificity for the determination of target DNA.

The fabrication reproducibility was carried out by measuring the target DNA at 1 pM to 50 fM with five different DNA biosensors. The relative standard deviation (RSD) for both concentrations was less than 5%, revealing that this method had good reproducibility.

To evaluate the stability of the proposed biosensor, we detected target DNA with the fabricated DNA biosensor, the modified electrode had been stored at 4 °C for 14 days, and compared with the fresh prepared one, only 3% decreases in the DPV current response had been detected. This indicated the good stability of the developed biosensor.

4. Conclusion

In summary, the proposed method combined the MB mediated CSDP with the isothermal and exponential amplification of HRCA for signal enhancement. The dual amplification led to an extremely high sensitivity with a detection limit as low as 8.9 aM. What is more, compared with the single signaling electrochemical sensor reported previously, the electrochemical sensing strategy developed in this paper was more specific because of the intrinsic

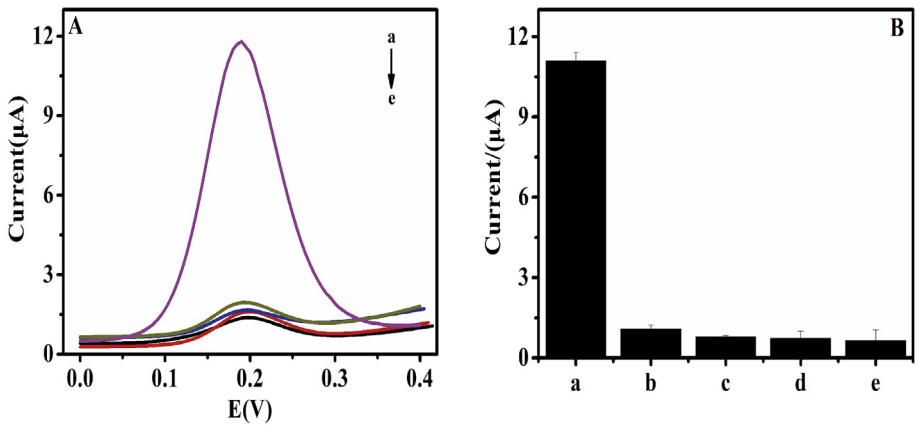

Fig. 6. (A) Typical DPV curves and (B) DPV peak currents respectively respond to 10 pM of target DNA (a), SM (b), DM (c), NC (d), and blank (e). The error bars represent the standard deviations in three parallel determinations for each concentration.

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functions of MB and polymerase. The improved sensitivity and specificity makes this method a promising alternative for development of integrated, portable and low cost DNA detection devices. Therefore, the biosensor we designed was expected to provide further applications in the study of bimolecular and early diagnosis of diseases.

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