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ORIGINAL PAPER

Voltammetric determination of attomolar levels of a sequence derived from the genom of hepatitis B virus by using molecular beacon mediated circular strand displacement and rolling circle amplification

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Abstract
The authors describe an electrochemical method for the determination of the single-stranded DNA (ssDNA) oligonucleotide with a sequence derived from the genom of hepatitis B virus (HBV). It is making use of circular strand displacement (CSD) and rolling circle amplification (RCA) strategies mediated by a molecular beacon (MB). This ssDNA hybridizes with the loop portion of the MB immobilized on the surface of a gold electrode, while primer DNA also hybridizes with the rest of partial DNA sequences of MB. This triggers the MB-mediated CSD. The RCA is then initiated to produce a long DNA strand with multiple tandem-repeat sequences, and this results in a significant increase of the differential pulse voltammetric response of the electrochemical probe Methylene Blue at a rather low working potential of −0.24 V (vs. Ag/AgCl). Under optimal experimental conditions, the assay displays an ultrahigh sensitivity (with a 2.6 aM detection limit) and excellent selectivity. Response is linear in the 10 to 700 aM DNA concentration range.

Keywords Electrochemical biosensor · Differential pulse voltammetry · Nucleic acid assay · Signal amplification · Phi29 DNA polymerase · T4 DNA ligase · Circular template

Introduction

Hepatitis B virus (HBV) is one of the most serious virus in the hepadnaviridae family [1]. HBV infection can induce a number of hepatic diseases and is one of the major causes of death in the world [2]. In order to control the spread of HBV, reliable and sensitive approaches are needed for the detection of the presence of HBV DNA sequences in the early infection stage. Typically, HBV DNA sequences are present in very small amount. Thus, analytical methods that are capable of identifying HBV DNA sequences and measuring their concentrations at biologically relevant levels in specimens are beneficial for studying and diagnosing disease. Up to date, a variety of techniques and various instrument-based approaches have been reported to detect HBV DNA sequences, such as liquid chromatography [3], polymerase chain reaction (PCR) [4], fluorescence spectrometry [5], electrochemiluminescence [2], and mass spectrometric analysis [6]. However, some of them are complicated, expensive, and time consuming for clinical diagnosis. Electrochemical techniques have been widely utilized for HBV DNA sequences due to their unique advantages of low operating cost, quick response, high sensitivity, and good stability. Yang et al. reported the electrochemical detection method for HBV DNA segment based on label-free hemin/G-quadruplex DNAzyme biosensor with the detection limit for target DNA of 0.5 pM [7]. There is still a big demand for the development of more reliable and efficient electrochemical sensor for highly selective and ultrasensitive analysis of HBV DNA segment.

Rolling circle amplification (RCA) is an isothermal enzymatic gene amplification approach [8, 9]. Owing to

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its simplicity, high specificity, and high sensitivity of RCA, it has been widely used for the amplification of specific nucleic acids in vitro cloning, library construction, and other molecular biology applications [10, 11]. During RCA process, the circular padlock DNA template for phi29 DNA polymerase is used to generate long stretch of continuous single-stranded DNA sequences [12]. The long linear single-stranded padlock DNA with splint target nucleotides is implemented as target decision step to make a circular DNA template [12]. The RCA in combination with padlock probes has various applications in biosensing, diagnosis, and genomics. Various electrochemical sensors using RCA technique have been reported. Yin et al. constructed a dual-signal electrochemical biosensor for determination of HIV-1 related gene based on the application of cascade toehold-mediated strand displacement reaction [13]. Sun et al. reported the sensitive determination of DNA via a target-induced strand-displacement reaction using quantum dot-labeled probe DNA [14]. The performance of RCA is cost-effective compared with PCR and is more time-saving than culture, which has been widely utilized to generate enhanced electrochemical signal for sensitive detection of numerous targets [15–17].

Molecular beacon (MB) mediated circular strand displacement (CSD) is another widely utilized isothermal DNA amplification method. MB mediated CSD can be used to design a cascade signal amplification strategy for ultrasensitive and highly specific determination of target DNA through improving the analytical performance of RCA-based biosensing strategies. In typical CSD process, an oligodeoxy-ribonucleotide prime can be extended for direct recycling of the target DNA based on the ability of DNA polymerase. Trace DNA is geometrically amplified, which becomes an essential step for DNA detection and quantification [18, 19]. During MB mediated CSD process, some nucleic acid strand can participate in the cyclic nucleic acid strand displacement polymerization due to the DNA complementary base recognition event. Since the hairpin structure of MB can bind efficiently with specific DNA targets, MB mediated CSD is excellent specificity for discriminating from mismatched sequences. Ding's group developed an electrochemical DNA sensor by combining MB mediated CSD, RCA, biotin-strepavidin system, and enzymatic amplification [20]. Li et al. reported an ultrasensitive electrochemical biosensor for specific detection of DNA based on MB mediated CSD polymerization and hyperbranched RCA [21]. As far as we known, there is no relative reports indicating that RCA technique integrating with MB medicated CSD has been used for HBV DNA sequences during electrochemical method until now.

Accordingly, we fabricated an electrochemical biosensor for ultrasensitive and highly selective determination of HBV DNA segment based on MB mediated CSD and RCA. The inherent properties of the excellent specificity of MB mediated specific target binding of CSD and the significant signal amplification of RCA can maintain the ultrahigh sensitivity and excellent selectivity of this electrochemical biosensing strategy. This electrochemical biosensor exhibits excellent analytical performance towards target HBV DNA segment with ultrahigh sensitivity, ultralow detection limit, excellent specificity, and good stability. This method demonstrates a promising electrochemical sensing platform for target DNA in clinical diagnosis, disease treatment, pathogen detection, and environmental monitoring in the future.

Materials and methods

Reagents

All oligonucleotides, phi29 DNA polymerase, T4 DNA ligase, and deoxyribonucleotides (dNTPs) were purchased from Shanghai Sangon Biological Engineering Technological Co. Ltd. (http://www.sangon.com/). All oligonucleotides were purified by high-performance liquid chromatography. The base sequences were list as below:

Molecular beacon (MB): 5'-SH-$(CH_2)_6$-ATC GAT TAC CGC AAG GAC GTC CCG CGC AGG ATC CAG TAC GTA ATC GAT-3′;

Primer DNA: 5'-ACA GGC GAA GAC AGG TGC TTA GT AAA ATC GAT TAC-3′;

Circular template: 5′-TGT CTT CGC CTG TCC GAT GCT CTT CCT TGA AAC TTC TTC CTT TCT TTC GAC TAA GCA CC-3′;

Target HBV DNA segment: 5'-CTG GAT CCT GCG CGG GAC GTC CTT-3′;

Single-base mismatch DNA (T1-DNA): 5'-CTG GAT CCT **C**CG CGG GAC GTC CTT-3′;

Double-base mismatch DNA (T2-DNA): 5'-CTG GAT CCT **C**CG CGG **C**AC GTC CTT-3′;

Non-complementary DNA (T3-DNA): 5′-TTA GGG TTA GGG TTA GGG TTA GGG-3′;

Hepatitis C virus (HCV) DNA segment (C-DNA): 5'-GGC GAC GCG GGA TCC GAC GTT-3′;

Human papilloma virus (HPV) DNA segment (P-DNA): 5'-CAA GCA GAA CCG GAC AGA CCC CAT-3′.

All oligonucleotides were dissolved in 10 mM tris(hydroxymethyl) aminomethane-HCl (Tris-HCl, pH 8.0) buffer containing 1 mM ethylenediamine tetracetic acid (EDTA) and stored at 4 °C. Other biological reagents were purchased from Aladdin (http://www.aladdin-e.com/us_en/). All chemical reagents were purchased from Sinopharm Chemical Reagent Factory (http://en.reagent.com.cn/). All chemical reagents were of analytical grade and used as received without any further purification.

Apparatus

All electrochemical experiments were performed on a Chenhua CHI-660E electrochemical workstation (http://www.chinstr.com/) equipped with a thermostatic bath. Three-electrode cell was employed. Gold electrode (GE) and modified GE (MB/GE) was used as working electrode, while an Ag/AgCl electrode served as the reference electrode and platinum wire was employed as the counter electrode. All pH measurements were made with Sartorius basic PB-10 pH meter (https://www.sartorius.com.cn/sartoriusCN/zh/CNY). Ultrapure water with resistivity of 18.2 MΩ cm was used through Millipore-Q Academic purification set (http://www.merckmillipore.com/).

Preparation of the gold electrode modified with molecular beacon (MB/GE)

MB/GE was prepared according to our reported method [22]. Prior to modification, bare GE was polished subsequently with 1.0 μm, 0.3 μm, and 0.05 μm alumina slurry on a polishing cloth, followed by rinsing with ultrapure water and ultrasonic cleaning with ethanol, 0.5 M H_2SO_4, and ultrapure water subsequently. After that, the electrode was soaked in the piranha solution (volume ratio of H_2SO_4 to H_2O_2 of 3 to 1) for 10 min. Then, the electrode was rinsed thoroughly with ultrapure water to eliminate other substances. For the preparation of MB/GE, 5.0 μL MB (5.0 μM) was incubated with 1.5 μL tris(2-carboxyethyl) phosphine hydrochloride (TCEP, 1 mM) for 30 min to cleave the disulfide bonds. After that, 5.0 μL MB was dropped on the prepared GE surface by drop casting method and then the electrode was incubated over night at 4 °C. The obtained MB/GE was rinsed with ultrapure water to remove the loose adsorbed materials. Then, 10 μL 6-mercaptohexanol (MCH, 1 mM) solution was dropped on the electrode for 1 h to block nonspecific binding sites at room temperature. Finally, the modified electrode was rinsed with ultrapure water and washing buffer in sequence before following operation.

Human samples preparation

Human serum samples were obtained from healthy male and female adult volunteers in our laboratory. All experiments were performed in compliance with the relevant laws and institutional guidelines, and informed consent was obtained for any experimentation with human volunteers. Human serum samples were centrifuged at 8000 g for 6 min to get rid of blood cells. In order to precipitate proteins in human plasma samples, 1.0 mL human sample was treated with 20 mL perchloric acid ($HClO_4$, 20% *v*/v) with vigorous stirring for 5 min and then was centrifuged at 10000 g for 20 min. The supernatant was transferred into 2 mL eppendorf tube. The pretreated human serum samples were stored at 4 °C. Before human serum samples detection, different concentrations of target DNA were added into the samples. After thoroughly mixing, the samples were diluted 50 times with ultrapure water to avoid the influences of the matrix effects of serum samples (charge or ionic strength) on methylene blue. Finally, the concentration of the diluted target DNA was determined by this method.

Procedures for DNA determination

For DNA determination, 10 μL target DNA with different concentrations was dropped onto the MB/GE surface. The electrode was incubated at 4 °C for 1 h and then rinsed with ultrapure water. Then, MB mediated CSD was performed by adding 10 μL reaction mixture (10 mM dNTPs, 1 mM primer DNA, and 4 U phi29 DNA polymerase) on MB/GE surface. The electrode was incubated at 37 °C for 2 h. After the MB mediated CSD reaction, the electrode was rinsed with washing buffer. Subsequently, 10 μL circular template (1 mM) was added onto the electrode surface and the electrode was incubated at 37 °C for another 30 min. The RCA reaction was performed after the addition of 20 μL RCA reaction mixture (100 nM dNTPs, 1 U of phi29 DNA polymerase, and 10 U T4 DNA ligase). The electrode was incubated at 37 °C for another 100 min. After washing with ultrapure water, the electrode was immersed in 5 mL methylene blue (10 mM) for 40 min. Finally, the electrochemical sensor was washed thoroughly with ultrapure water.

Differential pulse voltammetry (DPV) measurements were carried out in 10 mM Tris-HCl (pH 7.4) with experimental parameters as: potentials range from −0.50 V to 0.10 V; pulse amplitude 0.05 V; pulse width 0.05 s; pulse period 0.5 s; sample width 0.0167 s; quiet time 2 s; scan rate 0.05 V s^{-1}.The DPV response of methylene blue at a rather low working potential of −0.24 V (vs. Ag/AgCl) was used to detect target HBV DNA segment. Electrochemical impedance spectrometry (EIS) was carried out over the vibration frequency range from 10 kHz to 0.01 Hz with quiet time of 2.0 s and amplitude of 5 mV. EIS experimental curves were obtained in 5.0 mM $[Fe(CN)_6]^{3-/4-}$ solution containing 0.1 M KCl at 25 °C.

Gel electrophoresis

A 10% native polyacrylamide gel electrophoresis analysis of the products via the MB mediated CSD and RCA reactions was carried out in 1 × Tris-HCl-EDTA (pH 8.0) at 100 V constant voltage for about 1 h. After GelRed nucleic acid staining solution, gels were scanned using a Molecular Imager Gel Doc XR (http://www.bio-rad.com/).

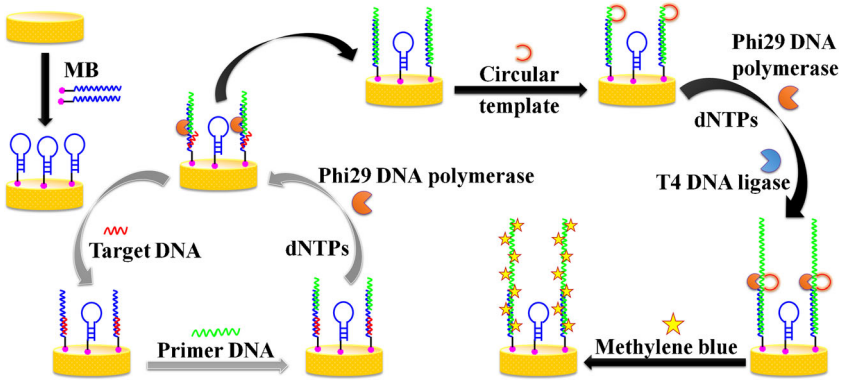
Results and discussion

Design of electrochemical biosensor

A schematic of the electrochemical biosensor is shown in Scheme 1. Firstly, molecular beacon (MB) is immobilized on the surface of GE though gold-thiol binding interaction. Upon the addition of target DNA, it can specifically recognize the loop portion of MB and tightly hybridize with the circular sequence of MB. This results in the variation of the MB conformation and the opening of the site for primer DNA hybridization. Following this, the primer DNA hybridizes with the 3′-terminal DNA sequences of MB. Therefore, the polymerization of DNA strand is initiated in the presence of dNTPs and phi29 DNA polymerase, resulting in the liberation of the target DNA. After that, the released target DNA repeatedly binds with another MB to trigger the MB mediated CSD process. The 5′-terminal sequences of primer DNA anchors the RCA circular templates on the surface of electrode through DNA hybridization. In the presences of dNTPs, T4 DNA ligase, and phi29 DNA polymerase, the RCA is initiated to produce massive long DNA strand with multiple tandem-repeat sequences. It has been reported that methylene blue specifically binds to the guanine bases of DNA molecules and is adopted as the widely used electrochemical redox probe during electrochemical sensing platforms [23]. Once the redox probe is attached on the prolonged primer DNA strand, the redox signal of methylene blue is increased significantly. However, if there is little or no target DNA, the hairpin structure of MB remains closed and the primer DNA can not hybridize with the 3′-terminal DNA sequences of MB. Under this circumstance, the RCA is not preceded and the primer DNA is not prolonged. Consequently, the redox signal of methylene blue is not increased dramatically. Compared with the redox signal variation of traditional protocols without signal amplification process, the redox signal variation is dramatically enhanced even at ultralow concentration of target DNA with RCA as the signal amplification strategy. Evidently, a relationship between the redox signal variation and target DNA concentration is constructed. The good selectivity of MB for discriminating from mismatched sequence ensures the high specificity of the cascade signal amplification strategy. The long DNA strand ascribed to RCA maintains the ultrahigh sensitivity of this electrochemical biosensor. Based on the MB mediated CSD and RCA strategies, an ultrasensitive electrochemical biosensor is successfully developed for ultrasensitive detection of target DNA.

Electrochemical characterization of the modified electrodes

The sensing interface of the modified electrodes was important for the achievable magnitude of target DNA response and was monitored by EIS. EIS is often used to study the change of the interfacial property of the modified electrode and to provide information on the impedance variations of electrode surface/electrolyte solution [22]. In a typical EIS curve, the linear part at lower frequencies implies the diffusion limited process and the semicircular portion at higher frequencies means the electron transfer limited process. The diameter of the semicircular portion, which is indicative of the electron transfer resistance (R_{et}), refers to the electron transfer kinetics of the redox probe at the electrode surface. Figure 1 shows the typical Nyquist plots of 5.0 mM $[Fe(CN)_6]^{3-/4-}$ redox probe at bare GE and different substances modified GE in 0.1 M KCl solution. Prior to the immobilization of MB, the R_{et} of bare GE was quite small (curve a in Fig. 1), due to the direct electron transfer of $[Fe(CN)_6]^{3-/4-}$ redox probe in the sensing system. However, the diameter of the semicircle increased obviously after the immobilization of MB on bare GE surface (curve b in Fig. 1). Such enhancement of R_{et} is attributed to the negatively charged phosphate skeleton of MB. The electron transfer of negatively charged $[Fe(CN)_6]^{3-/4-}$ redox probe from the electrode surface was prevented consequently. Moreover, the surface density of MB on GE surface was evaluated to 1.2×10^{12} molecules/cm^2 by using chronocoulometry method (Fig. S1), which reduced steric limitations on the surface for complete hybridization



Scheme 1 Fabrication process of the designed electrochemical biosensor and its application for ultrasensitive and highly selective detection of HBV DNA segment. MB: Molecular beacon

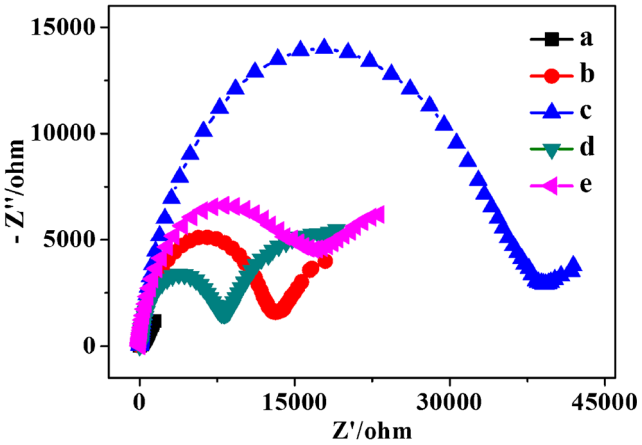


Fig. 1 EIS of 5.0 mM $[Fe(CN)_6]^{3-/4-}$ redox probe at bare GE (**a**), MB/GE (**b**), MB/GE after hybridization with 0.5 f. target DNA (**c**), MB mediated CSD (**d**), and RCA (**e**) in 0.1 M KCl with the frequencies swept from 10 kHz to 0.01 Hz

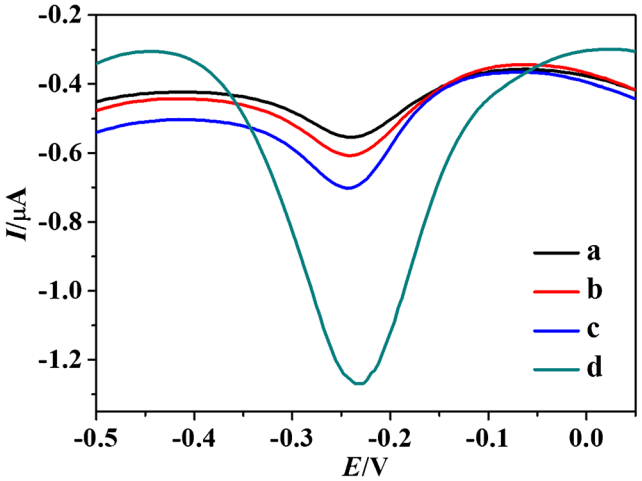


Fig. 2 Typical DPV curves of this electrochemical biosensor responding to blank control (**a**), 0.5 f. target DNA without (**b**) and with MB mediated CSD (**c**) and CSD/RCA (**d**)

[24]. Similarly, after the blocking of the electrode with MCH and binding of target DNA, the R_{et} was further increased due to the steric hindrance of double-stranded DNA (dsDNA) and repulsive effect on electron transfer on the electrode (curve c in Fig. 1). When MB mediated CSD was occurred, the released target DNA bonded with another MB and the hindrance of dsDNA on the electrode was decreased. The multiple dsDNA became rigid linear chain structure, so the R_{et} was decreased dramatically (curve d in Fig. 1) [25]. Finally, the RCA process produced multiple massive long single-stranded DNA (ssDNA). Due to the enhanced hindrance of the electron transfer process of long ssDNA molecules on the electrode, the R_{et} was increased consequently (curve e in Fig. 1). These results prove the assembly, hybridization, and amplification processes of the modified electrodes. Hence, the construction of the electrochemical biosensing interface for target DNA was successfully achieved.

The electrochemical responses of electrochemical biosensor to target DNA were further demonstrated from DPV responses of 0.5 f. target DNA in 10 mM Tris-HCl (pH 7.4). As shown in Fig. 2, methylene blue showed a specific DPV signal at peak position of around −0.24 V. Methylene blue exhibited a very tiny DPV peak current of −0.20 μA at the surface of MB/GE, which was regarded as the blank response (curve a in Fig. 2). When 10 μL target DNA (0.5 fM) was dropped onto the surface of MB/GE, the DPV peak current was increased to −0.21 μA without MB mediated CSD and RCA strategies (curve b in Fig. 2). After the MB mediated CSD process, methylene blue exhibited an obviously enhanced DPV peak current of −0.24 μA (curve c in Fig. 2). In the presences of both MB mediated CSD and RCA processes, a significant higher DPV peak current of −0.77 μA was obtained (curve d in Fig. 2), which was 3.7 times higher than the signal response (curve b in Fig. 2). This dramatic enhancement of the DPV peak current indicated the successful MB mediated CSD and the noticeable RCA signal amplification. Therefore, the developed electrode is a promising excellent electrochemical biosensing platform for target DNA.

The MB mediated CSD polymerization reaction was further confirmed with a 10% native polyacrylamide gel electrophoresis (PAGE) analysis. As shown in Fig. 3, MB and target DNA exhibited only one band (line a and line b

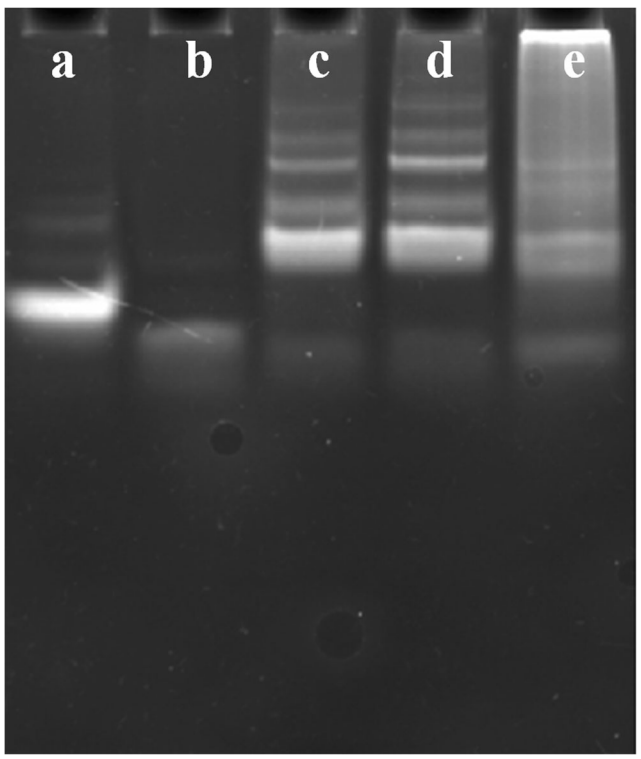


Fig. 3 PAGE analysis of the products: **a** target DNA; **b** MB; **c** MB, primer DNA, and target DNA; **d** MB, primer DNA, and target DNA in the presence of phi29 DNA polymerase and dNTPs; **e** MB, primer DNA, and target DNA via MB mediated CSD and RCA

in Fig. 3), respectively. After primer DNA and target DNA were added to the solution, new bands appeared and the mobility of the bands were reduced (line c in Fig. 3), which corresponded to the formed dsDNA. After phi29 DNA polymerase and dNTPs were further added into the mixture, the DNA bands with a slow migration speed were observed (line d in Fig. 3). This result is contributed to the novel dsDNA sequences resulting from the DNA polymerization that liberates the target DNA. In the presences of phi29 DNA polymerase, dNTPs, and T4 DNA ligase, the RCA is initiated to produce massive DNA sequences, which results in the slowed migration of the long DNA strands (line e in Fig. 3). This phenomenon is contributed to the displacement of target DNA, verifying, and polymerization process of MB mediated CSD and RCA.

Detection of HBV DNA

This electrochemical biosensor was developed for the ultrasensitive detection of target DNA. To achieve optimum sensing performance, some important experimental parameters were optimized: (a) surface density of MB; (b) pH value of the working solution; (c) the amount of polymerase; (d) the reaction time of the MB mediated CSD; (e) the reaction time of the RCA process; (f) the immersion time of methylene blue. Respective data and Figures are given in the Electronic Supporting Material (Fig. S2 to S7). The following experimental conditions were found to give best results: (a) A 5 μM MB; (b) a pH value of 7.4; (c) an amount of 4 U/10 μL phi29 DNA polymerase; (d) a 120 min of MB mediated CSD; (e) a 100 min of RCA process; (f) 40 min of methylene blue.

Under the optimum experimental conditions, DPV was employed for the determination of target HBV DNA segment by utilizing this electrochemical biosensor with MB mediated CSD and RCA strategies. Figure 4A displays the DPV responses of the modified electrode for increasing the concentration of target DNA under optimal experimental conditions. It was noticeable that the DPV peak current of methylene blue at around −0.24 V increased as the concentration of target DNA increasing from 0 f. to 1 fM. As exhibited in Fig. 4B, the DPV peak current difference was increased with the increase of the concentration of target DNA. As exhibited in the insert of Fig. 4B, good relationship was obtained between the DPV peak current difference and the target DNA concentration. When the concentration of target DNA was in the range of 10 aM to 700 aM, the DPV peak current difference was directly proportional to the concentration of target DNA with linear regression equation of ΔI (μA) = − 0.676 c (fM) − 0.297 and correlation coefficient of 0.9987. Due to the high loading of the methylene blue on the modified electrode surface, the detection limit of target DNA was estimated to be 2.6 aM (15.6 units/10 μL) according to the equation of three signal-to-noise ratio. As shown in Table 1, compared with some reported electrochemical approaches [26–33], this method exhibited the ultrahigh sensitivity and ultralow detection limit for target DNA. Compared with other rolling circle amplification techniques for DNA [11–14], our method avoids the complicated PCR process and the application of expensive nanoclusters. Furthermore, this convenient strategy shows ultrahigh sensitivity and ultralow detection limit compared with traditional rolling circle amplification methods.

This electrochemical biosensor showed good reproducibility for target DNA. The relative standard deviation (RSD) of same modified electrode response to 0.5 f. target DNA was 3.6% for twenty successive assays. The DPV peak current of same modified electrode was approximately 90.1% of its initial current response for 0.5 f. target DNA even after twenty days storage. These indicated the good fabrication reproducibility of this electrochemical biosensor. Additionally, the RSD of six different modified electrodes response to 0.5 f. target DNA was 1.1%. The excellent reproducibility of this electrochemical biosensor is promising for ultrasensitive determination of target DNA.

Selectivity and samples determination

The selectivity of this electrochemical biosensor was evaluated by detecting six different kinds of ssDNA sequences, including

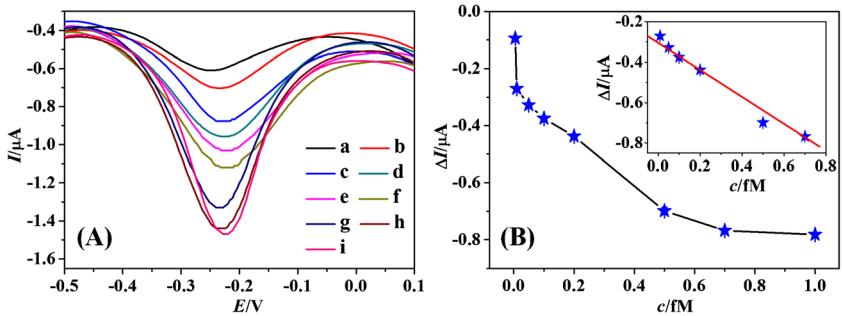


Fig. 4 **A** DPV responses of the modified electrode to target DNA at concentrations of 0 (a), 0.005 (b), 0.01 (c), 0.05 (d), 0.1 (e), 0.2 (f), 0.5 (g), 0.7 (h), and 1 f. (i). **B** DPV peak currents versus target DNA concentration from 0.01 f. to 0.7 fM. The insert was the corresponding calibration curve. Results were expressed as the average of three independent experiments. Error bars represent standard deviations

Table 1 Comparison of this electrochemical biosensor and other electrochemical methods for the determination of specific HBV DNA segment

Electrode	Technique	Linear range	Detection limit	Ref
Single walled carbon nanotube array coated with gold nanoparticles	EIS	1×10^{-18} M – 1×10^{-6} M	1×10^{-18} M	[26]
DNA probe-modified carbon paste electrode	DPV	0.22 ng L^{-1} – 5.40 ng L^{-1} or 3.51×10^{-14} M – 8.62×10^{-13} M	0.07 ng L^{-1} or 1.12×10^{-14} M	[27]
DNA probe-modified gold electrode	Chronocoulometry	1×10^{-10} M – 1×10^{-7} M	1×10^{-10} M	[28]
Oligonucleotide modified poly(4-aminophenol) film deposited graphite electrode	DPV	1.89×10^{-9} M – 1.89×10^{-6} M	2.61×10^{-9} M	[29]
O-Aminobenzoic acid, avidin, molecular beacon, and β-cyclodextrins functionalized gold nanoparticles modified silver carbon	DPV	4.0×10^{-13} M – 4.0×10^{-9} M	3.0×10^{-13} M	[30]
DNA and gold nanorods modified gold electrode	Cyclic voltammetry	1.0×10^{-12} M – 1.0×10^{-5} M	2.0×10^{-12} M	[31]
DNA modified gold electrode	Alternating current voltammetry	1.0×10^{-15} M – 1.0×10^{-9} M	1.0×10^{-15} M	[32]
DNA, multi-walled carbon nanotubes, and nanocrys zeo modified fluorine doped tin oxide glass electrode	Cyclic voltammetry	150–10^6 copies/mL	50 copies/mL	[33]
DNA modified gold electrode	DPV	1.0×10^{-17} M – 7.0×10^{-16} M	2.6×10^{-18} M	This work

the perfectly complementary target HBV DNA segment, single-base mismatched DNA sequence (T1-DNA), double-base mismatched DNA sequence (T2-DNA), non-complementary DNA sequence (T3-DNA), HPV DNA segment (P-DNA), and HCV DNA segment (C-DNA). As shown in Fig. 5, the DPV peak current responses to 1 f. T1-DNA (column b), 1 f. T2-DNA (column c), 1 f. T3-DNA (column d), 1 f. P-DNA (column e), and 1 f. C-DNA (column f) were quite small those were omitted within experimental errors. However, the DPV peak current response to 1 f. target DNA (column a) was about ten times bigger than those of other kinds of ssDNA sequences. Furthermore, the selectivity of this electrochemical biosensor was investigated by detection three biomacromolecules, such as thrombin, human serum albumin (HSA), and bovine serum albumin (BSA). The DPV peak current responses to 1 f. thrombin (column g), 1 f. HSA (column h), and 1 f. BSA (column i) were very small. However, the presence of 1 f. target DNA resulted in a remarkable increase in the DPV peak current response (column a), which was about thirty times as much as those of some biomacromolecules. These results demonstrated that the electrochemical biosensor displayed high selectivity for target DNA, which implied the potential application for real samples.

In order to confirm the feasibility and evaluate the applicability of this electrochemical biosensor to real samples, this method was used to detect target DNA in human serum samples. The recovery of target DNA in spiked human serum samples were detected by spiking three concentrations of target DNA (0.05 fM, 0.1 fM, and 0.2 fM) into human serum samples. As indicated in Table 2, the recovery values were in the range of 94.0% to 106.0%, indicating that this assay was potentially applied to realistic biological samples. These results demonstrated that this electrochemical biosensor was able to effectively detect target DNA with high selectivity

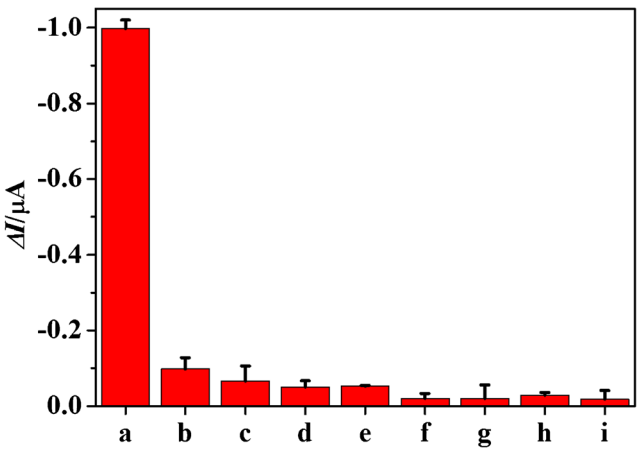


Fig. 5 DPV peak currents responding to 1 f. target DNA (a), 1 f. T1-DNA (b), 1 f. T2-DNA (c), 1 f. T3-DNA (d), 1 f. P-DNA (e), 1 f. C-DNA (f), 1 f. thrombin (g), 1 f. HSA (h), and 1 f. BSA (i). The error bars represent the standard deviations in three different measurements for each concentration

Table 2 Detection of the diluted target DNA in human serum samples ($n = 6$)

Sample	Added (fM)	Found (fM)	Recovery (%)	R.S.D. (%)
Human serum sample 1	0	0	–	–
	0.05	0.047–0.052	94.0–104.0	4.1
	0.1	0.096–0.101	96.0–101.0	3.2
	0.2	0.194–0.203	97.0–101.5	3.6
Human serum sample 2	0	0	–	–
	0.05	0.048–0.053	96.0–106.0	4.8
	0.1	0.097–0.100	97.0–100.0	3.4
	0.2	0.195–0.204	97.5–102.0	2.6
Human serum sample 3	0	0	–	–
	0.05	0.051–0.053	102.0–106.0	1.6
	0.1	0.098–0.106	98.0–106.0	1.3
	0.2	0.196–0.208	98.0–104.0	2.8

and good reproducibility. Since the target DNA is derived from the genom of HBV, this electrochemical biosensor has great potential for clinical analysis of HBV.

Conclusions

Herein, an effective electrochemical biosensing platform was developed for ultrasensitive and excellent selective detection of target DNA with sequence derived from the genom of HBV by the cascade signal amplification based on MB mediated CSD and RCA. MB was used to ensure the good selectivity of the sensor. To further improve the sensing performance of the strategy, RCA was employed to amplify the DPV current signal response. The data in the study demonstrated that the sensing performance reached the limit of detection of 2.6 aM (15.6 units/10 μL) in detection, representing a huge improvement. Since real HBV DNA is substantially longer and double stranded, the short target DNA will less present in clinical samples. The present electrochemical sensing platform may be applied for other important oligonucleotides in clinical diagnosis and disease treatment. Further research to develop a more facile modification protocol and highly practical fabrication procedures of the sensing electrode is ongoing in our group.

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

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