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# A sequence-specific DNA sensor for Hepatitis B virus diagnostics based on the host–guest recognition

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ABSTRACT

In this work, we demonstrate the applicability of an electrochemical supramolecular platform to detect Hepatitis B virus (HBV) sequences. A DNA molecular beacon was designed as the probe, and immobilized onto the electrodes through the biotin at the 3′-end, while the 5′-end of the probe was labeled with 4-(4-dimethyl aminophenylazo) benzoic acid (dabcyl). The β-cyclodextrins functionalized Au nanoparticles (Au-CDs) were employed as electrochemical signal provider. The probe DNA immobilized on the electrode kept the stem-loop configuration, which shielded dabcyl from docking with Au-CDs in solution due to the steric effect. While in the presence of the target DNA, the probe conformation was changed and a double-stranded DNA (dsDNA) molecule was formed through the hybridization. Consequently, Au-CDs were linked to dsDNA owing to the host–guest recognition between β-CD and dabcyl. Thus, the hybridization events could be sensitively transduced to electrochemical signals provided by Au nanoparticles. The designed sensor favored discrimination between the healthy and single-nucleotide polymorphisms (SNP)-containing sequences. Under optimized detection conditions, the proposed method showed high sensitivity and specificity with a detection limit of $3.00 \times 10^{-13}$ M for HBV DNA sequence.

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## 1. Introduction

Biosensors for DNA sequence detection have become increasingly important in the preventative therapy of genetic disorders, prognosis of cancer, and for the treatment of bacterial and viral infections [1–6]. Growing interest has been aroused in the development of simple, rapid and convenient methods for DNA sequence and mutant gene analysis to allow early and precise diagnoses of disease [7,5]. Among these methods, electrochemical DNA detection has the advantages of high sensitivity, compatibility for miniaturization, easy operation and low cost [8–10]. Many scientists focused on the development of electrochemical approaches to lower the complexity of the sensors by avoiding sample labeling [11,12].

The molecular recognition technology, defined as the supramolecular noncovalent interaction between the “host” and “guest” molecules, has been previously employed in the chemical sensing [13–15]. Cyclodextrins (CDs) consist of one well-known family of cyclic oligosaccharides, which has the shape of hollow truncated cone. They can form an inclusion complex with various guest molecules because of their special molecular structures of hydrophobic internal cavity and hydrophilic external surface [16,17]. Thus, CDs have the capability to selectively include specific compounds in the hydrophobic cavity in an aqueous solution [18]. The utilization of CDs opens the possibilities to create useful devices for electrochemical DNA sensors [19].

In this paper, we describe an electrochemical DNA sensor for the detection of Hepatitis B virus (HBV) based on the host–guest recognition. HBV causes acute and chronic hepatitis, and is closely associated with the development of hepatocellular carcinoma [20]. To date, the determination of HBV was mostly realized through the DNA-like full genomic sequence analysis and PCR-based detection. The former is a very expensive technique. The latter is suitable for clinic applications, but it needs expensive private kits and also needs some toxic agents. With external hybridization indicator, it is unable to identify minor amounts of mismatch sequence in a mixed solution. Furthermore, the capability in discriminating perfectly complementary DNA from single-base mismatches should be improved. In this case, electrochemical approaches were exploited to the detection and identification of the mutation of HBV [21–23], which made great contributions to achieve early and precise diagnoses of HBV with simple, inexpensive and rapid methods. However, in most studies, additional electroactive molecules were

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used as the hybridization indicator [24,25], which made the biosensors more complex. Therefore, electrochemical label-free methods are of particular interest for the analysis of HBV single-nucleotide polymorphisms (SNPs) sequences.

In our strategy, in order to improve the specificity of detection, a molecular beacon as DNA probe was designed, which exhibited thermodynamically preconditioned higher specificity for SNP than linear DNA [26–28], and has been successfully applied in the electrochemical DNA detection [29–32]. Our group has designed molecular beacon based host–guest recognition for specific DNA detection [33]. CdS nanoparticle modified cyclodextrins was used as both electrochemical signal provider and host–guest recognizer with specific molecular recognition characteristics. This host–guest recognition based electrochemical sensor has been able to detect DNA target with excellent differentiation ability. However, CdS nanoparticle is not environmentally friendly and apt to cause pollution. While, Au nanoparticles have large surface area and good bio-compatibility. They are excellent candidates for bioconjugation [34,35] and offer excellent electrochemical signals [36]. β-Cyclodextrin (β-CD) functionalized Au nanoparticles (Au-CDs), synthesized via thiol attachment according to the intriguing reactivity between the thiol group of β-CD and Au [37], were utilized to detect DNA hybridization in the solution based on host–guest recognition technology. The reduction signal of Au was observed as a result of the hybridization between the probe and the target. This sequence-specific DNA sensor favors discrimination between the fully matched and SNP-containing HBV DNA. This sensor is pronounced at high specificity, cost-effectiveness because of its lack of extra modification and external hybridization indicators. It was demonstrated that the presented method was highly sensitive and specific with a wide detection range of four orders of magnitude and a detection limit as low as $3.00 \times 10^{-13}$ M. This platform holds great promise in ultrasensitive sequence-specific analysis.

## 2. Materials and methods

### 2.1. Materials

The oligonucleotides were purchased from Sangon Biotech Corp. (Shanghai, China) with the following sequences:

Probe oligonucleotide with dabcyl group at its 5′-end and biotin group at its 3′-end: 3′-biotin-AAA CGC TCC CGT CGA TAT ACCTAC TAC ACC ATA ATT TTG AGC GAA A-dabcyl-5′;
Target oligonucleotide: 3′-TTA TGG TGT AGT AGG TAT ATC GAC-5′;
Mismatched oligonucleotide: 3′-TTA TGG TG$\underline{A}$ AGT AGG TAT ATC GAC-5′;
Random oligonucleotide: 3′-CGT GTT GAC AAA GAA GAC GTC AAG-5′.

Mercapto cyclodextrin, avidin, ethyl-3-(dimethylamino-propyl)-carbodiimide hydrochloride (EDC), n-hydroxy succinimide (NHS), o-aminobenzoic acid ($C_7H_7NO_2$, ABA) were purchased from Sigma–Aldrich. Chloroauric acid, trisodium citra, potassium ferricyanide/ferrocyanide ($[Fe(CN)_6]^{3-/4-}$), PBS buffer (0.1 M, pH 7.0) and other reagents were commercially available. They were all of analytical reagents grade and used without further purification. Ultrapure water was obtained from Aquapro system.

### 2.2. Instrumentation

Atomic force microscope images were performed on AFM Multimode 8 (Bruker Instrument, USA). UV–vis absorption spectra were recorded on a Varian Cary 50 UV–vis spectrophotometer (Varian, USA). Fourier transform infrared (FT-IR) spectra were obtained on a Nicolet Nexus 670 FT-IR spectrophotometer (Thermo Nicolet, USA).

Differential pulse voltammogram (DPV) and faradaic impedance spectra were performed using a CHI Instruments model 660 Electrochemical Analyzer (CH Instrument Inc., USA). The electrochemical system comprised of a glassy carbon working electrode ($D$ = 3.0 mm), an Ag/AgCl (saturated KCl) reference electrode and a platinum wire counter electrode.

### 2.3. Preparation of β-CDs functionalized Au nanoparticles

β-CDs modified Au nanoparticles (Au-CDs) were prepared according to the literature [38]. In brief, all glassware used in the following procedure were cleaned in a bath of freshly prepared mixture of $HNO_3$–HCl (3:1), then rinsed thoroughly in twice-distilled water and dried in air. 1% (w/w) of $HAuCl_4$ and sodium citrate solution need to be filtered through 0.22 μm microporous membrane filter prior to use.

100 ml of boiling aqueous solution containing 1% (w/w) $HAuCl_4$ and 0.06184 g SH-β-CDs was heated to reflux. Subsequently 2.5 ml of 1% (w/w) sodium citrate was injected quickly to the boiling solution [39–41] and stirred for 30 min. In process of time, the following colors appeared successively in the solution: grey, blue, purple and wine red. The mixture was heated under reflux for 30 min and then set aside to cool down to room temperature naturally. The free SH-β-CDs molecules were separated from the Au-CDs conjugates by selective diffusion through a semi-permeable membrane. Because the host–guest recognition could trigger the nonspecific of Au-CDs on the modified electrode, Au-CDs were pretreated with BSA before the intercalator binding process to prevent the nonspecific binding. The procedures were as follows: 50 μl Au-CDs were pretreated with 100 μl of 10% BSA for 1 h. Then it was centrifuged for 12,000 r/min, and redispersed in the solution. The resulting conjugates were stored in a dark glass vessel at 4 °C until required.

### 2.4. Modification of the electrode

The electropolymerization of o-aminobenzoic acid (ABA) onto the electrode surface was prepared as the literature [42]. It was performed by immersing the electrode in a solution containing 0.05 M ABA and 1 M $H_2SO_4$, and cyclic voltammogram ran for eight cycles between 0 V and 1.0 V at a scan rate of 40 mV/s. Then the poly(ABA)-coated electrode was rinsed with large amounts of water to remove any nonspecifically adsorbed substance. The probe was covalently immobilized on the electrode via the carboxyl groups of the poly(ABA) film, which were first activated by covering the electrode surface with 2.0 μl of 4 mM EDC and 2.0 μl of 1.0 mM NHS in 30 mM PBS buffer and standed at room temperature for 15 min. Then it was followed by adding 2.0 μl of $1.00 \times 10^{-5}$ M avidin to the activation solution and incubating for 3 h at room temperature. The unreacted reagents were washed off with 0.1 M PBS buffer. 5.0 μl of $4.00 \times 10^{-6}$ M probe oligonucleotide was added and standed for 1 h. The modified electrode was rinsed with water to remove any non-immobilized probe for further use.

### 2.5. DNA hybridization and host–guest recognition

For the DNA sequence detection, the assay procedure was initiated by immersing the probe-modified electrodes in the hybridization buffer solution, containing 10 μl of the target DNA and 10 μl of Au-CDs. 10 μl of 0.80 M NaCl was added to keep the salinity and the solution was stirred for 3 h at 42 °C to carry out the hybridization reaction. Then, the electrode was washed thoroughly with PBS.

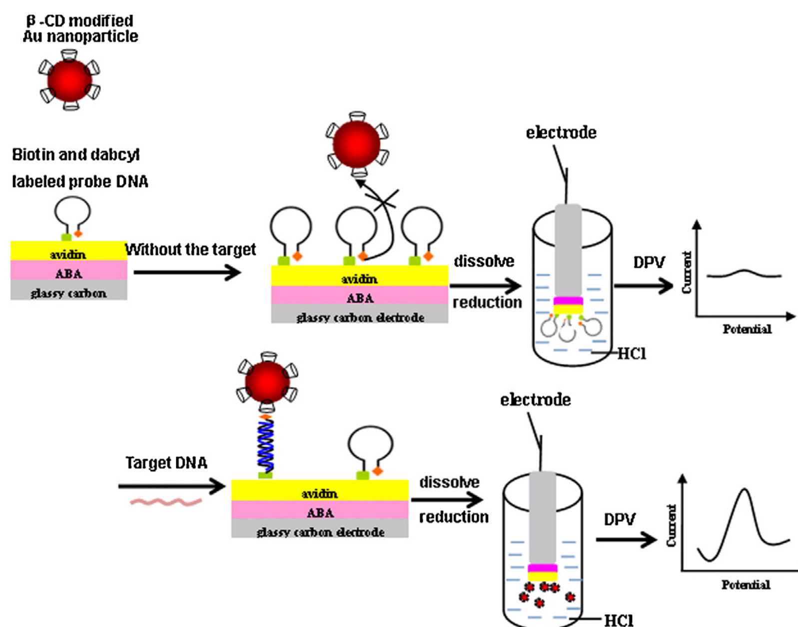


**Fig. 1.** Schematic illustration of electrochemical sensor for HBV sequence detection based on the host–guest recognition.

*2.6. Electrochemical detection*

All electrochemical experiments were directly performed in an electrochemical cell. Before the detection, the electrode was dipped into 100 μl of 0.1 M HCl solution to dissolve the Au nanoparticles. Then, +1.25 V was applied for 120 s to carry out the electrochemical pre-oxidation of Au. Differential pulse voltammetry (DPV) scanning was followed on in the range from +0.70 V to +0.20 V (Incr E: 0.004 V; Amplitude: 0.05 V; Pulse width: 0.05 s; Pulse period: 0.2 s). An analytical signal at a potential of +0.47 V was collected due to the reduction of $AuCl_4^-$, which relates to the amount of the Au nanoparticles on the electrode.

The measurements of faradaic impedance spectra were performed in the presence of 0.01 M $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) mixture (0.1 M PBS) as redox probe, and the frequency range were from $1.0 \times 10^5$ Hz to 1.0 Hz at the open circuit potential of +0.22 V versus Ag/AgCl.

## 3. Results and discussion

*3.1. Principle of HBV sequence-specific detection*

A general problem in DNA gene mutation analysis is the relatively low specificity of linear DNA probes for single-nucleotide polymorphisms. However, the structures of molecular beacon can provide a better discrimination between the intact and mutated sequences [43]. In our paper, the DNA molecular beacon, composed of a self-complementary stem and a loop, was immobilized in their folded state onto the electrode. Its stem-loop structure is formed through the base pairing and the sequences in the loop were complementary to the target HBV specific sequence. β-cyclodextrin (β-CD) functionalized gold nanoparticles (β-CD-Au) was synthesized and the resulting β-CD-Au was used to detect DNA hybridization in the solution based on host–guest recognition technology. The reduction signal of gold was observed as a result of the specific hybridization between the probes and their targets (Fig. 1).

As can be seen from Fig. 2, before hybridization, the probe remained in a "closed" state, and prevented dabcyl from entering the cavity of β-CD, resulting in only small background current signal (Fig. 2, curve a). In the presence of the complementary target, the probe was unfolded and transformed into a straight dsDNA molecule. In this "open" state, the dabcyl molecule was forced away from the electrode surface and thus could be captured. Therefore, Au nanoparticle was brought onto the electrode surface. HCl solution was applied to dissolve the Au nanoparticles and then the electrochemical preoxidation of Au was carried out. Differential pulse voltammetry (DPV) scanning was followed on and an analytical signal at a potential of +0.47 V was collected due to the reduction of $AuCl_4^-$, which relates to the amount of the Au nanoparticles on the electrode and a marked electrochemical signal of Au was obtained (Fig. 2, curve b).

*3.2. Characterization of Au-CDs*

The size and morphology of synthesized Au-CDs was measured by AFM and UV–vis spectrum. The typical AFM image and particles size distribution of Au-CDs are illustrated in Fig. 3A. The average diameter of Au-CDs is approximately 50 nm and excellent monodispersed in aqueous solution. The UV–vis spectrum shows the absorption maxima at 531.5 nm (Fig. 3B). The particle diameter

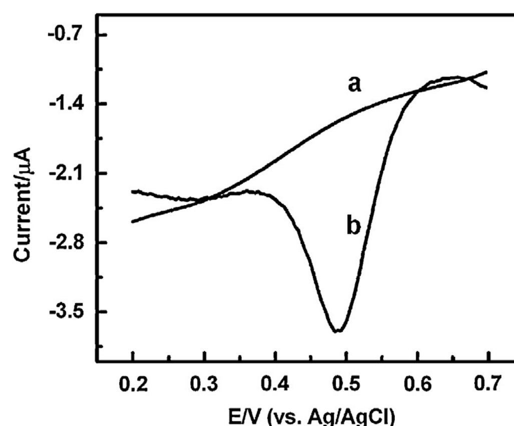


**Fig. 2.** DPV responses of the probe without target DNA (a) and in the presence of $1.00 \times 10^{-9}$ M target DNA (b).

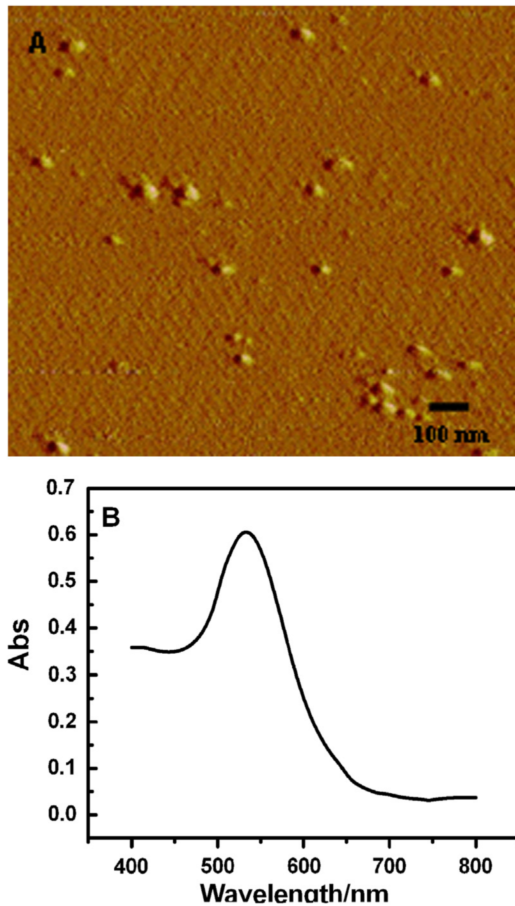


**Fig. 3.** AFM image (A) and UV–vis spectra (B) of Au-CDs nanoparticles.

($d$) ranging from 35 to 100 nm can thus be calculated from the peak position according to the following formula [44]:

$$D = \frac{\ln[(\lambda_{spr} - \lambda)/6.53]}{0.0216}$$

where $D$ is the diameter of the particle, $\lambda_{spr}$ is the experimental peak positions, and $\lambda$ is the fit parameters determined from the theoretical values (when $D > 25$, $\lambda = 512$).

The average diameter obtained from the formula is in accordance with the AFM image.

The formation of Au-CDs was characterized by FT-IR spectra. As shown in Fig. 4, the stretching vibration peak of S–H in

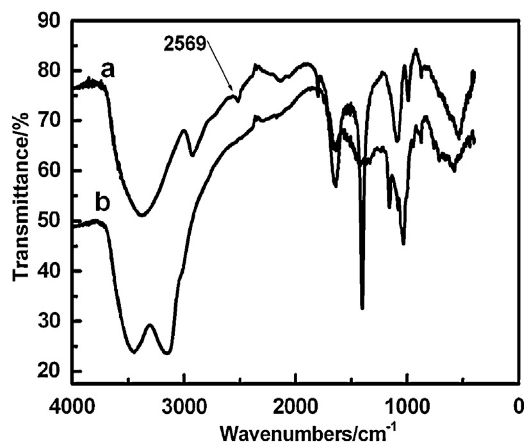


**Fig. 4.** FT-IR spectra of SH-β-CD (a) and Au-CDs (b).

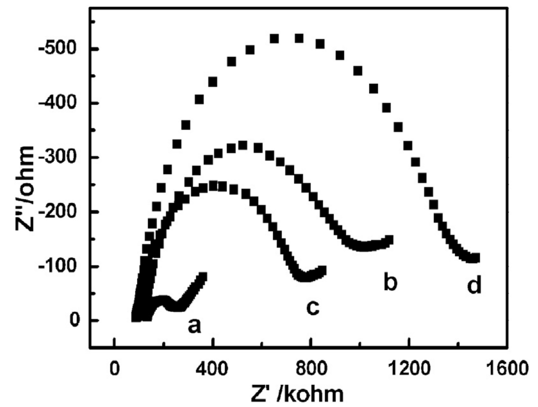


**Fig. 5.** Faradaic impedance spectra corresponding to GCE (a), ABA/GCE (b), avidin/ABA/GCE (c), probe DNA modified avidin/ABA/GCE.

SH-β-CD is located at 2569 $cm^{-1}$ (curve a) and the characteristic SH stretching vibration absorption peak disappears after the conjugation between SH-β-CD and Au nanoparticles (curve b), confirming that β-CD were modified to the surface of Au through Au–S covalent bonds. This is consistent with the results reported in the literature [45], which demonstrated the successful formation of β-CD functionalized Au nanoparticles.

### 3.3. Characterization of the modified electrode

In this work, poly(ABA)-coated electrode was used for the probe immobilization. Thus, prior to the probe coupling reaction, it is necessary to ensure that the ABA polymer film is formed on the electrodes. Faradaic impedance spectroscopy [46,47] is employed here to evaluate the immobilization of ABA at a bare electrode. As shown in Fig. 5, in comparison with the bare electrode (curve a), an apparent increase in the electron-transfer resistance (Rct) of the electroactive marker was recorded after the interaction of ABA with the bare electrode, indicating the successful deposition of the ABA support layer (curve b). The increase in Rct value is due to the presence of increased negative charge from carboxyl groups of ABA. After the ABA was coated, the electroactive positions on the electrode were occupied and the coating would consequently suppress the electron transfer of the electrode. After activation with EDC/NHS, the negative charge of carboxyl groups is declined resulting in a decrease in Rct (curve (c)) [48]. The followed immobilization of the probe DNA (curve d) led to the dramatic changes in the increment of charge transfer resistance in the spectra, demonstrating that the adsorption of the probe DNA obstructed charge transfer. The probable reasons are the annular structure and negative electricity of DNA, which block the electrochemical reaction on the electrode.

### 3.4. Elimination of the nonspecific binding

The pretreatment of Au-CDs with BSA could prevent the nonspecific binding on the probe/avidin/ABA/GCE electrode. Compared to the reduction current of unpretreated Au-CDs (Fig. 6, curve b), that of pretreated one decreases remarkably (Fig. 6, curve a). That is to say, nonspecific interactions are greatly eliminated by the pretreatment of BSA. This process can avoid the strong nonspecific interactions between Au-CDs and the electrode, and the detection limit of DNA hybridization detection can be improved greatly.

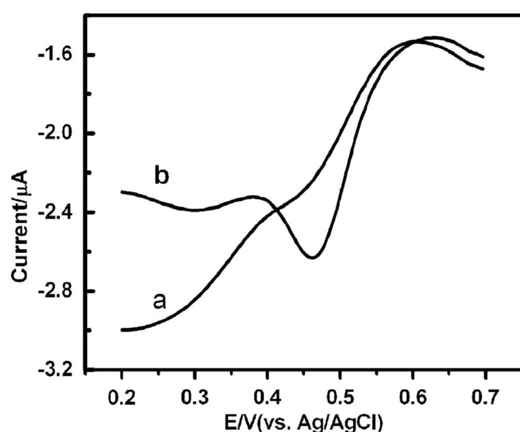


**Fig. 6.** DPV responses of Au from probe/avidin/ABA/GCE electrodes incubated with Au-CDs (b) and BSA pretreated Au-CDs (a).

### 3.5. Optimization of experimental conditions for DNA sensing

The DNA sensing was strongly influenced by the experimental conditions, such as time, salinity and temperature. The time for the hybridization and host–guest recognition was firstly examined, and Fig. 7A presents the DPV responses of the captured Au nanoparticles with different periods of time. Clearly, the peak current increased rapidly as the time increased from 1 h to 3 h, and then continued to increase but at a much slower rate. Thus, 3 h was chosen for the experiment.

The temperature has also an influence on the hybridization rate and the host–guest recognition. The effect of temperature was investigated in the range of 27–57 °C (Fig. 7B). The results showed that the signal was enhanced with the increasing temperature from 27 °C to 42 °C, and the strongest response was obtained around 42 °C. Then, it decreased obviously. This might be attributed to the fact that the temperature has a complex effect on the efficiency of the hybridization: ds DNA may denature at a higher temperature. Thus, 42 °C was used for the research.

Fig. 7C shows the effect of $Na^+$ concentrations from 0.4 to 1.6 M on the electrochemical DNA sensing. The signal increased significantly as the $Na^+$ concentration increased up to 0.8 M, reflecting improved hybridization performance. This owes to the fact that there is a strong electrostatic repulsion between the DNA strands, and $Na^+$ may neutralize the charge. Thus, 0.1 M PBS (pH 7.0) containing 0.8 M $Na^+$ was chosen as reaction buffer.

### 3.6. Specific detection of HBV sequence

The designed probe of molecular beacon allowed favorable discrimination between the fully matched and the T to A mutated HBV gene sequence. A series of hybridization experiments were carried out to evaluate the specificity of the sensor with different target oligonucleotides, including complementary DNA, one-base mismatched DNA, and non-complementary DNA. As shown in Fig. 8, the non-complementary DNA produced a negligible DPV signal (curve c), which was similar to the background signal from non-specific adsorption of Au-CDs on the modified electrode (curve d). The signal intensity of the non-complementary and one-base mismatched sequence was only 27.11% and 42.50% of that of the complementary one, respectively. This is due to the fact that a structural rearrangement of the DNA molecular beacon into the double-stranded helix was induced by hybridization with the complementary DNA. While for the mismatched sequence, the binding with molecular beacon was much weaker. Thus, most of the molecular beacons still remained in the "closed" state, resulting in the lower signal. These results exhibited the hybridization specificity

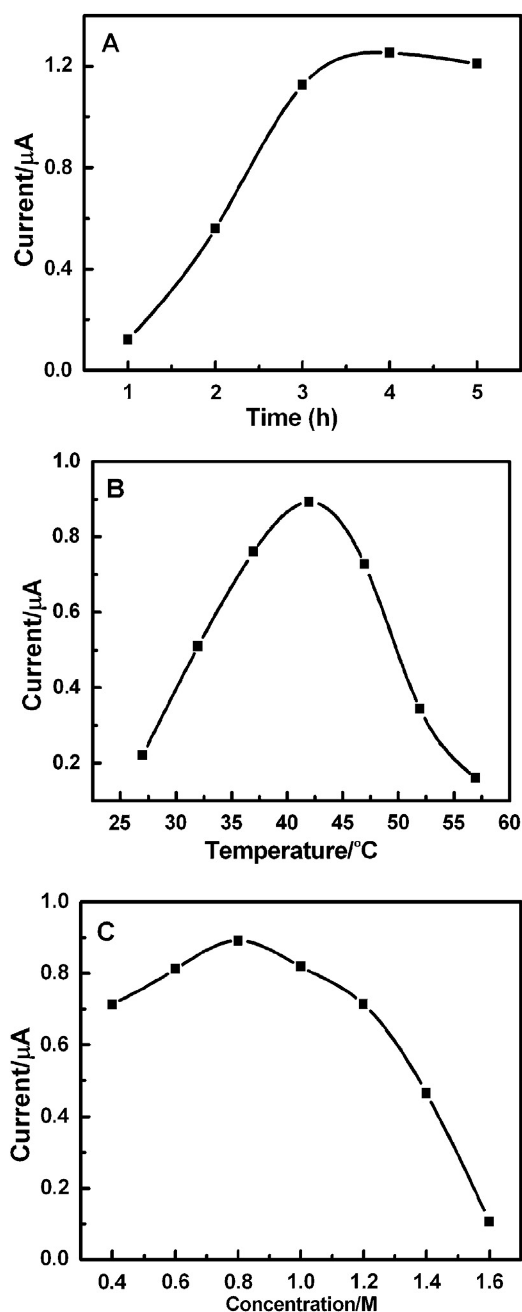


**Fig. 7.** (A) Effect of the time from 1 h to 5 h; (B) effect of the temperature varying from 27 °C to 57 °C; (C) effect of the salinity varying from 0.4 M to 1.6 M with 10 μl of Au-CDs and 10 μl of $1.00 \times 10^{-9}$ M DNA.

of the sensor for the target sequence due to the intrinsic recognition ability of DNA molecular beacon [28,49]. The significant current difference demonstrated that this DNA sensor was able to discriminate SNP DNA with excellent specificity.

### 3.7. Quantitative analysis of the target DNA

The hybridization was transduced to an electrochemical signal from the captured Au nanoparticles by the host–guest recognition. The signal increased with the rising amount of the complementary sequence presented in the hybridization solution. A series of corresponding DPV responses of Au nanoparticles were obtained as shown in Fig. 9A and we found that the DPV signals were logarithmically related to the target concentration in the range of

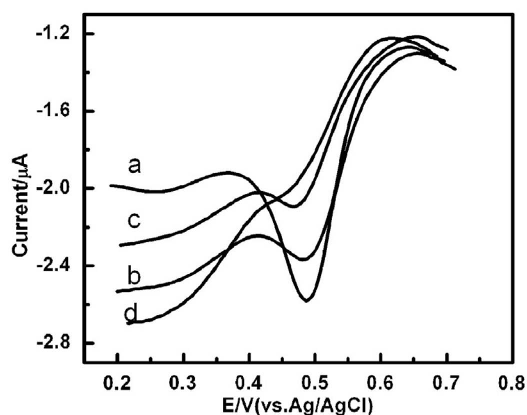


**Fig. 8.** DPV responses of Au after the DNA probe hybridized with $1.00 \times 10^{-9}$ M complementary target DNA (a), one-base mismatched sequence (b), non-complementary sequence (c) and 0.1 M PBS without DNA sequences (d).

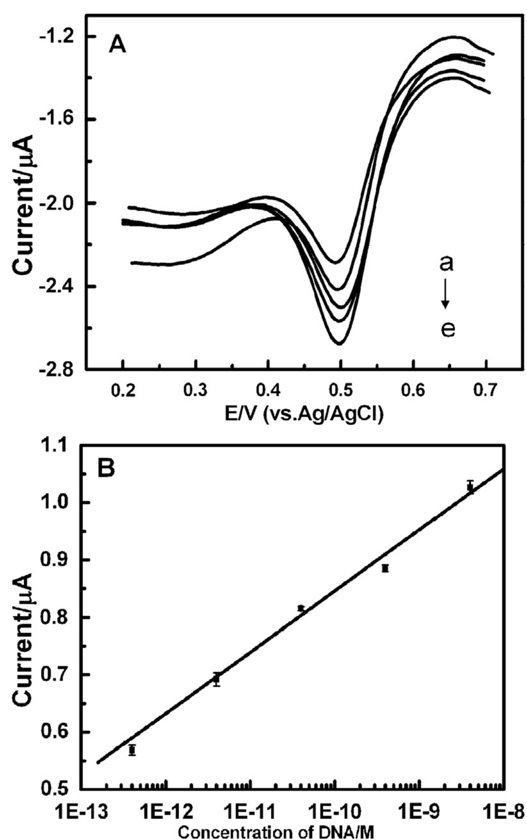


**Fig. 9.** (A) DPV responses of Au on the probe/avidin/ABA/GCE electrode after the addition of target DNA in different concentrations (curves a–e: $4.00 \times 10^{-13}$ M, $4.00 \times 10^{-12}$ M, $4.00 \times 10^{-11}$ M, $4.00 \times 10^{-10}$ M, $4.00 \times 10^{-9}$ M). (B) The plot of the reduction peak current of Au versus the logarithm of target DNA concentration. Error bars show the standard deviations of measurements taken from independent experiments with at least five distinct sensors.

$4.0 \times 10^{-13}$–$4.0 \times 10^{-9}$ M (Fig. 9B) with a regression equation of $i_{pa}$ (μA) = 1.9128 + 0.1067 lg$c_{DNA}$ (M) and the linear correlation coefficient of 0.997. Target DNA at a concentration as low as $3.0 \times 10^{-13}$ M was able to be detected using this sensor. Compared with other DNA detection methods [50,51], the proposed one is superior in wide linear range and it is able to discriminate a single-base difference in the target DNA with high specificity.

## 4. Conclusions

We constructed a host–guest recognition based electrochemical sensor for sequence-specific HBV DNA detection. A new derivative – Au-CDs was prepared and characterized. Conformation-based DNA stem-loop mode gave high specificity to the sensor for DNA sensing, and Au nanoparticle offered superior electrochemical signals for quantifying target HBV DNA. It was demonstrated that the proposed sensor had a wide detection linear range from $4.00 \times 10^{-13}$ M to $4.00 \times 10^{-9}$ M with a detection limit of $3.00 \times 10^{-13}$ M, indicating that this sensor has high sensitivity for the determination of sequence-specific DNA. Moreover, it achieves impressive specificity with successful discrimination between intact and SNP-containing HBV sequences. Thus, the designed DNA molecular beacon system favors discrimination between the fully matched and SNP-containing DNAs, which is strongly required in HBV sequence-specific DNA assays.


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