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Ultrasensitive electrochemical biosensing for DNA using quantum dots combined with restriction endonuclease

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A universal and sensitive electrochemical biosensing platform for the detection and identification of DNA using CdSe quantum dots (CdSe QDs) as signal markers was designed. The detection mechanism was based on the specific recognition of *MspI* endonuclease combined with the signal amplification of gold nanoparticles (AuNPs). *MspI* endonuclease could recognize its specific sequence in the double-strand DNA (dsDNA) and cleave the dsDNA fragments linked with CdSe QDs from the electrode. The remaining attached CdSe QDs can be easily read out by square-wave voltammetry using an electrodeposited bismuth (Bi) film-modified glass carbon electrode. The concentrations of target DNA could be simultaneously detected by the signal of metal markers. Using *mycobacterium tuberculosis* (*Mtb*) DNA as a model, under the optimal conditions, the proposed biosensor could detect *Mtb* DNA down to 8.7 × 10^{-15} M with a linear range of 5 orders of magnitude (from 1.0 × 10^{-14} to 1.0 × 10^{-9} M) and discriminate mismatched DNA with high selectivity. This strategy presented a universal and convenient biosensing platform for DNA assay, and its satisfactory performances make it a potential candidate for the early diagnosis of gene-related diseases.

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Introduction

The performances of electrochemical biosensing depend on their signal-transduction methods. Recently, enzymes and quantum dots (QDs)-based platforms for detecting DNA and protein have been proposed.[1,2] CdSe and PbS QDs were employed as labels to simultaneously detect rabbit immunoglobulin G antigen and carcino-embryonic antigen.[3] In addition, Marin *et al.* have constructed a biosensing platform for the detection of cystic fibrosis-related DNA using CdS QDs as a signal label.[4] Although the responses of enzymes are rapid, their activity is easily affected by the environment and chemicals. Compared to enzymes, QDs have received more attention because of their characteristics of signal enhancement, inherent miniaturization, low detection limits, low cost, low power requirements, and especially because they have excellent stability against environment and chemicals. Moreover, the signal response could be generated from QDs themselves.[3] Herein, CdSe QDs were applied to our design to improve sensing performance.

Restriction endonuclease, as an analytical tool, has been used in frequently DNA assays. Restriction endonuclease can interact with its DNA recognition sites more specifically than other DNA-binding proteins. Biosensing platforms combined with endonuclease for DNA detection was recently reported.[5] In most cases, a mercury film modified electrode was preferred for stripping analysis due to its high reproducibility and sensitivity.[6] However, the toxicity of mercury in combination with the inconvenience of handling has limited its application in biosensors. Some electrode materials have been proposed as substitutes for mercury, such as platinum,[7] silver,[8] iridium,[9] antimony,[10] and bismuth.[11] Among these, bismuth is an environmentally friendly material with relatively low toxicity, and it exhibits a performance comparable to mercury in stripping analysis. Therefore, a bismuth-based electrode has become a highly attractive and suitable substitute for the on-site environmental monitoring and clinical testing of several toxic metals.[12]

Tuberculosis (TB) caused by *mycobacterium tuberculosis* (*Mtb*) bacillus is one of the leading causes of death from infectious diseases. There were almost 8.8 million incident cases of TB in 2010.[13] At present, the most widely used method for diagnosing *Mtb* is the traditional microbial culture-based tests.[14] However, this assay has huge limitations because it is time-consuming and cannot detect viruses during the early stage of infection. Therefore, *Mtb* DNA determination is a valuable alternative assay.

Different techniques for DNA detection have been developed, including fluorescence,[15] photoelectrochemistry,[16] electrochemistry,[17] and colorimetry.[18] Among these techniques,

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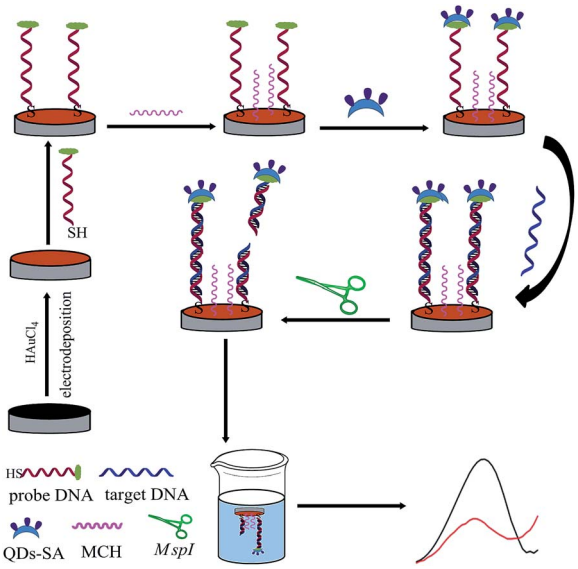

Scheme 1 Fabrication process of the electrochemical biosensor.

electrochemical methods have attracted considerable attention due to their inexpensive instrumentation, fast analysis time and excellent compatibility with miniaturization techniques.

In this work, a novel electrochemical biosensing platform for *Mtb* DNA was designed using CdSe QDs as a label combined with *MspI* endonuclease and AuNPs to improve the selectivity and amplify the signal (Scheme 1). A molecule of *MspI* endonuclease has a molecular mass of 29 kDa.[19] It recognizes the duplex symmetrical sequence 5′-CCGG-3′ and catalyzes the double-strand DNA (dsDNA) cleavage between the two cytosines, but does not act on single strand DNA.[20] After the glass carbon electrode was modified with AuNPs, the biotin-labeled probe DNA was immobilized on the AuNP-modified glass carbon electrode, the CdSe QDs–streptavidin (SA) label was linked to probe DNA *via* specific recognition. Upon probing DNA on the modified electrode hybridized with target DNA, *MspI* endonuclease could recognize its specific sequence in the duplex DNA and cleave the dsDNA fragments linked with CdSe QDs from the electrode, resulting in a decrease in the electrochemical signal. The higher the concentration of target DNA, the lower the electrochemical signal. Based on the variation of current before and after digesting the DNA hybrid, a novel signal-off electrochemical DNA biosensor was developed (Scheme 1). This proposed electrochemical biosensor exhibited good specificity, high sensitivity, satisfactory reproducibility and a wide linear concentration range.

Experimental section

Chemicals and reagents

1 μM stock solution of CdSe QD-coated streptavidin (CdSe QDs–SA) (the ratio of CdSe QDs–streptavidin is 3 : 1) was purchased from Beijing Zhongkewuyuan Biotechnology Co., Ltd (Beijing, China). *MspI* endonuclease was obtained from New England Biolabs (USA). Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) and 6-mercapto-1-hexanol (MCH) were purchased from Sigma-Aldrich Company. Potassium monohydrogen phosphate, potassium dihydrogen phosphate, bismuth nitrate pentahydrate, hydrochloric acid, nitric acid, acetic acid stock solutions were purchased from Guoyao Chemical Reagent Co., Ltd (China). Chloroauric acid ($HAuCl_4 \cdot 4H_2O$) was obtained from Shanghai Reagent Company (Shanghai, China). All other reagents were of analytical grade. All of the synthetic oligonucleotides purified with high-performance liquid chromatography were purchased from Sangon Inc. (Shanghai, China) and their base sequences were as listed below.

Probe DNA: 5-SH-$(CH_2)_6$-GGTCTTCGTGGCCGGCGTTCA-$(CH_2)_6$-biotin-3′

Target DNA: 5′-TGAACGCCGGCCACGAAGACC-3′

Mismatch1 (M1): 5′-TGAACGCC*T*GCCACGAAGACC-3′

Mismatch2 (M2): 5′-TGAACGCCGGCCA*T*GAAGACC-3′

Aqueous solutions were prepared with ultrapure water (≥18.25 Ω), and phosphate buffered saline (PBS) (0.01 M, pH 7.4) was used as the washing buffer. The different concentrations of DNA used in this work were obtained by dilution with (0.01 M, pH 7.4) Tris–HCl solution.

Apparatus

Square-wave voltammetric (SWV) stripping measurements were performed on a CHI 660D electrochemical workstation (CH Instrument Co., USA). All the experiments were carried out at room temperature using a conventional three-electrode system with a platinum wire as auxiliary, a Ag/AgCl electrode as reference and a Bi film modified glass carbon electrode as the working electrode in 0.1 M HAc–NaAc buffer solution (pH 4.6) containing Cd^{2+} in different concentrations. Electrochemical impedance spectroscopy (EIS) was carried out on an Autolab potentiostat/galvanostat PGSTAT302N (Metrohm, Netherlands) and controlled by Nova 1.8 software under open-circuit conditions in 0.1 M KCl solution containing 5 mM $K_3Fe(CN)_6$/$K_4Fe(CN)_6$ (1 : 1). The voltage frequencies ranged from 0.1 Hz to 10 0000 Hz. The morphologies of the AuNPs were characterized by a JSM-7600F field emission scanning electron microscopy (SEM) (JEOL, Japan) at an accelerating voltage of 10 kV.

Fabrication of the biosensor

The fabrication process is outlined in Scheme 1. The glass carbon electrodes (GCEs, 3 mm in diameter) were freshly polished prior to use with 0.3 μm alumina slurries and thoroughly rinsed with ultrapure water, then ultrasonically rinsed in ethanol and water for 2 min, and dried in air. The AuNPs on the pretreated GCEs were prepared by scanning at −0.5 V in H_2SO_4 solution of pH 2.0 containing 5 mM $HAuCl_4$ for 120 s. To fabricate the biosensor, 5 μL of 1 μM biotin-labeled probe DNA was dropped onto the AuNPs-modified GCEs and kept overnight at 4 °C to prepare probe DNA/AuNPs-modified GCEs through interaction of S–Au. Then, the probe DNA/AuNPs-modified GCEs were rinsed with ultrapure water and washing buffer solution. Subsequently, the probe DNA/AuNPs modified GCEs were treated with 10 μL of 1 mM MCH for 40 min to occupy the remaining bare sites and to obtain a well-aligned DNA

monolayer.[21] Using this two-step process to form a probe DNA/MCH mixed monolayer, nonspecifically adsorbed DNA was largely removed from the surface. Moreover, the probe DNA/MCH mixed monolayer was found to be stable, and hybridization reactions were found to be completely reversible and specific. After rinsing with ultrapure water and 0.01 M PBS, the MCH/probe DNA/AuNPs-modified GCEs were incubated with 5 μL CdSe QDs–SA solution at 37 °C for 50 min. Due to the biotin–SA specific interaction, the CdSe QDs–SA could be firmly linked with biotin-labeled probe DNA. After the CdSe QDs–SA/MCH/probe DNA/AuNPs-modified GCEs were rinsed and dried at room temperature, 5 μL target DNA of different concentrations was pipetted onto the CdSe QDs–SA/MCH/probe DNA/AuNPs-modified GCEs with centrifugal tubes upside down and incubated at 37 °C for 2 h. Then, the target DNA/CdSe QDs–SA/MCH/probe DNA/AuNPs-modified GCEs were thoroughly swilled to remove unbound target DNA and dried at room temperature. Finally, *MspI* cleavage was performed by dipping the target DNA/CdSe QDs–SA/MCH/probe DNA/AuNPs modified GCEs in Tris–HCl buffered saline (0.01 M, pH 7.4) containing 20 U μL^{-1} *MspI*, 50 mM KAc and 10 mM $Mg(Ac)_2$ for 2 h at 37 °C. After washing with ultrapure water and PBS solution, the *MspI*/target DNA/CdSe QDs–SA/MCH/probe DNA/AuNPs modified-GCEs were dipped into 0.1 M HNO_3 solution for 2 h. The sensing electrodes were immersed in 100 μL of 0.1 M HNO_3 solution to detect the electrochemical signal of Cd^{2+}; then, the HNO_3 solution was transferred to 400 μL 0.1 M HAc–NaAc buffer solution. Therefore, the final volume of the solution was 500 μL. During this process, the residual CdSe QDs on the electrode were dissolved as Cd^{2+} from the AuNPs-modified GCE, and the signal of Cd^{2+} could be detected by a bismuth film-modified GCE using SWV.

Preparation of the bismuth film modified glass carbon electrode

The GCEs (3 mm in diameter) were polished with 0.3 μm alumina powder. They were thoroughly rinsed with ultrapure water, followed by successive sonication in water and ethanol for 2 min. To obtain Bi film-modified GCEs, the pretreated GCEs were immersed in air-saturated bismuth nitrate solution (pH 2.0, pH value adjusted by acetic acid) at Bi(III) ion concentration of 1.25 mg mL^{-1} and applied potential of −1.0 V for 120 s. Once the bismuth film was plated, the electrodes were gently rinsed with water and placed in the fresh measurement solution.

DNA measurement conditions

The CdSe QDs remaining on the biosensors were dissolved as Cd^{2+} by dipping the biosensor in 100 μL of 0.1 M HNO_3 solution for 2 h. Then, the HNO_3 solution was transferred into 400 μL 0.1 M HAc–NaAc buffer solution (pH 4.6). The analytical procedure involved a 1 min pretreatment at +0.6 V, 2 min electrodeposition at −1.1 V for two times (10 s stirring was applied between each accumulation period). Subsequently, Cd^{2+} was stripped by scanning from −0.9 to −0.3 V using SWV measurements with 4 mV potential steps, 15 Hz frequency and 25 mV amplitude.

Results and discussion

Optimization of experimental condition

The morphologies of AuNPs are controlled by the rates of nucleation and growth, which are determined by the pH values of $HAuCl_4$ solution. Different pH of gold precursor baths result in different forms of gold complex, and therefore lead to the different reduction rates of gold and different morphologies of final gold nanostructures. The pH values were adjusted to a fixed value with NaOH and HCl solutions and were aged for more than 12 h. The different morphologies of gold nanostructures reflect different gold loading and relative surface area of the modified electrode. Thus, the effect of pH from 1.0 to 6.0 on the biosensor performance was investigated in this work. As shown in Fig. 1A, the maximum current response appeared at pH 2.0. Therefore, pH 2.0 was selected as the pH value of $HAuCl_4$ solution for DNA biosensor fabrication.

The incubation time of CdSe QDs–SA and biotin-labeled probe DNA on the glass carbon electrode was another important parameter in optimizing the current response. It can be seen that the current response was enhanced with increasing incubation time, which then tended to a plateau after 50 min (Fig. 1B); this might be attributed to the fact that biotin-labeled probe DNA on the glass carbon electrode was saturated with CdSe QDs–SA. Thus, 50 min was selected as the optimal incubation time for the DNA biosensor fabrication.

Characterization of the electrodeposited Au nanoparticles

To verify the formation of the AuNPs, an SEM image of AuNPs was recorded and is shown in Fig. 2. As can be seen, the deposited film was homogenous, and the particles were well dispersed and remained relatively stable without aggregation, which was beneficial for immobilizing biomolecules to fabricate the biosensor.

Electrochemical characterization of the biosensor

The fabrication process of the biosensor was monitored by EIS. EIS is an effective method for the surface analysis because it does not damage the biomolecular layers on the electrode. In EIS, the semicircle diameter represents the electron-transfer resistance (R_{et}), which dominates the electron transfer kinetics of the redox probe at the electrode surface; the linear part at lower frequencies dominates the diffusion-limited process.

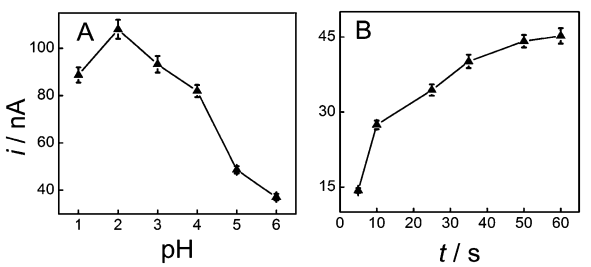

Fig. 1 Effects of the pH of $HAuCl_4$ and incubation time of the biotin-labeled probe DNA with QDs–SA on the electrochemical response.

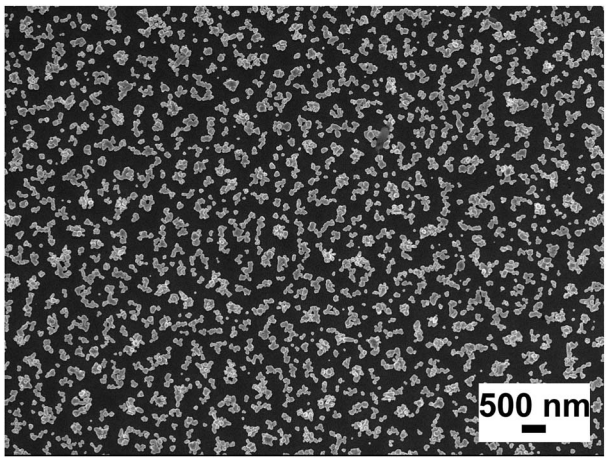

Fig. 2 SEM image of the AuNPs.

Fig. 3 shows the Nyquist plot of the different modified GCEs in 0.1 mol L^{-1} KCl solution containing 5 mmol L^{-1} $K_3Fe(CN)_6$/$K_4Fe(CN)_6$ (1 : 1) at open circuit potential with the frequency varied from 0.1 Hz to 10 0000 Hz. The bare glass carbon electrode showed a small R_{et} of about 68 Ω (curve a), after AuNPs were electrodeposited, a smaller R_{et} value of 56 Ω (curve b) was witnessed. The reason may be that the AuNPs accelerated the interfacial electron transfer between the $[Fe(CN)_6]^{3-}$/$[Fe(CN)_6]^{4-}$ redox probe and the glass carbon electrode surface. The self-assembly of probe DNA and MCH on the AuNPs-modified glass carbon electrode surface made the R_{et} increase to 400 Ω because the negatively charged phosphate backbone of the probe DNA on the modified glass carbon electrode produced an electrostatic repulsion force to $[Fe(CN)_6]^{3-}$/$[Fe(CN)_6]^{4-}$ (curve c). Subsequently, the R_{et} enhanced to 800 Ω (curve d), indicating the successful linkage of CdSe QDs–SA to the biotin-labeled probe DNA through the interaction between biotin and SA. Upon the hybridization of the immobilized probe DNA with the complementary target DNA, the R_{et} further increased to 1000 Ω (curve e), suggesting an efficient DNA hybridization on the modified glass carbon electrode surface. However, R_{et} dramatically decreased to 600 Ω after incubation with *MspI* endonuclease. The reason for this may be inferred as follows: after hybridization with target DNA, a duplex structure was formed on the glass carbon electrode surface. *MspI* endonuclease could identify its specific sequence in the duplex DNA and cleave the duplex DNA fragments linked with CdSe QDs away from the glass carbon electrode surface. The decrease of electron transfer resistance confirmed the successful cleavage of duplex DNA fragments. These results were in good agreement with those obtained from SWV measurements (Fig. 4B). As discussed above, EIS proved that the biosensor was constructed as described in Scheme 1.

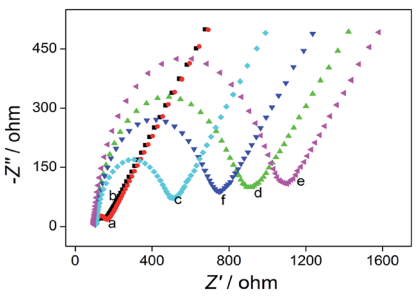

Fig. 3 Nyquist diagrams of impedance spectra obtained at bare GCE (a), AuNPs (b), MCH/probe DNA/AuNPs (c), CdSe QDs–SA/MCH/probe DNA/AuNPs (d), target DNA/CdSe QDs–SA/MCH/probe DNA/AuNPs (e) and *MspI*/target DNA/CdSe QDs–SA/probe DNA/MCH/AuNPs (f) modified GCEs.

Feasibility of the proposed biosensor

Due to the unique benefits, such as easy preparation, good biocompatibility and stability, AuNPs have been frequently used in electrochemical field to enhance the signal. In this work, the amplification of AuNPs was verified by comparing the signal on the gold electrode with that on the AuNPs modified glass carbon electrode. As shown in Fig. 4A, the signal on the AuNPs-modified glass carbon electrode (curve a) was much more improved than the signal on the bare gold electrode (3 mm in diameter) (curve b). Cyclic voltammetry was employed to characterize the gold nanostructure by estimating the real surface area *via* scanning in 0.5 M H_2SO_4. Based on the charge consumed during the formation of the surface oxide monolayer a value of 386 μC cm^{-2} was reported.[22] The electrodeposited Au-modified electrode surface area was 0.17 cm^2, which was larger than the surface area of the gold electrode, leading to an enhanced response.

As shown in Fig. 4B, compared with the signal from the CdSe QDs–SA/MCH/probe DNA/AuNPs modified glass carbon electrode (curve a), the signal from target DNA/CdSe QDs–SA/MCH/probe DNA/AuNPs modified glass carbon electrode (curve b) remained almost unchanged. However, after digestion by *MspI* endonuclease, the electrochemical response of Cd^{2+} obviously declined (curve c), suggesting that *MspI* endonuclease could cleave the dsDNA fragment linked with QDs from the glass carbon electrode surface, and only the dsDNA fragment can be cleaved from the electrode. The remaining single-stranded probe DNA linked with CdSe QDs left the electrode. Therefore, there is a specific SWV peak in the blank. This phenomenon

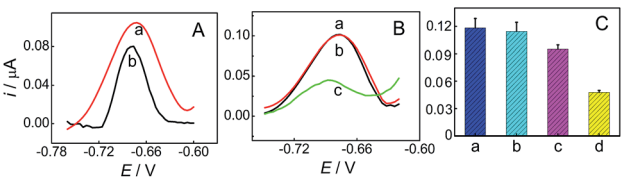

Fig. 4 (A) SWV of target DNA/CdSe QDs–SA/MCH/probe DNA/AuNPs modified GCE (a), target DNA/CdSe QDs–SA/MCH/probe DNA modified gold electrode (b); (B) SWV of CdSe QDs–SA/MCH/probe DNA/AuNPs (a), target DNA/CdSe QDs–SA/MCH/probe DNA/AuNPs (b), *MspI*/target DNA/CdSe QDs–SA/MCH/probe DNA/AuNPs (c) modified GCEs. (C) The response of the biosensor without target DNA (a), with 10^{-10} M M1 DNA (b), 10^{-10} M M2 DNA (c) and 10^{-10} M target DNA (d).

verified that the decrease of the signal was caused by *MspI* endonuclease cutting off CdSe QDs–SA-linked DNA strands rather than the probe DNA hybridizing with target DNA.

Specificity of the biosensor

To use this developed biosensor in clinical diagnosis, it was essential to minimize a false positive response. Therefore, the selectivity of this biosensor was measured (Fig. 4C). The CdSe QDs–SA-labeled probe DNA was hybridized with M1DNA and then treated by *MspI* endonuclease. The electrochemical signal of Cd^{2+} (column b) remained nearly invariant compared with the signal in the absence of target DNA (column a), suggesting that *MspI* endonuclease could not cleave the probe DNA/M1DNA hybrid because the hybrid does not contain the specific recognition sequence of the *MspI* endonuclease. When the M2 DNA was used to hybridize with the probe DNA and then incubated with *MspI* endonuclease, the response declined almost 20% (column c) in comparison with column a. Moreover, when the complementary target DNA was used to hybridize with the probe DNA and then treated by *MspI* endonuclease, the current decreased about 60% (column d) in comparison with column a. These results demonstrated that the designed biosensor could distinguish even single-base mismatched DNA. Therefore, it would be useful for the high-selectivity analysis of DNA.

Biosensing for DNA

The analytical performance of the developed biosensor was evaluated. As seen from in Fig. 5A, a well-defined peak located at −0.68 V for the oxidation of Cd was observed in the SWV, and the peak currents of Cd^{2+} decreased with increasing concentrations of target DNA. There was a linearity between the peak currents, and the negative logarithm of target DNA concentrations was in the range of $1.0 \times 10^{-14} - 1.0 \times 10^{-9}$ M (Fig. 5B), which was wider than $1.0 \times 10^{-16} - 1.0 \times 10^{-15}$ M assisted by nicking endonuclease[23] and $2 \times 10^{-11} - 3 \times 10^{-10}$ M assisted by exonuclease III[24] for signal amplification by electrochemical biosensors. The detection limit in the present work was 8.7×10^{-15} M at signal-to-noise rate of 3, which was lower than 7.8×10^{-10} M using the electrochemical biosensor prepared by a nanostructured chitosan–zirconium-oxide composite[25] and 4.0×10^{-11} M using a personal glucose meter for DNA detection.[26] Thus, the biosensor showed both a wide linear response range and high sensitivity in the determination of *Mtb* DNA.

The reproducibility of the designed biosensor was investigated by detecting 10^{-10} M target DNA using five independent electrodes; the relative standard deviation was 6.2%, indicating satisfactory reproducibility. The biosensor also exhibited satisfactory stability; when the biosensor was stored in 4 °C for 8 days, 92.7% of its initial current response was maintained, suggesting acceptable stability.

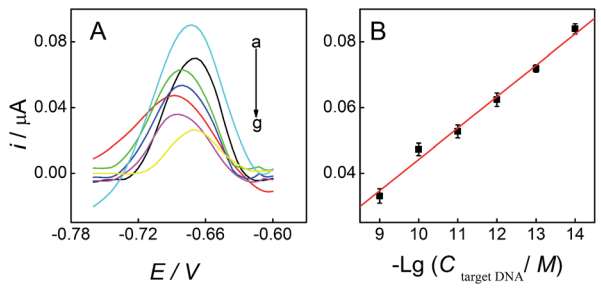

Fig. 5 (A) SWV of the *MspI*/target DNA/CdSe QDs–SA/MCH/probe DNA/AuNPs modified glass carbon electrode in the presence of different concentrations of target DNA (0, 10^{-14} M, 10^{-13} M, 10^{-12} M, 10^{-11} M, 10^{-10} M, 10^{-9} M from top to bottom) in 0.1 M HAc–NaAc buffer solution (pH 4.6). (B) The linear relationship between the currents (*i*) and the negative logarithm of target DNA concentrations.

Conclusions

A universal and sensitive electrochemical biosensing platform based on the specificity of *MspI* endonuclease and signal amplification of AuNPs, as well as using the environmentally friendly Bi film for the detection and identification of DNA, was developed. *MspI* endonuclease recognized its specific sequence in the dsDNA and cleaved the dsDNA fragments linked with CdSe QDs–SA from the glass carbon electrode; the remaining attached signal markers can be easily read out by SWV using the electrodeposited bismuth film-modified glass carbon electrode. Using *Mtb* DNA as a model, an ultrasensitive electrochemical biosensor for detecting *Mtb* DNA is proposed. The designed biosensor exhibited good performance characteristics, such as wide linear response range, high sensitivity, good specificity and satisfactory reproducibility. The Use of *MspI* endonuclease and Bi film for the monitoring and identification of DNA for developing a biosensing platform and constructing a biosensor with extended applications can provide a promising prospect for the early diagnosis of gene-related disease.

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