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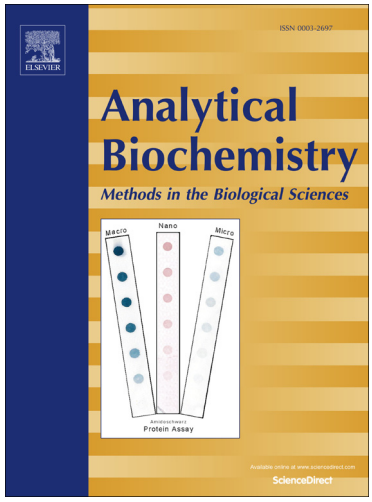

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Ultra-sensitive biosensor for K-ras gene detection using enzyme capped gold nanoparticles conjugates for signal amplification

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Abstract

In this study, an ultra-sensitive hairpin DNA–based electrochemical DNA biosensor for the K-ras gene detection is described. Gold nanoparticles (Au-NPs) and horseradish peroxidase (HRP)-streptavidin capped Au-NPs (HAS) conjugates are used for signal amplification. Initially, hairpin DNA dually labeled with thiolated at its 5' end and with biotin at its 3' end is immobilized on the surface of Au-NPs modified electrode and the hairpin DNA is in "closed" state, hence the HAS conjugates are shielded from being approached by the biotin due to steric hindrance. However, in the presence of target DNA, the target DNA hybridizes with the loop structure of hairpin DNA and causes conformational change, resulting in biotin forced away from the electrode surface, thereby becomes accessible for the HAS conjugates. Thus the HAS conjugates are linked to the electrode surface via the specific interaction between biotin and streptavidin. Electrochemical detection was performed in phosphate buffered saline (PBS) containing tetramethylbenzidine (TMB) and H_2O_2. Under optimal conditions, the peak current differences (ΔI) are linear with the target DNA in the range from 0.1 f M to 1 nM with a detection limit of 0.035 fM. Furthermore, this biosensor also demonstrates its excellent specificity for single base mismatched DNA.

Keywords: Electrochemical biosensor; hairpin DNA; Enzyme; Signal amplification; Gold nanoparticles; Conjugates

Introduction

To our knowledge, carcinoma of the pancreas (PC) is a threatening disease and the death ratio of PC is considered as the fourth all over the world [1]. The K-ras oncogene is one of the three members of the human ras gene family and naturally occurring mutation is located on codon 12, so it is regarded as a 'signature' of PC [2]. Thus, the detection of K-ras mutation will provide an early diagnosis and monitor of the disease. Therefore, it is necessary to develop a sensitive method for the detection of K-ras gene.

As is well known, electrochemical DNA biosensors have been considered as a sensitive method for specific gene detection due to high sensitivity, selectivity, fast response [3], [4], and [5]. Up to date, various approaches have been designed to construct highly sensitive electrochemical DNA biosensor. Among the strategies, signal amplification strategies have played a key role in the sensitive determination of DNA [6], [7], [8], [9], [10] and [11]. From the previous reports, signal amplification from nanomaterials including Au-NPs, carbon nanotubes and graphene have been reported [12], [13], [14] and [15]; Enzyme amplification strategy is often utilized owing to its prominent biocompatible ability and supercatalytic property [16], [17], and [18]. For instance, Wang and co-workers have reported a sensitive DNA detection method based on carbon nanotube and enzymes amplify detectable signal [19]; Chen and co-workers developed an hairpin LNA probe based on enzyme-amplified biosensor for the detection of PML–RAR fusion gene [20]; Fan and co-workers reported a stem-loop probe dually labeled with biotin and digoxigenin to bind HRP linked-anti-dig oxigenin antibody for enzymatically amplifying the electrochemical signal [21]. Yu and co-workers have built an enzyme-based electrochemical DNA sensor, which exhibits excellent discrimination ability for single mismatches [22]. These high sensitive biosensors obtained mainly benefit from enzyme catalyze ability. From the previous reports, what's noteworthy is that the immobilization amount of enzyme in sensing system is limited. We wondered if larger amount of enzyme could be introduced in the sensing system, is it possible for the sensitivity of sensor further enhanced?

Based on consideration above, we designed a highly sensitive electrochemical biosensor based on hairpin DNA and enzymatic amplification protocol. As is well known, molecular beacon, which is composed of a hairpin-like DNA stem-loop structure, has been widely used for specific

DNA detection because of it has excellent specificity for detecting nucleic acid targets. Previously, we fabricated molecular beacon based impedance biosensor for the sequence-specific detection of DNA [23]. The biosensor exhibits high sensitivity. However, the impedance biosensor is limited in real analysis due to time-consuming and low reproducibility. In this study, Au-NPs are deposited on the electrode surface for improving immobilization amount of hairpin DNA probe; the HRP-streptavidin capped Au-NPs (HAS) conjugates are used for signal amplification. In absence of the target DNA, hairpin probe is in the "closed" state, which shields biotin from being approached by HAS conjugates. In the presence of target DNA, the target DNA hybridized with the probe and causes significant conformational change from stem-loop structure to linear chain structure. Therefore, the biotin is forced away from the surface of the electrode and becomes accessible to the HAS conjugates, thus the HAS conjugates are captured on the electrode surface firmly through the specific interaction between biotin and streptavidin. By virtue of large amount of HRP capped on the HAS introduced in this sensing system, the detect signal is clearly amplified, resulting in sensitivity of the biosensor improved evidently. Under optimal conditions, the biosensor fabricated is able to detect 0.035 fM target DNA. More importantly, the DNA biosensor has been applied in human serum samples for complementary target DNA analysis and the results is satisfactory, which provide the possibility to detect K-ras gene in serum samples.

Materials and methods

Reagents

Chloroauric acid ($HAuCl_4 \cdot 4H_2O$), PEG (Poly (ethylene glycol) 20000 were obtained from Shanghai Chemical Reagent Co. Ltd (Shanghai, China,). Bovine serum albumin (BSA) was purchased from the Sinopharm Chemical Reagent Co., Ltd (Shanghai, China,). 3,3′;5,5′-tetramethylbenzidine (TMB) was purchased from Aladdin Ltd. (Shanghai, China,). Streptavidin, HRP conjugated and various oligonucleotides sequences used in this study were purchased from Sangon Inc. (Shanghai, China). Their base sequences are shown as follows:

Probe sequence: 5'-SH-$(CH_2)_6$-GAA-TTC-TAC-GCC-ACC-AGC-TCC-AGA-ATT-C -Biotin-3'

Complementary sequence: 5'-TGG-AGC-TGG-TGG-CGT-A -3'

Single-base mismatch sequence (□): 5'- TGG-AGC-TGA-TGG-CGT-A-3'

Single-base mismatch sequence (□): 5'-TGG-AGC-TGT-TGG-CGT-A -3'

Non-complementary sequence: 5'-GCT-GCA-GAT-ACA-ATG-C-3'

The single-base mismatch sequences are the mutation types whose one base is different from K-ras gene on codon 12. All oligonucleotides stock solutions were prepared with 0.01 M phosphate buffered saline (PBS, pH 7.40) and were stored in a refrigerator. The buffers employed in this study were as follows: hybridization buffer solution (0.01 M PBS + 0.1 M NaCl, pH 7.40) and electrochemical test solution (0.1 M PBS, pH 7.40). All chemicals were of analytical grade and used without further purification. All solutions were prepared with twice-quartz-distilled water.

Apparatus

Electrochemical measurements were performed on a CHI 650C electrochemical analyzer (CH Instruments, Inc., China). The three-electrode system consisted of gold electrode or modified electrode as working electrode, a platinum wire as auxiliary electrode, and a saturated calomel electrode (SCE) as reference electrode. The morphology of electrode surface were investigated by scanning electron microscopy (SEM) using a JEOLJSM-6700F microscope (Hitachi, Japan).

Electrochemical impedance spectra (EIS) was obtained in 0.10 M PBS containing 5.0 mM $[Fe(CN)_6]^{3-/4-}$ (1:1) at a pH of 7.40. The frequency ranged from 0.1 to 100 kHz at a formal potential of 0.18 V, and the amplitude of the alternate voltage was 5 mV.

All measurements were performed at room temperature in a 10.0 mL electrolytic cell with 5.0 mL solutions, from which oxygen was removed by purging with high-purity nitrogen for 10 min, and a blanket of nitrogen was maintained over the solution during the measurements.

Preparation of the HAS conjugates

Au-NPs was prepared by reduction of $HAuCl_4 \cdot 4H_2O$ with trisodium citrate according to previous literature [24]. Briefly, a 100.0 mL sample of aqueous $HAuCl_4$ (0.01%) was brought to

the boil, and then 4.0 mL of 1% trisodium citrate was added rapidly with vigorous stirring. The mixed solution was kept boiling for 15 min until the solution became wine red and continued heated for 10 min. Then, the solution was cooled to room temperature while stirring. The obtained colloidal gold solution was placed into clean bottles and stored at 4 °C for further used.

HAS conjugates were prepared according to the literature [25] with minor modification. Firstly, 1.0 mL Au-NPs solution (pH 8.2) was centrifuged at 10000 rpm for 25 min and the supernatant was removed. Next, 10.0 μL Streptavidin–HRP (1.0 mg/mL) was injected into the Au-NPs solution (The final volume was adjusted to 100.0 μL) and was stirred for 30 min and stood for 2 h. Secondly, the Au-NPs bioconjugate (stabilized with 0.5 % PEG20000) was then purified by centrifugation at 9000rpm and washes 3-4 times with 10.0 mM PBS buffer (pH 7.2, 0.5% BSA, 2.5% sucrose and 0.1 % PEG20000). Lastly, the HAS conjugate obtained was re-dispersed and was stored at 4°C for further application.

Fabrication of electrochemical DNA biosensor

Prior to modification, the bare gold electrode (2.0 mm in diameter) was polished to a mirror-like surface with 1.0, 0.25 and 0.05 μm gamma alumina suspensions and then sequentially cleaned ultrasonically in 95% ethanol and twice distilled water for 5 min. Next, it was allowed to dry with a N_2 stream for further use.

Au-NPs were electrodeposited on the surface of gold electrode at −0.2 V for 300 s by amperometry in 5.0 mM $HAuCl_4$ solution containing 0.1 M KNO_3. The obtained electrode was denoted as Au-NPs/Au.

Subsequently, five microliters of 1.0×10^{-5} M probe was dropped on the surface of the Au-NPs modified electrode and kept it for 12 h in a refrigerator. Thus, the single strand DNA(ssDNA)/Au-NPs/Au electrode was achieved.

Electochemical detection of target DNA

Hybridization event was performed by placing the ssDNA/Au-NPs/Au electrode into hybridization buffer solution containing various concentrations of target DNA for 2 h at 40 °C. After that, it was transferred to a beaker containing 1% BSA solution for 1 h at 37 °C to prevent

any possibile non-specific adsorption, followed by washing with 0.1% Tween to remove the excess blocking reagent. Finally, five microliters HAS conjugates solution was dropped on the modified electrode surface (double strand DNA (dsDNA) /Au-NPs/Au) above and was incubated for 60 min at 4 °C in the humidified chamber. After being rinsed with PBS solution, the biosenor was then placed in the pH 7.5 PBS containing 0.01 M TMB and 0.02 M H_2O_2, and the electochemical detection started 20 seconds later. The reduction peak current in Differential Pulse Voltammetry (DPV) at the potential of about −0.212 V was recorded and the concentration of target DNA was quantified as the relationship between the peak current difference ($\Delta I = I_{dsDNA} - I_{ssDNA}$) and the concentration of complementary DNA. Here, $I_{ss\text{-}DNA}$ and $I_{ds\text{-}DNA}$ denote the reduction peak current before and after the probe electrode hybridized with the complementary DNA, respectively. DPV parameters were as follows: potential range: 0.60V−0.20 V; amplitude: 0.05 V; pulse width: 0.05 s; sample width: 0.0167 s，pulse period: 0.2 s and quiet time: 2 s.

Results and discussion

Design strategy of the biosensor

The fabrication procedure of DNA biosensor is shown in Fig. 1. After the modification of gold electrode, the hairpin probe labeled with thiolated at 5' end and biotin at 3' end is immobilized on the electrode via Au-S binding. Initially, the hairpin probe is in “closed” state in absence of complementary target, and inhibits HAS conjugates accessing to electrode. In the presence of complementary target, the stem-loop structure is broken and turns into rigid linear structure, resulting in the biotin exposed to the HAS conjugates. Hence the HAS is fixed on the electrode surface firmly via the specific interaction between biotin and streptavidin. Therefore, the larger numbers of HRP are introduced on electrode surface, leading to signal amplification significantly.

Fig. 1

The investigation of the biosensor assembled process

SEM and CV techniques were employed to investigate of the assembled process of the

biosensor. Fig. 2A and 2B showed the surface morphologies of bare electrode and Au-NPs modified electrode, respectively. The obvious difference could be readily oberverd. After the electrode electrodeposited at −0.2 V for 300 s in 0.1 M KNO_3 solution containing 5.0 mM $HAuCl_4$, the spherical Au-NPs was observed clearly and surfaces looks like multi-layers of Au-NPs structure, which is beneficial to improve the immobilized amounts of hairpin probe on the electrode surface.

In this study, we employed CV technique to evaluate the relative active area of electrode surface, The effective surface areas of electrodes could be obtained from the coulombic integration of the reductive waves of gold oxide [26]，Fig. 2C showed CVs of bare and Au-NPs modified electrode in 0.5 M H_2SO_4 solution. It was clearly to observe that the peak current at Au-NPs modified electrode was much higher than that at bare Au electrode, which revealed the Au-NPs increase the effective surface area of electrode significantly.

Fig. 2

EIS characterization of the fabricated biosensor

EIS was used to investigate assembled process of the biosensor and the results were shown in Fig 3. Generally, the impedance spectra include a semicircle and a linear portion. The semicircle portion at higher frequencies corresponds to the electron-transfer-limited process and the electron transfer resistance (R_{et}) equals the semicircle diameters. In contrast to bare gold electrode (curve a), the R_{et} of Au-NPs/Au electrode decreased obviously (curve b), indicating that Au-NPs facilitated the electron transfer of $[Fe(CN)_6]^{3-/4-}$. When probe DNA was immobilized on electrode surface, the R_{et} was increased clearly (curve c). The increasement of R_{et} was ascribed to the electrostatic repel interaction from negative charges DNA and $[Fe(CN)_6]^{3-/4-}$ block the electron transfer of $[Fe(CN)_6]^{3-/4-}$. By the same token, the R_{et} were further increased after the probe DNA hybridized with the target DNA (curve d). When the hybridized electrode was incubated with HAS conjugates, a larger semicircle (R_{et}) was observed (curve e), indicating the protein layer of HAS hinder interfacial electron transfer. These results were identical to that we expected.

Fig. 3

Signal amplification of HAS conjugates

For investigating the signal amplification from the HAS conjugates, a sets of contrast test from the ssDNA/Au-NPs/Au and dsDNA/Au-NPs/Au electrodes were designed and the results obtained are shown in Fig 4. It could be observed that a significantly increased signal was obtained at the HAS/dsDNA/Au-NPs/Au (curve c) in contrast to the HAS/ssDNA/Au-NPs/Au (curve a), implying the HAS conjugates have been linked to the surface of hybridized electrode (dsDNA/Au-NPs/Au). It was meaningful that when the dsDNA/Au-NPs/Au electrode is incubated with HRP solution and HAS solution under the same condition respectively, the signal value of HRP/dsDNA/Au-NPs/Au (curve b) is less than half of that of HAS/dsDNA/Au-NPs/Au, indicating the prominent amplification ability is from the HAS conjugates.

Fig. 4

Optimization of the experiment conditions

Optimization of the experiment conditions are closely related to performance of sensing. In this study, various parameters (Such as pH, incubation time) were optimized in the presence of 1.0×10^{-12} M complementary sequence. Fig 5a shows the influence of pH on response signal. It could be observed that the signal are linearly increased as the pH increase from 5.5 to 7.5 and linearly decreased over 7.5, indicating that the catalytic ability of HRP in HAS conjugates is highest at the point of pH 7.5. Fig 5b shows the relationship between signal and amount of HAS conjugates, it was observed that the signal increased significantly with the volumes of HAS conjugates increased from 2.0 to 5.0 μL and keep a constant over 5.0 μL. It is that the amount of HAS reached saturation on the electrode surface when the HAS is 5.0 μL. Fig 5c shows the influence of different incubation time on response signal. It could be observed that the signal is linearly increased as the incubation time increased from 20 to 70 min, and kept a constant from then on. So, 60 min is selected in this experiment.

Fig. 5

Selectivity of the DNA biosensor

In this study, we also investigated the selectivity of the DNA biosensor using various DNA sequences including single-base mismatched, three-base mismatched and complementary sequences, and the results are shown with Normalized histogram in Fig. 6. It could be observed that the complementary sequence obtained the highest electrochemical response, the single-base mismatched sequences obtain almost a quarter of signal of the complementary sequence and the three-base mismatched sequence obtained the negligible response signal that almost equal to the background signal, indicating this biosensor has good selectivity.

Fig. 6

Analytical performance

In this study, we selected the changes of peak current value (ΔI) as the analytical signal to investigate the relationship between the analytical signal and concentrations of the complementary target DNA under optimal conditions. Fig. 7A shows the DPV response of the biosensor in different concentrations of target DNA. It could be clearly observed that the signal increases with the increasing of concentration of target DNA and was linearly related to the logarithm of the concentration of the target DNA in the range of 0.1 fM to 1 nM (Seen Fig.7B). The regression equation can be expressed as ΔI (μA) =8.8481+0.4182 log C (unit of C is M) (the regression coefficient is 0.9963) and the detection limit is 0.035 fM (S/N=3). We compared this work with some electrochemical DNA biosensor previously reported, and this biosensor exhibits wider linear range and lower limit of detection (seen Table 1). The results demonstrated that biosensor could be used for DNA detection with good sensitivity.

Fig. 7

Table 1

Reproducibility and stability of the biosensor

Reproducibility of the DNA biosensor was also investigated. In this study, five different biosensors were independently fabricated under the same experimental conditions and were used

to detect 1.0×10^{-11} M complementary DNA. The signals obtained are 4.664, 4.65, 4.628, 4.61, 4.588 μA, respectively. The relative standard deviation (R.S.D.) was 0.55 %, indicating the DNA biosensor had good reproducibility. The stability of the biosensor was recorded the response value of five biosensors above-mentioned everyday during a week and results obtained are shown in Fig.S1. These datas obtained during a week showed no obviously changes (a ~ e), which exhibited the excellent stability of the biosensor.

Real samples assay

In this study, we selecte serum samples from a normal people (it is provided by Yiji Shan Hospital of Wuhu, Anhui） for this analysis. The serum is treated with 4 % sodium citrate and centrifuged for 5 min at 10,000 rpm. Then, 100 μL treated blood serum from the supernate is injected into 900 μL hybridization buffer solution containing 10 μL 1.0×10^{-10} M or 1.0×10^{-11} M target DNA. The detection is performed according to the section of 'Electrochemical measurements' and the recovery obtained are within 90.67 % to 92.81 % (seen in Table S1). This datum showed that the biosensor could be applied to the determination of the K-ras gene in serum samples.

Conclusions

In the assay, we developed a simple and reliable biosensor for DNA detection with high sensitivity and specificity. The biosensor fabricated applies to a short single-stranded oligonucleotide model target, and that application to double-stranded genomic DNA has not be demonstrated. However the recovery investigation in serum sample provides the possibility to qualitatively and quantitatively detect K-ras gene in pancreatic cancer.

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Caption to figures

Fig.1. Schematic representation of the fabrication procedure of DNA biosensor

Fig.2. SEM images of (A) the bare Au electrode, (B) the Au-NPs/Au electrode obtained by electrodepositing at the potential of 200 mV (vs. Ag/AgCl) in 5mM $HAuCl_4$ for 300 s, (C) Cyclic voltammograms of (a) the bare Au electrode, (b) the Au-NPs/Au electrode in 0.5M H_2SO_4 solution.

Fig.3. Nyquist plots of the biosensor at the different assembled stages in the presence of 5.0 mM $[Fe(CN)_6]^{3-/4-}$ at the pH of 7.40. Bare Au (a), Au-NPs/Au (b), single strand DNA (ssDNA)/Au-NPs/Au (c), double strand DNA (dsDNA)/Au-NPs/Au (d) and horseradish peroxidase (HRP)- streptavidin capped Au-NPs conjugates (HAS) /dsDNA/ Au-NPs/Au (e).

Fig.4. The contrast test of investigation of HAS amplification ability are measured in PBS solution containing 0.02 M H_2O_2 and 0.01M 3,3′;5,5′-tetramethylbenzidine (TMB). (a) HAS/ssDNA/Au-NPs/Au (b) HRP/dsDNA/Au-NPs/Au (c) HAS/dsDNA/Au-NPs/Au.

Fig.5. Optimization of experiment conditions in the presence of 1.0×10^{-12} M complementary sequence: (A) The pH of detection solution (B) The plot of the ΔI *vs.* the volumn of HAS conjugates (C) The plot of the ΔI *vs.* different incubation time of HAS conjugates.

Fig.6. Normalized histograms of the current changes of the biosensor in the presence of different targets: complementary sequence (T1); single-base mismatched sequence (T2 (Ⅰ) (Ⅱ)); three-base mismatched sequence (T3); non-complementary sequence (T4).

Fig.7. (A) DPVs of the biosensor in the presence of various target DNA concentrations in PBS solution containing 0.01 M TMB and 0.02 M H_2O_2: 1.0×10^{-16} M (a); 1.0×10^{-15} M (b); 1.0×10^{-14} M (c); 1.0×10^{-13} M (d); 1.0×10^{-12} M (e); 1.0×10^{-11} M (f); 1.0×10^{-10} M (g); 1.0×10^{-9} M (h).

(B) Logarithmic plot for the ΔI *vs*. the concentration of the target DNA.

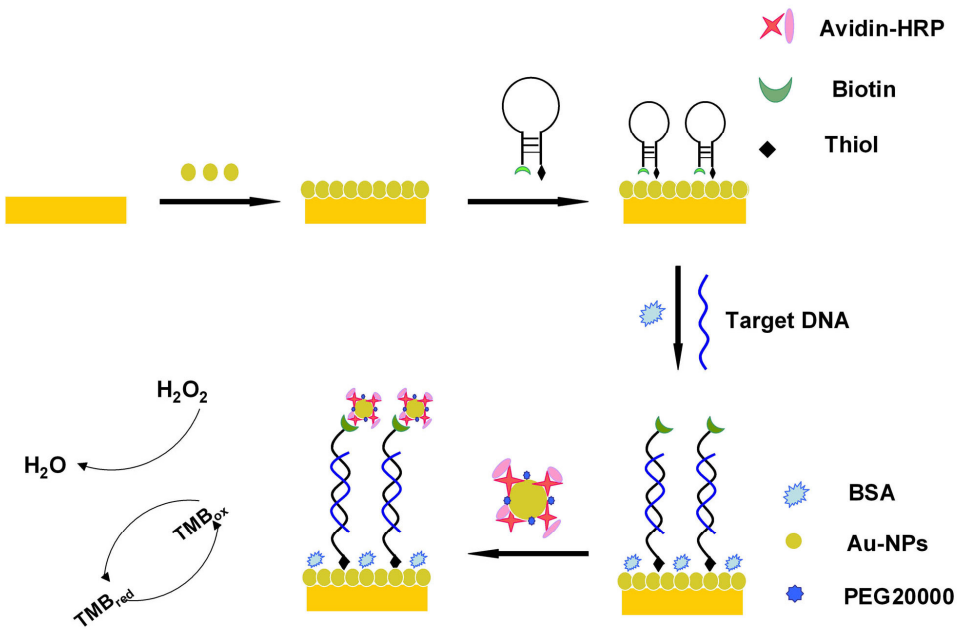

Fig.1

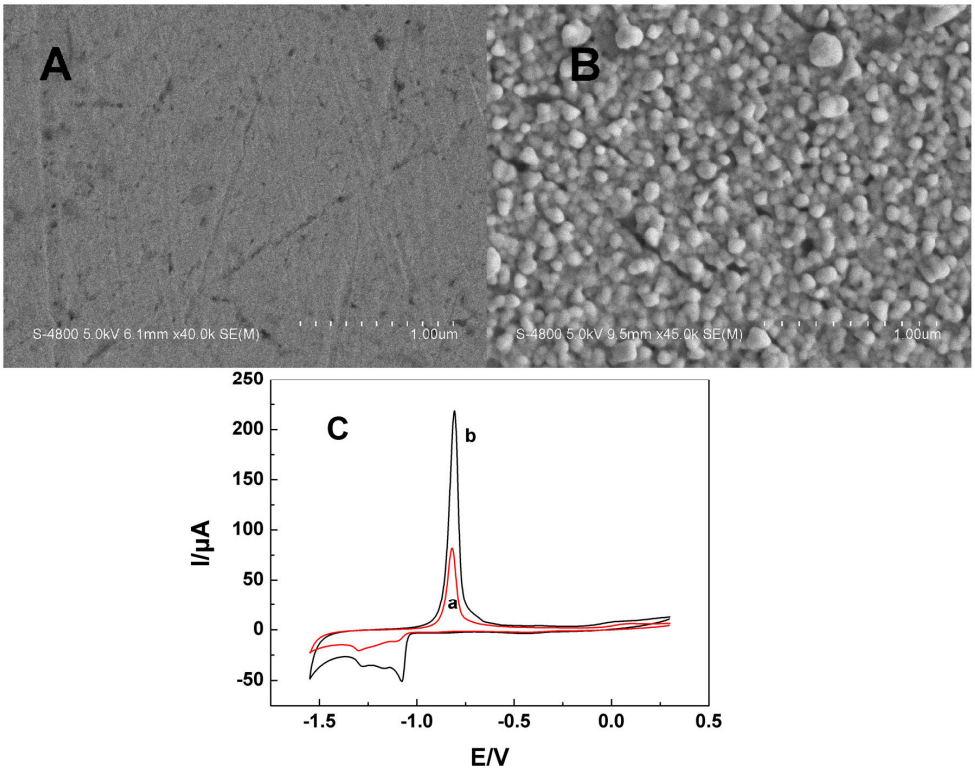

Fig. 2

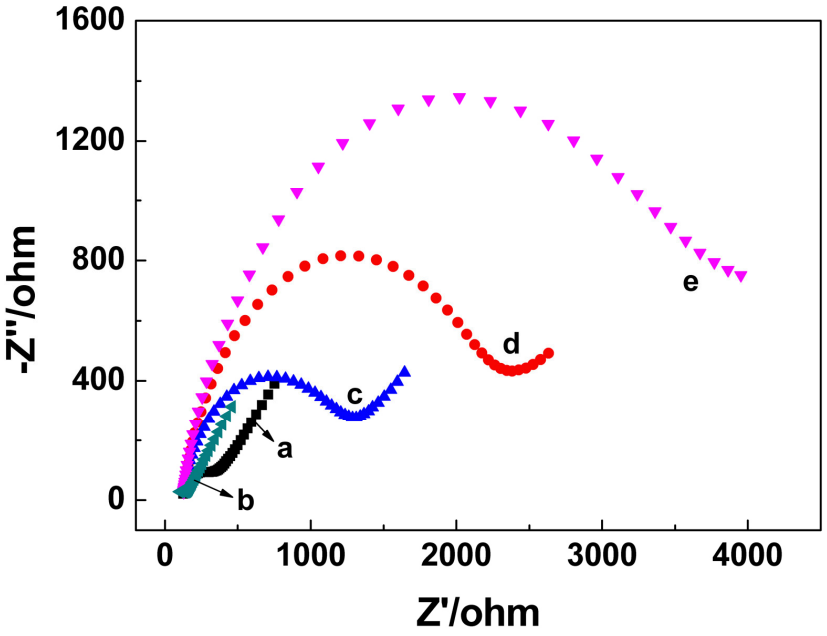

Fig.3

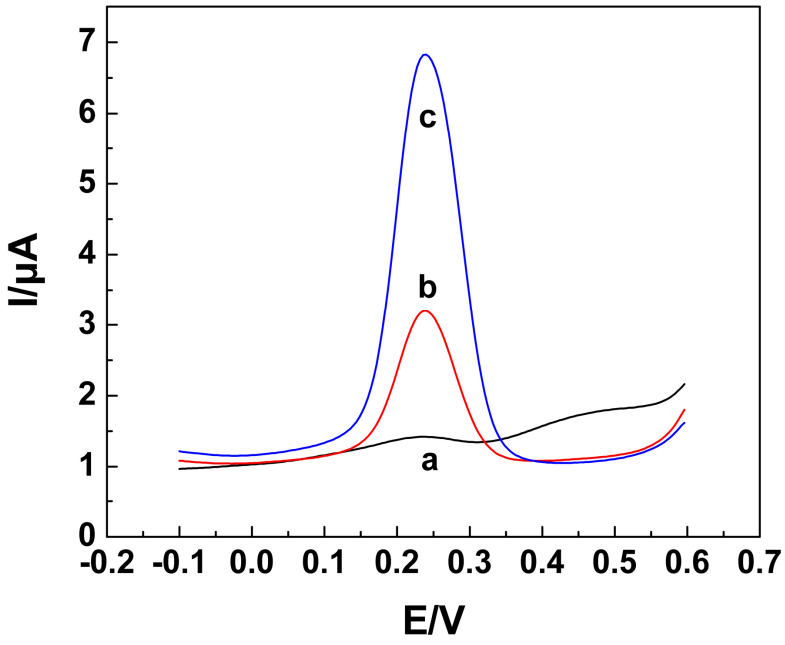

Fig. 4

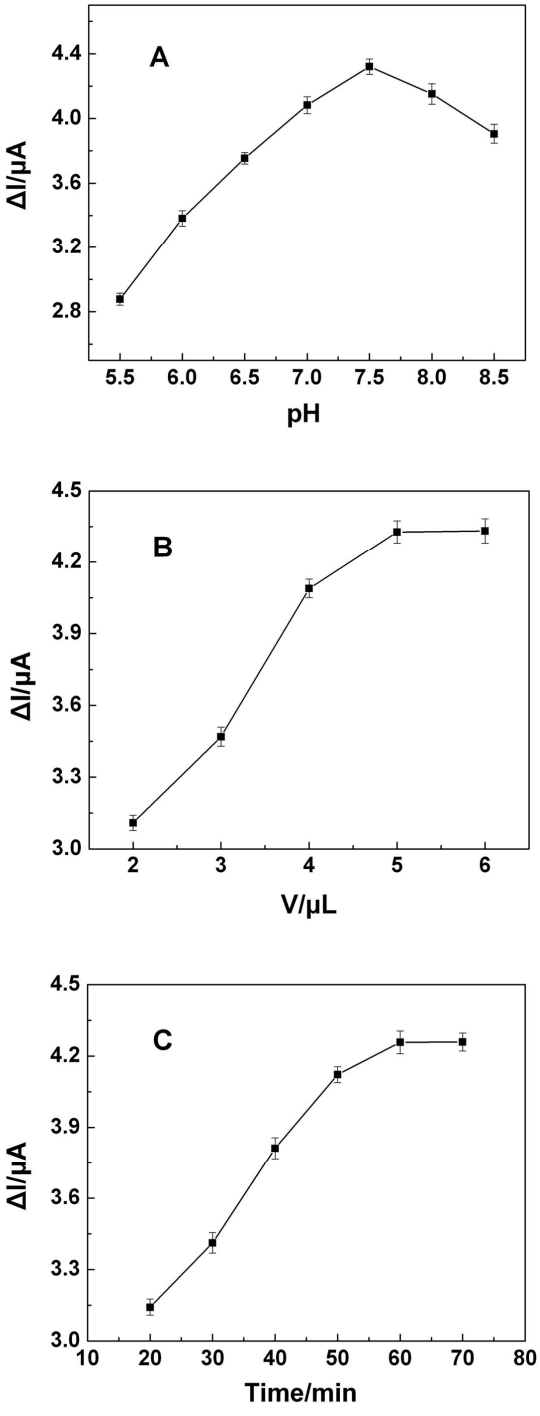

Fig.5

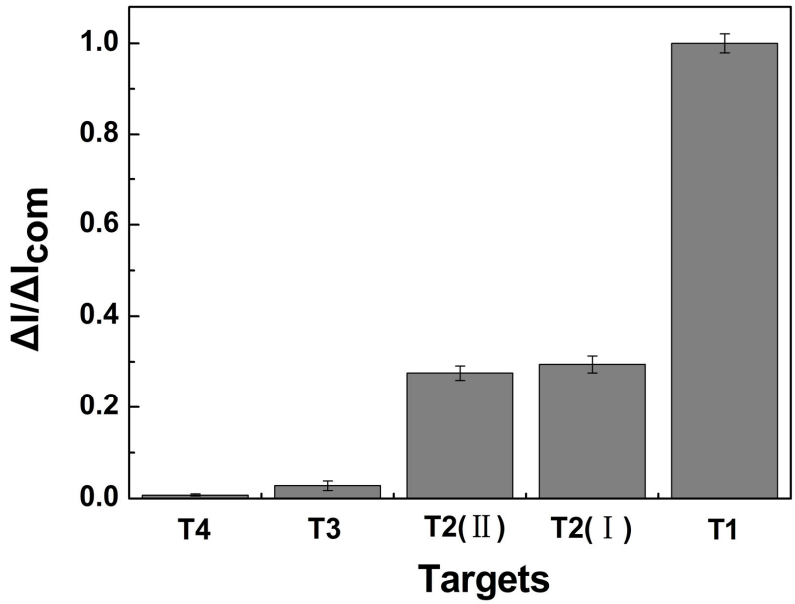

Fig.6

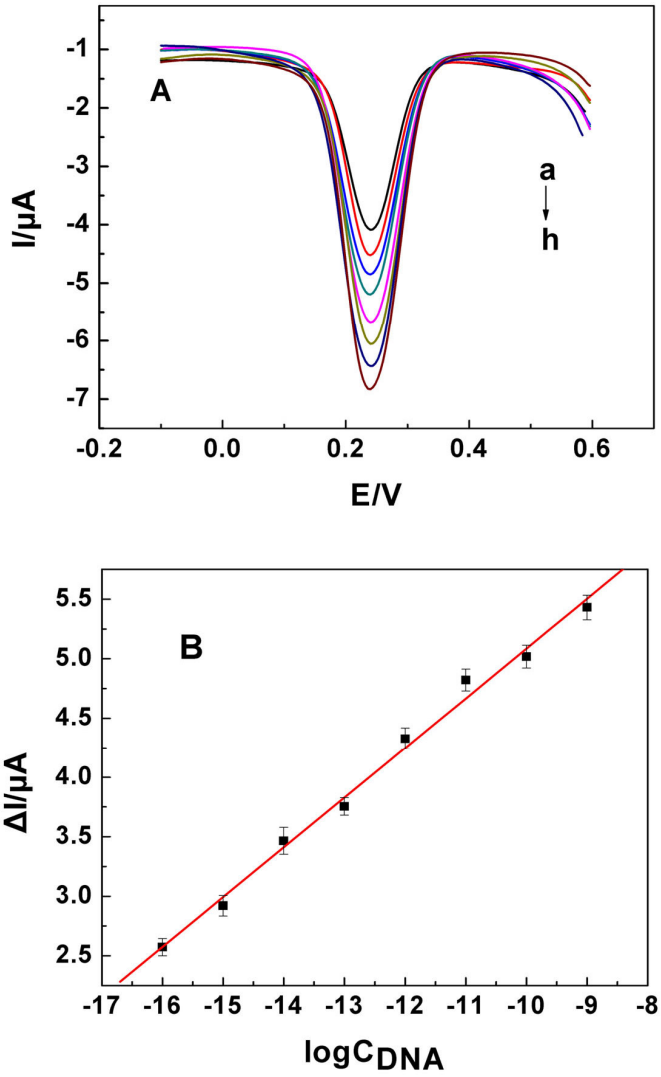

Fig.7.

Probe DNA	Signal amplification	Detection method	Linear range(M)	Detection limit(M)	Single-nucleotide polymorphism(SNP) differentiation	References
Linear DNA	yes	CC[1]	5.0×10^{-13}—1.0×10^{-11}	1.0×10^{-14}	not reported	[27]
Linear DNA	no	DPV	1.0×10^{-13}—1.0×10^{-8}	3.5×10^{-14}	good	[9]
Hairpin DNA	no	CV[2]	1.0×10^{-11}—1.0×10^{-5}	1.0×10^{-11}	not reported	[28]
Hairpin DNA	yes	CA[3]	1.0×10^{-9}—1.0×10^{-11}	6×10^{-12}	good	[7]
Hairpin DNA	yes	EIS[4]	1.0×10^{-15}—1.0×10^{-7}	3.5×10^{-16}	good	[23]
Hairpin DNA	yes	DPV	1.0×10^{-16}—1.0×10^{-9}	3.5×10^{-17}	good	This work

1: Chronocoulometry; 2: Cyclic voltammetry;

3: Chronoamperometry; 4: Electrochemical impedance spectroscopy.

Table 1 .Comparison of the different electrochemical DNA biosensors