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PII: S0168-1656(15)30121-8
DOI: http://dx.doi.org/doi:10.1016/j.jbiotec.2015.09.015
Reference: BIOTEC 7242

To appear in: *Journal of Biotechnology*

Received date: 4-6-2015
Revised date: 7-9-2015
Accepted date: 15-9-2015

Please cite this article as: Pan, Hong-zhi, Yu, Hong-wei, Wang, Na, Zhang, Ze, Wan, Guang-cai, Liu, Hao, Guan, Xue, Chang, Dong, Electrochemical DNA biosensor based on a glassy carbon electrode modified with gold nanoparticles and graphene for sensitive determination of Klebsiella pneumoniae carbapenemase.Journal of Biotechnology http://dx.doi.org/10.1016/j.jbiotec.2015.09.015

Title:

Electrochemical DNA biosensor based on a glassy carbon electrode modified with gold nanoparticles and graphene for sensitive determination of *Klebsiella pneumoniae* carbapenemase

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Sources of support: This study was supported by grant from National Natural Science Fund of China 81273129.

Highlights:

1. We fabricate a electrochemical DNA biosensor based on a glassy carbon electrode modified with gold nanoparticles and graphene.
2. The highly sensitive electrochemical DNA biosensor could successfully determination of Klebsiella Pneumoniae Carbapenemase.
3. This electrochemical DNA biosensor showed high sensitivity, excellent selectivity and good stability with a very low detection limit.
4. It might has a promising future for detection of Klebsiella Pneumoniae Carbapenemase. and solve the actual problem of early diagnosis in clinic.

Electrochemical DNA biosensor based on a glassy carbon electrode modified with gold nanoparticles and graphene for sensitive determination of Klebsiella pneumoniae carbapenemase

【Abstract】 We describe the fabrication of a sensitive electrochemical DNA biosensor for determination of Klebsiella Pneumoniae Carbapenemase (KPC). The highly sensitive and selective electrochemical biosensor for DNA detection was constructed based on a glassy carbon electrode (GCE) modified with gold nanoparticles (Au-NPs) and graphene (Gr). Then Au-NPs/Gr/GCE was characterized by scanning electro miroscope (SEM), cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). The hybridization detection was measured by diffierential pulse voltammetry (DPV) using methylene blue (MB) as the hybridization indicator. The dynamic range of detection of the sensor for the target DNA sequences was from 1×10^{-12} to 1×10^{-7} mol/L, with a detection limit of 2×10^{-13} mol/L. The DNA biosensor had excellent specificity for distinguishing complementary DNA sequence in the presence of non-complementary and mismatched DNA sequence. The results demonstrated that the Au-NPs/Gr nanocomposite was a promising substrate for the development of high-performance electrocatalysts for determination of KPC.

【Key words】Graphene; Gold Nanoparticles; Electrochemical DNA Biosensor;

Klebsiella Pneumoniae Carbapenemase; Determination;

Introduction

Over the past 30 years, *Klebsiella pneumoniae* (Kp) has been repeatedly identified as the most common enterobacterial species to spread extended-spectrum β-lactamase (ESBL) genes in healthcare facilities. However, as carbapenem use has become more frequent carbapenem-resistent bacteria have been identified with increasing frequency in recent years, *Klebsiella pneumoniae* carbapenemase (KPC), produced most commonly by Kp, is currently the most prevalent carbapenemase found in the clinical laboratory[1]. since KPC was first found in America[2], KPC disseminated rapidly around the world and Carbapenem resistance is becoming a major threat[3-5], the detection of KPC should be taken seriously in clinic laboratory. Routine susceptibility tests may be unreliable, Various culture-based methods(modified Hodge test and susceptibility testing), these methods are often time-consuming and yield results only after additional cultivation for 24 or even 48 h[6]. Automated susceptibility testing systems may fail to detect KPC-mediated resistance in clinical isolates[7], molecular diagnostic techniques such as real-time PCR and Triplex PCR have been shown to be sensitive and accurate methods for identifying the presence of carbapenemase genes[8,9] and these methods are relatively complex and require specialized, expensive instruments. This study is aimed to detect *Klebsiella. pneumoniae* carbapenemase and provide the basis for the rational use of antibiotic in clinic.

DNA biosensors are important in diagnostic tests for mutation and early cancer detection, analysis of gene sequences,forensic investigation, and assessment of medical treatment[10-11]. To date, a wide variety of DNA biosensors based on different detection techniques has been developed. Among these techniques, electrochemical DNA biosensors are particularly attractive because of their low cost, high sensitivity and miniaturization[12].

Graphene (Gr) has stimulated intense research interest because of its unique physical and chemical properties, such as high surface area, high electrical conductivity, good chemical stability, and strong mechanical strength[13]. Gold nanoparticles (Au-NPs) have good conductivity and high electrochemical catalytic activity[14]. It has been reported that the integration of carbon-based materials and metal nanoparticles usually shows synergistic effects in electrocatalytic applications[15], so there is a reason to expect the integration of Gr and Au-NPs will obtain the similar effect on the electrooxidation of KPC. We proposed an improved an electrochemical DNA

biosensor, Au-NPs and Gr modified glassy carbon electrode, MB was used as the hybridization indicator, which can be applied to the detection of KPC. The electrochemical signal was detected by DPV and showed good linear relationship with complementary DNA sequence.

1. Experimental

1.1Apparatus

The cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) were performed with a model CHI660A electrochemical workstation (Shanghai Chenhua Instruments, Shanghai, China). Electrochemical measurements were performed using a three-electrode system consist of an Ag/Agcl reference electrode, a platinum wire auxiliary electrode and a modified GCE (3mm in diameter), (All from Incole Union Technology Co. Ltd, Tianjin, China). Scanning electron microscope (SEM) images were obtained using Hitachi Fe-SEM S4800. Ultrasonic cleaning machine (from Kunshang. Co. Ltd). Solution PH was measured on pH meter (from Leici, Shanghai, China).

1.2 Reagents

Grephene was purchased from JCNANO Co.(Nanjing, China). Methylene blue (MB) and $HAucl_4 \cdot 4H_2O$ were purchased from Sigma (USA). Potassium ferricyanide and potassium ferrocyanide were purchased from Fuchen Chemical Reagent Co. (Tianjin, China). The buffers involved in this work are as follows: 0.1mol/L phosphate buffer (PH 7.0). 0.5% sodium dodecyl sulfate (SDS) solution. TE buffer solution contained 10mmol/L Tris-HCl and 1mmol/L $EDTANa_2$ (PH 8.0). Saline-sodium citrate buffer (2×SSC) contained 300mmol/L NaCl and 30mmol/L $Na_3C_6H_5O_7$ which were purchased from GuangFu Technology Co.Ltd (Tianjin, China). All chemicals were analytical grade. Deionized water purified with Millipore system (Millipore Ltd. China) was used throughout this study.

Oligonucleotide sequence (ssDNA): SH-$(CH_2)_6$-5'-TCT GGA CCG CTG GGA GCT GG-3' was used as probe;

Complementary sequence: 5'-CCA GCT CCC AGC GGT CCA GA-3'

One-base mismatched sequence: 5'-CCA GCT CCC ATC GGT CCA GA-3'. Three-base mismatched sequence: 5'-CTA GCT CCC ATC GGT CCA TA-3'. Non-complementary sequence: 5'-TTC ATG TTT CAT AAG TTG AG-3'.

All oligonucleotides used in this work were purchased from Sangon Biotech Co. Ltd.

(Shanghai, China) and preserved in -20ºC dry powder, centrifuged at 3500rpm for 10min before used. Stock solution of probe DNA were prepared in TE buffer solution, other oligonucleotides were prepared in 2×SSC buffer solution.

2. Methods

2.1 Preparation of the GCE

Prior to modification, the bare GCE was polished to a mirror-like surface with 0.05 μm and 1μm alumina slurry on micro-cloth pads. The electrode was then rinsed thoroughly with ultrapure water and cleaned ultrasonically sequentially in ultrapure water, absolute ethanol and acetone for 5 min. Finally the bare electrodes were scanned in 0.2 mol/L KNO_3 and 1mmol/L $K_3Fe(CN)_6$ between −0.1 to 0.6 V at a scan rate of 50 mV/s until a reproducible potential difference less than 70 mV. All electrodes were washed with ultrapure water and dried in the nitrogen atmosphere for further using.

2.2 Preparation of Au-NPs and Au-NPs-Gr modified GCE

For electrode preparation, 2 mg Gr was dispersed in 2.0 mL N,N-Dimethylformamide using an ultrasonic bath for 30 min to give a black suspension. Then 10 μL of the suspension was placed on the GCE surface by micropipette and left to dry at room temperature to obtain Gr/GCE. Subsequently the pretreated Gr/GCE was dipped in 0.05mol/L H_2SO_4 mixed with 5×10^{-3} mol/L $HAucl_4 \cdot 4H_2O$, then Au-NPs were fabricated on Gr/GCE by potentiostatic deposition method to acquire the Au-NPs self-assembled Gr/GCE. After that, Au-NPs/Gr/GCE was activated by CV twice to stability from -0.2 to 1.6 V at 50 mV/s in 0.5 mol/L H_2SO_4

2.3 Immobilization of probe DNA

Immobilization of the probe DNA (ssDNA) on the Au-NPs/Gr/GCE surface is described as follows. 10 μL of 1.0×10^{-6} mol/L probe DNA was dropped onto the surface of Au-NPs/Gr/GCE, left to dry naturally at room temperature. It was then rinsed with TE buffer and ultrapure water for three times to remove unfixed probe DNA. As such, The probe modified electrode was donated as ssDNA/Au-NPs /Gr/GCE. Then it was stored in a refrigerator at 4 ºC

2.4 Hybridize with complementary DNA

The ssDNA/Au-NPs/Gr/GCE was immersed into 2×SSC buffer solution containing complementary DNA for 60 min at 50 ºC. Then it was rinsed with 0.5% SDS and

deionized water respectively to remove the unbound complementary DNA. The obtained electrode was denoted as dsDNA/Au-NPs/Gr/GCE. Electrochemical detection of DNA hybridization process was performed by immersing ssDNA/Au-NPs/Gr/GCE and dsDNA/Au-NPs/Gr/GCE into 0.1mol/L PBS solution fixed with MB (2×10^{-5} mol/L) and were stired gently for 25 min respectively. After that, the modified electrodes were rinsed with deionized water. Finally the modified electrodes were scanned with DPV ranged from -0.8 V to 0.2 V at 50 mV/s in 0.1mol/L PBS buffer solution, using MB as an electrochemical indicator. In this study, variations of oxidation peak current of MB was used to quantify the concentration of complementary DNA.

3. Results

3.1 Characterization of electrode

3.1.1 Morphological characteristics of Gr/GCE, Au-NPs/GCE and Au-NPs/Gr/GCE

The SEM was employed to investigate the surface morphological characteristics of Gr/GCE, Au-NPs/GCE and Au-NPs/Gr/GCE. Figure 1A clearly shows that the Gr showed the typical crumpled and flake structure, indicating that Gr have been successfully cast on the electrode surface without aggregation and greatly increased the surface area of the GCE. Figure 1B showed that Au-NPs which diameter were between 50 nm to 70 nm have been equably modified onto GCE. After Au-NPs were introduced onto the surface of Gr film, the SEM images in Figure 1C of Au-NPs/Gr/GCE clearly show that Au-NPs are not homogeneously distributed due to may be some aggregation of Au-NPs and charge variation on the Gr surfaces, mainly edges of Gr surface contain more Au-NPs due to negative charges of carboxyl groups. The main driving forces for the formation of Au-NPs/Gr is strong electrostatic interaction between the positively charged Au-NPs and negatively charged Gr and also there is option for attachment of Au-NPs on the Gr surfaces by the reaction of primary amine groups on Au-NPs and epoxide groups on the Gr surfaces[16].

Figure 1. SEM Images of different electrodes.

3.1.2 Characterization of different electrodes

Figure 2 shows the CV behavior for different modified electrodes in 1 mmol /L $[Fe(CN)_6]^{3-/4-}$ aqueous solution containing 0.2 mol /L KNO_3 at a scan rate of 50 mV/s. Compared with bare electrode (E), the peak currents of at Au-NPs/GCE (F) and Gr/GCE (D) were increased, which indicated that Au-NPs and Gr have excellent conductivity and catalysis. When Au-NPs/Gr nanocomposite was assembled on the surface of GCE, the peak currents are significantly higher and more reversible on the Au-NPs/Gr/GCE, indicating that Au-NPs/Gr nanocomposite was a kind of ideal electrical conducting material and accelerated the electron transfer of $[Fe(CN)_6]^{3-/4-}$. It also showed in figure 2 that ssDNA/Au-NPs/Gr/GCE (C) exhibited peak currents reduced, the probable reason is the electrostatic repulsion occurred between negative-charged phosphate of ssDNA and $[Fe(CN)_6]^{3-/4-}$ which resist electron transfer. The reduction of the peak currents on the dsDNA/Au-NPs/Gr/GCE were preceded by the hybridization of the probe DNA with the target DNA, this can attributed to increased negative charges on electrode surface after hybridization of the probe DNA with the target DNA, resulting in repelling of $[Fe(CN)_6]^{3-/4-}$, Also, immobilization of the probe DNA and target DNA lead to increased density on the electrode surface and prevent electron transfer between the electrode surface and electroactive species.

Figure 2 Cyclic voltammograms of different electrodes in 1 mmol /L $[Fe(CN)_6]^{3-/4-}$ aqueous solution containing 0.2 mol /L KNO_3 at a scan rate of 50 mV/s.

3.1.3 Cyclic voltammograms of MB on different electrodes

It had been reported that MB interacted in a different way with ssDNA and dsDNA[17]. Figure 3 compares the cyclic voltammograms (CVs) of the bare GCE, Au-NPs/Gr/GCE, dsDNA/Au-NPs/Gr/GCE and ssDNA/Au-NPs/Gr/GCE. Four different electrodes were immersed in 0.1mol/L PBS solution fixed with MB, then scanned by CV at a scan rate of 50 mV/s in blank PBS solution. It can be seen that three obvious redox peaks of MB were observed at Au-NPs/Gr/GCE (curve B), dsDNA/Au-NPs/Gr/GCE (curve C) and ssDNA/Au-NPs/Gr/GCE (curve D) except bare GCE. It demonstrated that MB was immobilization on Au-NPs/Gr/GCE, dsDNA/Au-NPs/Gr/GCE and ssDNA/Au-NPs/Gr/GCE in different density. Many free guanine

bases of ssDNA were exposed out and had a strong affinity with MB, hence a large amount of MB accumulation occurred at the surface of ssDNA/Au-NPs/Gr/GCE. Therefore, the highest MB oxidoreduction signal was observed with ssDNA probe on Au-NPs/Gr/GCE. However a significant decrease in voltammetric peak current of MB was observed after complementary DNA sequence was allowed to hybridize with ssDNA/Au-NPs/Gr/GCE (curve C). The declined of current signal after hybridization was probably the inaccessibility of the guanine bases in dsDNA, the reason for it may be because there was no free guanine base exposed out in dsDNA, so different binding mode of MB with ssDNA/Au-NPs/Gr/GCE and dsDNA/Au-NPs/Gr/GCE resulted in variation in electrochemical responses.

Figure 3. The cyclic voltammograms of different electrodes in MB solution.

3.2 Optimization of experimential conditions

3.2.1 Optimization of MB concentration

Figure 4 depicted the plot of the peak currents of ssDNA/Au-NPs/Gr/GCE with varying MB concentrations from 1×10^{-6} mol/L to 2×10^{-5} mol/L, the current increased significantly with an increasing MB concentration up to 2×10^{-5} mol/L. When MB concentration continued to increase, the current increased slowly and finally flattened out, this result arose from MB aggregatiom on the surface of ssDNA/Au-NPs/Gr/GCE.

Figure 4. Relationship of the increasing current peak of ssDNA/Au-NPs/Gr/GCE with MB concentrations

3.2.2 Optimization of adsorption time

We investigated the effect of adsorption time on the response signal. ssDNA/Au-NPs/Gr/GCE was immersed in 2×10^{-5} mol/L MB solution then scanned in 0.1mol/L

PBS solution. The results were shown in Figure 5, it can be observed that the response signal increased significantly with increasing adsorption times from 1 min to 35 min. When the adsorption time exceeded 25 min, the response signal remained relatively constant, indicating that adsorption had reached saturation. Thus, 25 min adsorption time was selected in this study.

Figure 5. Amperometric response of ssDNA/Au-NPs/Gr/GCE in MB solution being stirred for successive additions of adsorption time.

3.2.3 Optimization of hybridization temperature and time

The effect of temperature on the biosensor response was investigated，the peak current of MB increased significantly with the temperature changed from 25ºC to 50ºC, at higher temperatures, the current response decreased slowly because of the denaturation of complementary DNA sequence. Then, the effect of DNA hybridization time on the peak current of MB was investigated. The designed biosensor exhibited a clearly increasing response with increasing hybridization times from 10 to 60 min and remained stable after 60 min, indicating that the hybridization reaction was completed during 60 min. Thus, we chose the hybridization temperature of 50ºC and the hybridization time of 60 min in subsequent experiments.

3.3 Performance evaluation of electrochemical biosensor

3.3.1 Analytical performance of the biosensor

The differential pulse voltammetry method is usually used for the determination of test samples because of its high sensitivity. Under optimal conditions, analytical performance of this electrochemical DNA biosensor was investigated by varying the complementary DNA concentration according to the procedures described above. As shown in Figure 6. The different current value obtained in the DPV response of MB after hybridization of ssDNA probe with complementary DNA sequence was recorded. The current response showed excellent correlation with the amount of complementary DNA sequence in the range from 1×10^{-12} mol/L to 1×10^{-7} mol/L (Figure 7). The regression equation is $\Delta I=10.789\log C+50.524$ (unit of C is mol/L , unit of I is μA) $R^2=0.9976$, The detection limit is 2.0×10^{-13} mol/L. (based on 3σ).

Error bars show good reproducibility of the electrode for stripping voltammetry.

Figure 6. Figure 6. DPV of MB accumulated on the ssDNA after its hybridization with different concentration of the target sequence. Target concentration A-N: 1.0×10^{-7} mol/L、5.0×10^{-8} mol/L、1.0×10^{-8} mol/L、5.0×10^{-9} mol/L、1.0×10^{-9} mol/L、5.0×10^{-10} mol/L、1.0×10^{-10} mol/L、5.0×10^{-11} mol/L、1.0×10^{-11} mol/L、5.0×10^{-12} mol/L 、1.0×10^{-12} mol/L.

Figure 7. The current peaks changes with the purposes of the logarithm of DNA concentration. Error bars = ± relative standard deviation.

3.3.2 Selectivity of electrochemical biosensor

Interference from extraneous electroactive compounds could be a great challenge in the development of the high-performance electrochemical DNA biosensor. The designed Au-NPs/Gr/GCE was further tested to demonstrate its capability for the determination of the different DNA sequences in 1×10^{-8} mol/L were detected by ssDNA/Au-NPs/Gr/GCE. The signals were showed in Figure 8. A:Complementary sequence; B:One-base mismatched sequence; C:Three-base mismatched sequence; D:Non-complementary sequence; The response of the one-base mismatched sequence was only 22% that of the complementary sequence, indicating that the sensor exhibited good performance to discriminate the perfectly complementary target and one-base mismatched sequence. Meanwhile, the peak current of three-base mismatched sequence showed only 7% that of the complementary sequence, which is almost as low as the non-complementary sequence. It was indicated that this electrochemical DNA biosensor has excellent selectivity to complete complementary DNA sequence.

Figure 8. The current peaks changes of the different target DNA.

3.3.3 Reproducibility and stability of the biosensor

Reproducibility of the response is an important characteristic for the modified electrode, which should be explored for analytical determinations. Five different biosensors were fabricated independently under the same conditions, and then they were employed to detect complementary DNA in 1×10^{-9} mol/L according to the procedures described above. The relative standard deviation (RSD) was 4.3%, indicating that the electrochemical DNA biosensor has excellent reproducibility.

As a performance parameter of the biosensor long-time stability needs to be tested, the probe DNA modified electrode was first stored in the refrigerator at 4ºC over 15 days and then examined via DPV after its hybridization with complementary target DNA. The results showed that the analytical performance of the biosensor tested has no obvious decline (RSD≤5%).

4. Conclusion

To summarize, we have introduced a strategy for DNA hybridization detection by employing an ssDNA/Au-NPs/Gr/GCE. The electrochemical DNA biosensor showed high sensitivity, excellent selectivity and good stability with a very low detection limit. It should be pointed out that the electrochemical DNA biosensor can effectively recognize the complementary DNA. It might has a promising future for detection of KPC and solve the actual problem of early diagnosis in clinic.

5. Acknowledgments

This study was supported by grant from National Natural Science Fund of China 81273129. The authors greatly appreciated this support.

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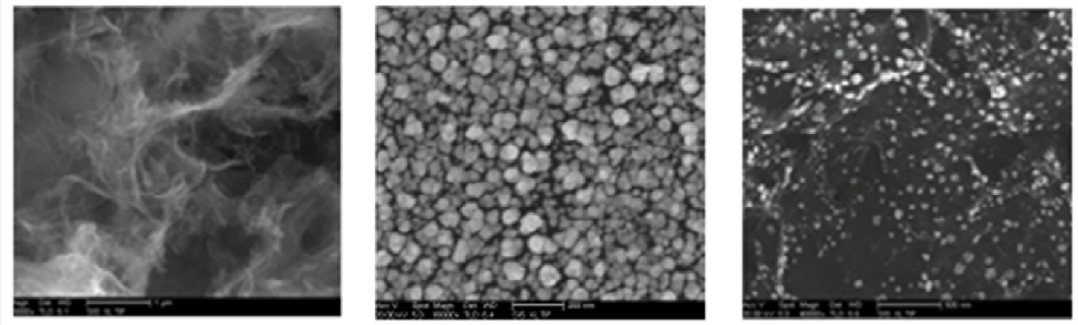


Figure 1. SEM Images of different electrodes. A: Gr/GCE B: Au-NPs/GCE C: Au-NPs/Gr/GCE

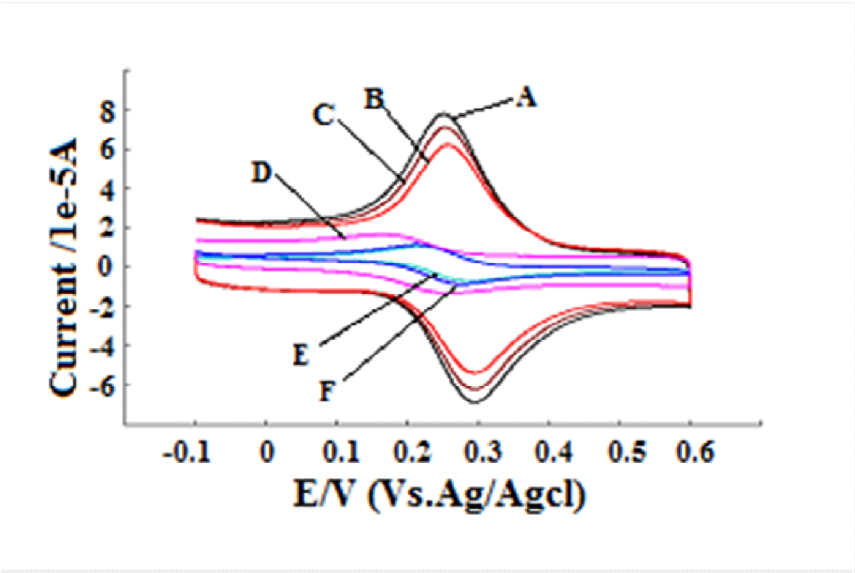


Figure 2. Cyclic voltammograms of different electrodes in 1 mmol /L $[Fe(CN)_6]^{3-/4-}$ aqueous solution containing 0.2 mol /L KNO_3 at a scan rate of 50 mV/s. A:Au-NPs/Gr/GCE; B: dsDNA/Au-NPs/Gr/GCE; C: ssDNA/Au-NPs/Gr/GCE; D:Gr/GCE; E:bare electrode; F:Au-NPs/GCE

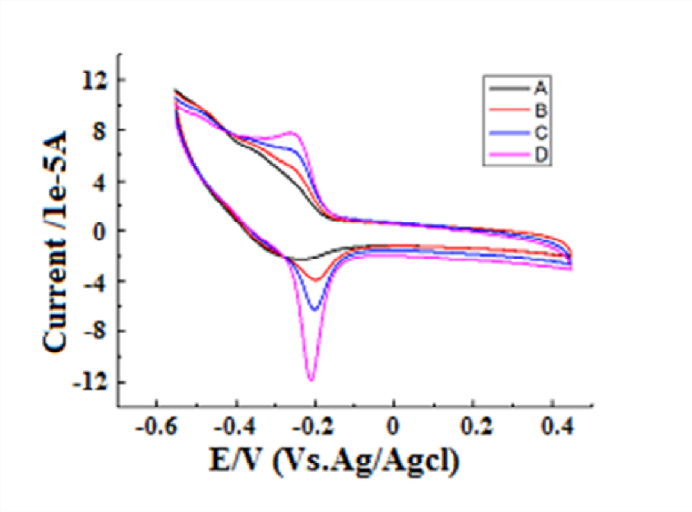
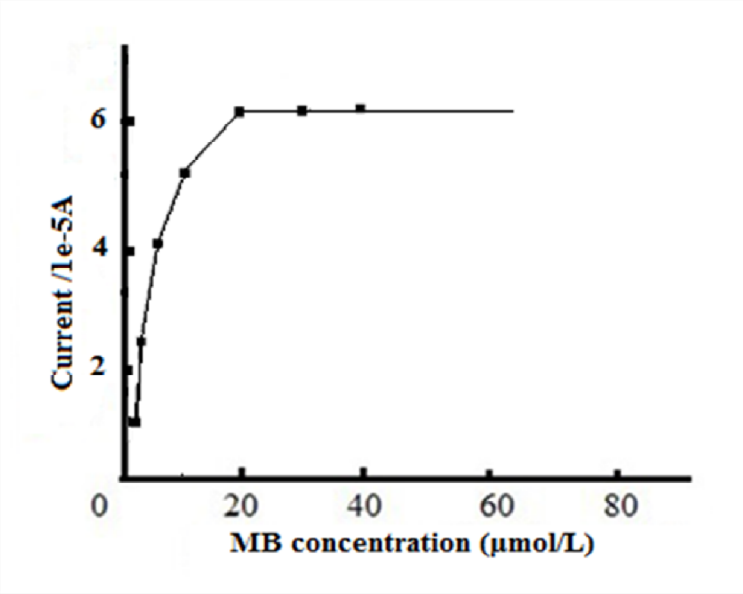


Figure 3. The cyclic voltammograms of different electrodes in 0.1mol/L PBS solution fixed with MB(2×10^{-5} mol/L). A:bare GCE. B:Au-NPs/Gr/GCE. C:dsDNA/Au-NPs/Gr/GCE. D:ssDNA/Au-NPs/Gr/GCE.



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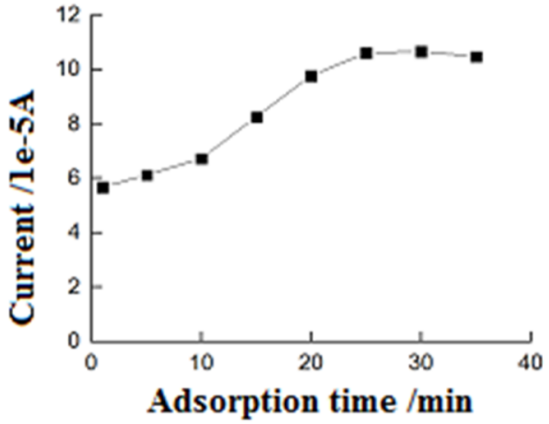

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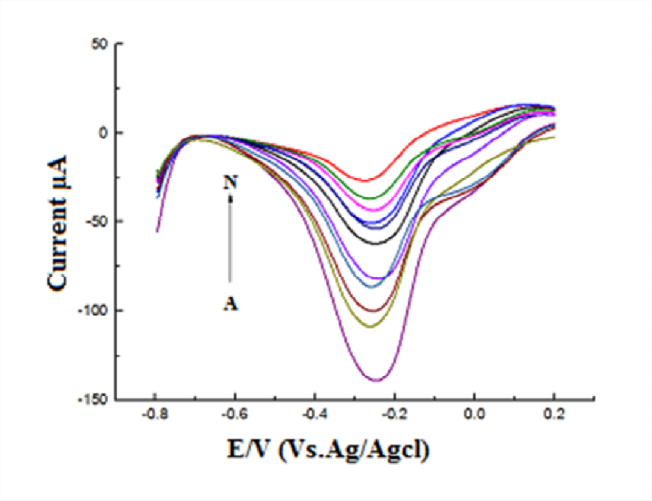

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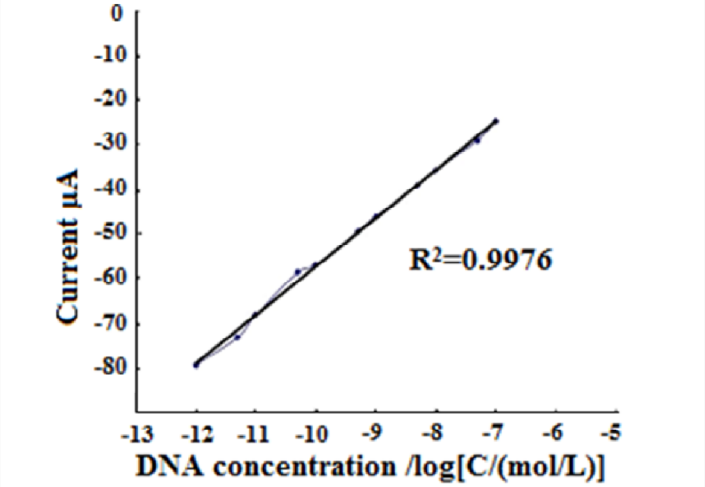

Figure 7:The current peaks changes with the purposes of the logarithm of DNA concentration. Error bars = ± relative standard deviation.

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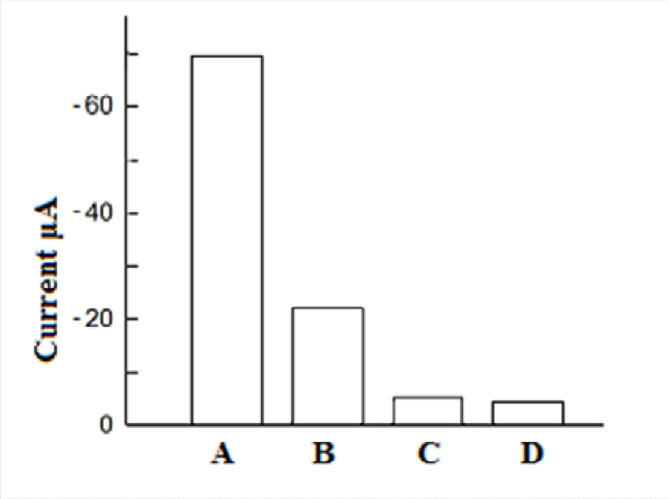

Figure 8. The current peaks changes of ssDNA/Au-NPs/Gr/GCE after hybridization with the different target DNA. A:Complementary sequence B;One-base mismatched sequence C:Three-base mismatched sequence: D:Non-complementary sequence.