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Zirconia/Poly(L–cysteine)/RGO-Modified Electrode for Sensing the CaMV 35S Gene Sequence

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Herein, a zirconia (ZrO_2)/poly(L–cysteine)/reduced graphene oxide (RGO)-modified electrode was reported. A ZrO_2 film was electrodeposited onto the poly(L–cys)/RGO electrode surface. Probe DNA was modified on the electrode surface via the interaction of DNA phosphate groups and ZrO_2. The electrochemical response changes of methylene blue were used as indicators for detecting target DNA. The performance of the biosensor was studied by electrochemical methods; methylene blue had an obvious change in its electrochemical signal when hybridized with completely matched target DNA compared with its signal when hybridized with mismatched target DNA. In addition, the biosensor had high sensitivity for completely matched target DNA, and the detection signals showed ideal linearity with a target DNA concentration range of 1.0×10^{-13} mol/L to 1.0×10^{-9} mol/L. The detection limit was 2.6×10^{-14} mol/L (*S/N* = 3). Experiments showed that the proposed biosensor had excellent reproducibility and stability and could be used to successfully assay the CaMV 35S gene sequence in human serum samples.

Keywords: CaMV 35S gene; Biosensor; Zirconia; RGO; Methylene blue

1. INTRODUCTION

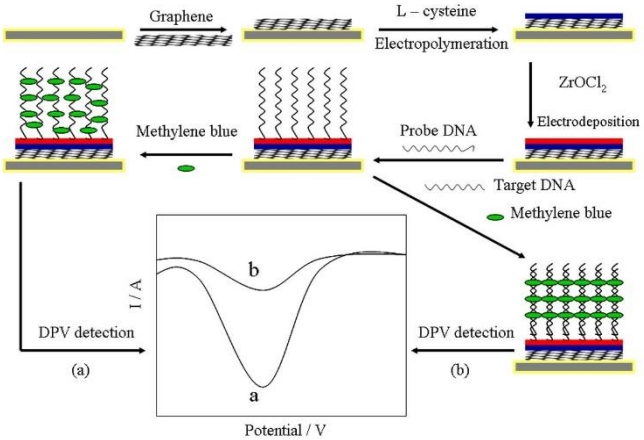
In recent years, the development of reliable and specific transgenic sequence detection methods has received great attention. Different methods including fluorescent,[1,2] radio-chemical,[3] surface plasmon resonance spectroscopy, [4] and electrochemical methods [5-9] have been reported. Electrochemical DNA biosensors have received wide attention due to their fast response, low cost, small size, and ease of integration and miniaturization. The key to electrochemical DNA biosensors is to develop an electrode surface suitable for the modification of probe DNA. Therefore, finding electrodes modified with different materials with good performance for the assay of target DNA is

highly desirable.

ZrO_2 is a thermally stable inorganic oxide that has no toxicity to DNA and possesses a strong affinity for groups with oxygen. [10-11] Therefore, ZrO_2 is an ideal surface modifier for electrodes intended for immobilizing DNA. Liu reported a strategy to immobilize probe DNA by ZrO_2 and a sol–gel technique. [12] Jiao developed a synergistic film of ZrO_2 for probe DNA immobilization. [13] Fang prepared a zirconia film-modified electrode for detecting DNA. [14] Wang detected DNA by a sphere-like CeO_2-ZrO_2-modified electrode. [15] Although the ZrO_2 film can provide an ideal interface for probe DNA immobilization and enhance biosensor performance, the sensitivity of the biosensor still requires improvement. Recently, a strategy has been developed in which suitable materials are used to fabricate ZrO_2 nanocomposites to improve the performance of biosensors. [16-20]

Reduced graphene oxide (RGO) is an ideal material for fabricating electrochemical sensors owing to its outstanding properties, such as its large specific surface area, strong electrical conductivity, and good biocompatibility. RGO-based nanocomposites are attractive for a wide range of applications in electrochemical biosensors because of their unique properties. [21-25] Recently, RGO-ZrO_2 nanocomposites have been successfully used to develop electrochemical biosensors with excellent performance. [26-28] However, RGO-ZrO_2 nanocomposites have not been introduced in electrochemical DNA sensors, where they can greatly increase the sensitivity of the biosensors to meet the needs of DNA assays.

Herein, we present a good strategy for probe DNA immobilization based on a ZrO_2/poly(L–cysteine)/RGO film-modified electrode, which provides a good target molecular recognition interface (**Scheme 1**). An electrode modified with RGO film was obtained via a simple dropping method. L–cysteine (L–cys) was electropolymerized onto the RGO film-modified electrode surface to form a poly(L–cys) film to increase the stability of the RGO film and provide a good interface for the electrodeposition of ZrO_2. Probe DNA was modified on the ZrO_2 at the electrode surface by the reaction between the phosphate group and ZrO_2. A probe DNA-modified ZrO_2/poly(L–cys)/RGO/GCE was used to detect target DNA by electrochemical methods, and methylene blue (MB) was used as an electrochemical indicator. The test results indicate that the proposed biosensor is highly selective and sensitive for detecting the CaMV 35S gene sequence. In addition, it can be applied to successfully detect the CaMV 35S gene sequence in human serum samples.



Scheme 1. Schematic representation of the preparation of biosensors and their application in DNA assays.

2. EXPERIMENTAL

2.1. Materials

$ZrOCl_2 \cdot 8H_2O$ and L–cys were purchased from Alfa Aesar (AR, Tianjin, China). Reduced graphene oxide (Chengdu Institute of Organic Chemistry, Chinese Academy of Sciences) was used without further purification. KCl, NaOH, $NaNO_3$, and H_3PO_4 were purchased from Nanjing Chemical Reagent Co., Ltd. (AR, Nanjing, China). All solutions were prepared by using doubly distilled water. The DNA sequences used in this work were synthesized by Shanghai Sangon Bioengineering (Shanghai, China) (**Table 1**). The solutions of DNA were prepared by using PBS (pH 7.0) and stored at 4 °C. The PBSs used in this work were PBS1 (0.1 M NaCl + 0.1 M PBS pH 7.0) and PBS2 (0.1 M PBS pH 7.0).

Table 1. The sequences of DNA and RNA used in this work.

Names	From 5′ to 3′
Probe DNA	TCT TTG GGA CCA CTG TCG
Complementary target DNA	CGA CAG TGG TCC CAA AGA
Noncomplementary target DNA	GCT TCC ATC GAG ATC GTC
One-base mismatched target DNA	CGA CAG TCG TCC CAA AGA

2.2. Apparatus

A CHI660A electrochemical workstation (Shanghai Chenhua Instruments, China) was used to record cyclic voltammetry (CV) and differential pulse voltammetry (DPV) curves. The three-electrode system was composed of a working electrode, a saturated calomel electrode (SCE, reference electrode), and a platinum wire (counter electrode). Experiments were performed in the presence of 5 mM $[Fe(CN)_6]^{3-/4-}$ as a redox probe to record the electrochemical impedance spectra (EIS) of different electrodes (frequency: 1-10^5 Hz; AC voltage amplitude: 5 mV). Nitrogen was used to remove oxygen in solution.

2.3. Fabrication of a ssDNA/ZrO_2/poly(L – cys)/RGO/GCE

Before modification, the GCE was polished to a mirror-like surface by using emery paper and 1.0, 0.3, and 0.05 μm alumina slurries. The GCE was then rinsed with doubly distilled water and sonicated in HNO_3 (1:1), acetone and water (each for 1 min). An RGO solution (1.0 mg/mL) in DMF was obtained by sonicating for approximately 30 min. The above solution (10 μL) was dropped onto the pretreated GCE surface, and it was dried at room temperature to form the RGO-modified electrode. Loosely adsorbed RGO was removed by immersing the electrode in water for 5 min.

The poly(L–cys)/RGO/GCE was prepared by CV with a potential window of -1.8 to +3.0 V sweeping for six cycles in PBS2 containing 0.01 mol/L L–cys with a scan rate of 100 mV/s. ZrO_2 was modified on the poly(L – cys)/RGO/GCE via CV scanning with a scan rate of 20 mV/s in a potential window between -1.1 V and +0.7 V for 5 cycles in an aqueous solution containing 5 mmol/L $ZrOCl_2$ and 0.1 mol/L KCl. Probe DNA was immobilized via Au-S bonds by dropping 10 μL of a 10 μmol/L probe DNA solution on the ZrO_2/poly(L–cys)/RGO/GCE surface. After 12 h at 4 ℃, any excess and mobile probe DNA was removed by immersing into PBS2 for 5 min. The final electrode was defined as an ssDNA/ZrO_2/poly(L–cys)/RGO/GCE.

2.4. Electrochemical assay of target DNA

Target DNA was detected by the ssDNA/ZrO_2/poly(L–cys)/RGO/GCE in its solution for 35 min at 40 ℃. After that, the nonspecifically bound DNA was removed by immersing in PBS2 for 5 min and rinsing three times. The final electrode was then exposed to PBS2 containing MB for 15 min. The oxidation peak current of the DPV change ($\triangle I = I_{ssDNA} - I_{dsDNA}$) of MB before and after DNA hybridization was used as the detection signal.

3. RESULTS AND DISCUSSION

3.1. Electropolymerization of L–cys

CV was utilized to prepare a poly(L–cys) film-modified electrode. Fig. 1 shows the repetitive CV sweeping of the RGO/GCE in PBS2 containing 0.01 mol/L L–cys. For the first cyclic scan, a pair of redox peaks appeared, and larger peaks were found with continuous scanning. When scanning to six cycles, the peak currents hardly increased, indicating that L-cys was successfully electropolymerized onto the RGO/GCE surface.

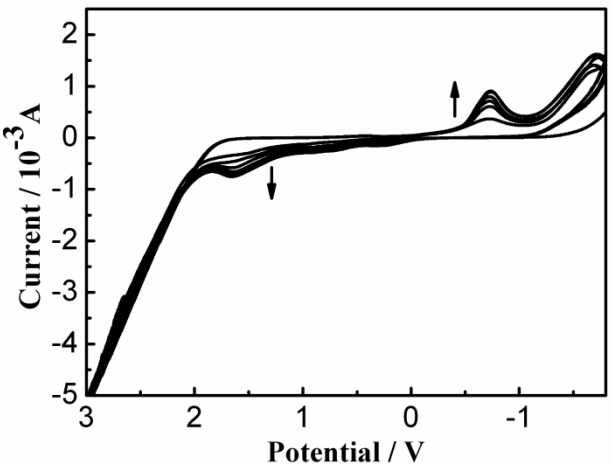


Figure 1. Repetitive CVs of 0.01 mol/L L–cys in PBS2 at the RGO/GCE. Scan rate: 100 mV/s.

3.2. Immobilization of the biosensor and the response to the target

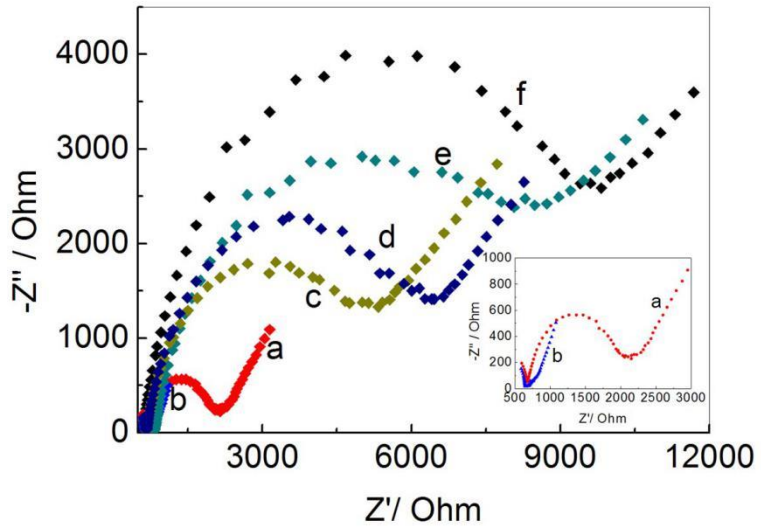


Figure 2. The Nyquist plots of impedance for the bare GCE (a), RGO/GCE (b), poly(L–cys) /RGO/GCE (c), ZrO_2/poly(L–cys)/RGO/GCE (d), ssDNA/ZrO_2/poly(L–cys)/RGO/GCE (e), and dsDNA/ZrO_2/poly(L–cys)/RGO/GCE (f).

Fig. 2 shows the Nyquist plots of impedance for different electrodes: the bare GCE (a), RGO/GCE (b), poly(L–cys)/RGO/GCE (c), ZrO_2/poly(L–cys)/RGO/GCE (d), ssDNA/ZrO_2/poly(L–cys)/RGO/GCE (e), and dsDNA/ZrO_2/poly(L–cys)/RGO/GCE (f). We observed a small electron transfer resistance (R_{et}) for RGO/GCE, and R_{et} continuously increased as the poly(L–cys) film and the subsequent ZrO_2 film were deposited on the electrode surface. After the probe DNA was linked, R_{et} increased, which was attributed to the negatively charged phosphate backbone of DNA repelling the $[Fe(CN)_6]^{3-/4-}$ anions. After the reaction between probe DNA and target DNA on the electrode surface, R_{et} further increased, which was attributed to the formation of the more negatively charged dsDNA. This shows that the biosensor can be successfully constructed and effectively detect target DNA.

Fig. 3 shows the CVs of different electrodes in PBS2 (3.0×10^{-2} mol/L MB): the bare GCE (a), ZrO_2/poly(L–cys)/RGO/GCE (b), ssDNA/ZrO_2/poly(L–cys)/RGO/GCE (c), and dsDNA/ZrO_2/poly(L–cys)/RGO/GCE (d). It can be seen from Fig. 3 that compared to the ZrO_2/poly(L–cys)/RGO/GCE, the peak currents become larger for the ssDNA/ZrO_2/poly(L–cys)/RGO/GCE. After exposing the ssDNA/ZrO_2/poly(L–cys)/RGO/GCE to complementary target DNA, the peak currents decreased, exhibiting experimental results that are similar to previous reports. [14] This result indicates that DNA hybridization events can result in changes in the electrochemical response of MB and can be used to detect target DNA.

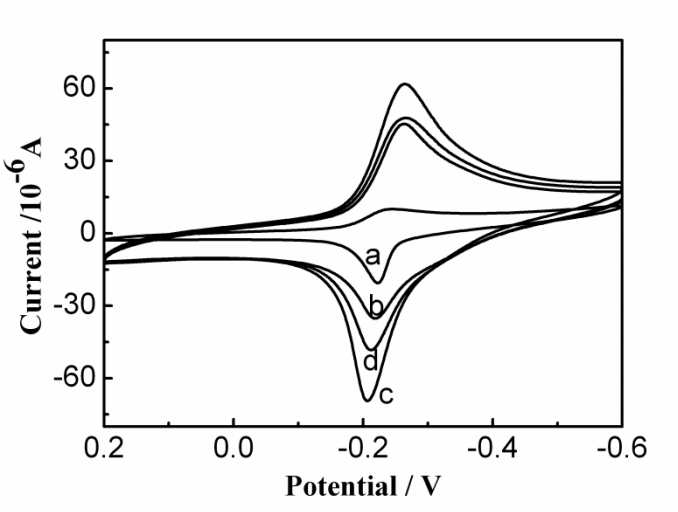


Figure 3. The CVs of different electrodes in PBS2 (3.0×10^{-2} mol/L MB): the bare GCE (a), ZrO_2/poly(L–cys)/RGO/GCE (b), ssDNA/ZrO_2/poly(L–cys)/RGO/GCE (c), and dsDNA/ZrO_2/poly(L–cys)/RGO/GCE (d).

3.3. Optimization of experimental conditions

The effect of temperature and time on DNA hybridization was studied by DPV. A concentration of 1.0×10^{-6} mol/L of the complementary target DNA was used. Fig. 4 shows the influence of the hybridization temperature (30, 35, 40, 45, 50, and 55 ℃) on △I. When the temperature was 40 ℃, △I reached a maximum. Therefore, 40 ℃ was chosen as the optimal temperature in this work.

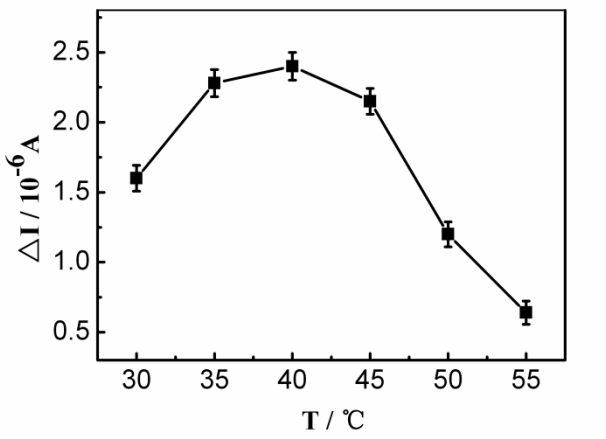


Figure 4. The influence of temperature on △I.

Fig. 5 shows the effect of the incubation time on △I. △I significantly increased with increasing incubation time from 10 to 35 min. After 35 min, △I almost no longer increased. Thus, 35 min was selected as the optimal incubation time.

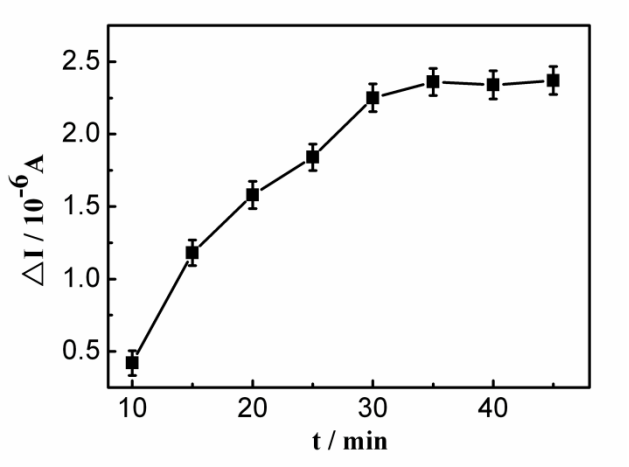


Figure 5. The effect of the incubation time on △I.

3.4. Accumulation time of MB

The accumulation time of MB was optimized (c_{MB} = 1.0×10^{-6} M). It can be seen from Fig. 6 that the peak current of MB obviously increased until 15 min, which indicates that the enrichment amount of MB reached saturation. Thus, 15 min was considered the optimal accumulation time.

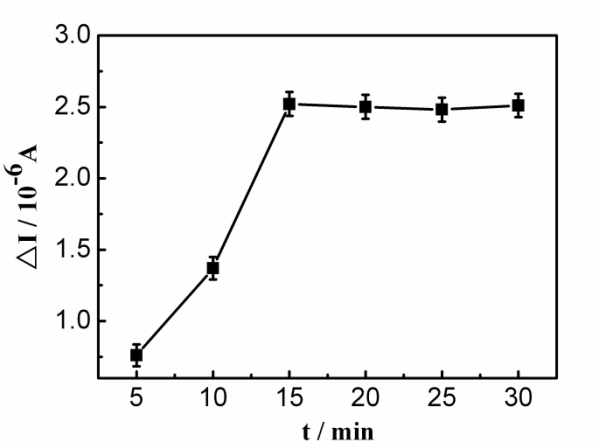


Figure 6. The effect of the accumulation time of MB on △I.

3.5. The sensitivity and selectivity of the sensor

Fig. 7 shows that the DPV response decreased with increasing target DNA concentration. △I becomes sufficiently linear with the logarithm of the target DNA concentration in the range from 1.0×10^{-13} mol/L to 1.0×10^{-9} mol/L (parallel sample number: 3). The regression equation was determined to be $\triangle I$ (μA) = 6.4668 + 0.4569 logc_{DNA} (*c:*M) (R=0.9936), and the detection limit was found to be 2.6×10^{-14} mol/L (*S/N* = 3).

The linear range and detection limit are the key factors for DNA biosensors. To verify the application of the proposed biosensor, we compared its performance with some previously constructed similar biosensors. The results are shown in **Table 2**, and the biosensor designed in this work has a

lower detection limit and a wider linear range than the biosensors in other works.

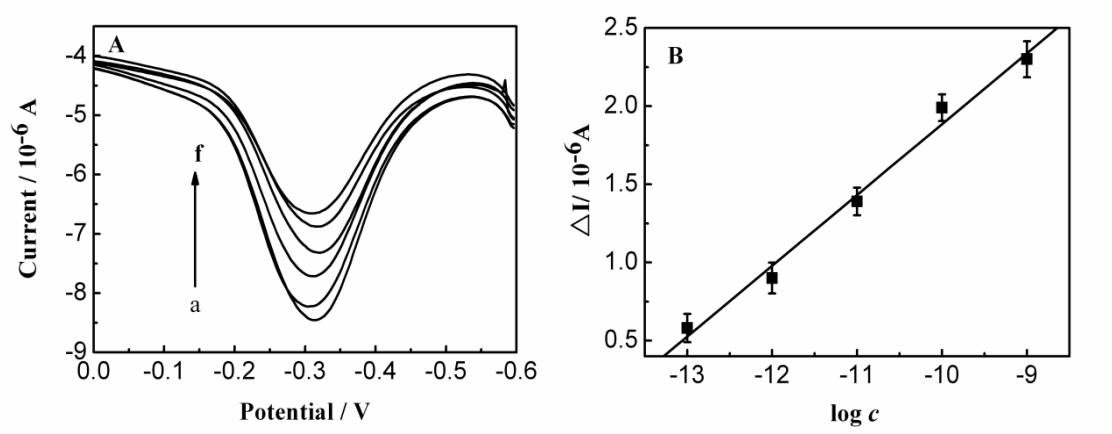


Figure 7. A) DPV curves of the biosensor in PBS2 (a), and after incubation with different concentrations of target DNA: 0 (a); 1.0×10^{-13} M (b); 1.0×10^{-12} M (c); 1.0×10^{-11} M (d); 1.0×10^{-10} M (e); 1.0×10^{-9} M (f). B) △I vs. the logarithm of the concentrations of target DNA.

Table 2. Comparison of the linear ranges and detection limits of previously constructed similar DNA biosensors.

Sensor	Electrochemical technique used	Linear range	Detection limit	References
ssDNA/AuNPs/PNR/GCE	DPV	0.01 nM– 17 nM	4.2 pM	[29]
PNA/poly(JUG-co-JUGA)/GCE	SWV	10 nM– 100 nM	10 nM	[30]
ssDNA/PICA/GCE	CV	3.34 nM – 10.6 nM	1.0 nM	[31]
ssDNA/ZrO_2/SWNTs/PDC/GCE	EIS	10 pM – 1.0 μM	1.38 pM	[32]
ssDNA/AuNPs/GO/GCE	DPV	60 pM -0.6 nM	27pM	[33]
ssDNA/ZrO_2/poly(L–cys)/RGO/GCE	DPV	0.1 pM-1.0 nM	26 fM	This work

The selectivity of the biosensor was studied by recording △I obtained from incubating with various kinds of target DNA in PBS2. The results are shown in Fig. 8. After incubating with noncomplementary or one-base mismatched target DNA, △I was 0.428 μA and 1.581 μA, respectively, which correspond to 18.64% and 68.86% of the △I for complementary target DNA (2.296 μA). This indicates that the biosensor had an excellent selectivity to distinguish various sequences of target DNA.

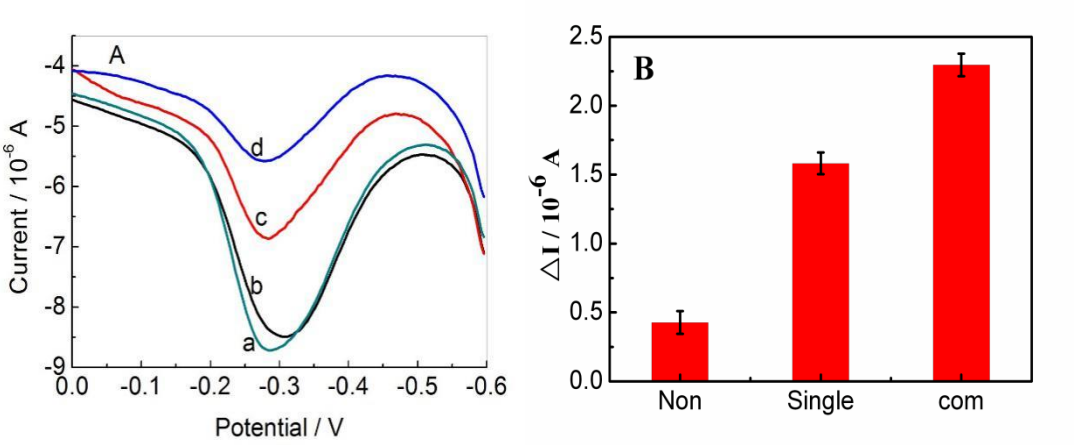


Figure 8. A) DPV curves of the biosensor in PBS2 (a), incubated with noncomplementary (b), one-base mismatched (c), and complementary (d) target DNA. B) Electrochemical signal of the sensor for detecting various targets of DNA. Target DNA: 1.0 μM.

3.6. Reproducibility, stability and regeneration

The reproducibility was tested by using six independently prepared biosensors to detect 0.1 nM complementary target DNA. A relative standard deviation (R.S.D.) of 3.7% for △I was obtained, which suggests sufficient reproducibility of the biosensors. The stability of the biosensor was studied by continuously scanning for 20 cycles, and the biosensor was then stored in the refrigerator at 4 °C for five days. After that, it was reused to detect target DNA, and approximately 89.2% of the original response was maintained.

The regeneration of the biosensor was researched by incubating it with 0.5 M NaOH for 10 min, denaturing dsDNA at the surface of the electrode to reform the probe DNA recognition interface. The results indicate that the biosensor could be successfully regenerated up to four times.

3.7. Sample detection

The CaMV 35S gene sample detection method was described in a previous report. [34] The preparation process of sample was as follows: the blood serum was prepared by treating blood with 4% sodium citrate and centrifuging for 5 min at 10,000 rpm. Finally, 500 μL of blood serum was diluted by using 2.0 mL PBS1. The test results exhibit recoveries of 96.4% and 95.3% and are listed in **Table 3**.

Table 3. Analysis results of target DNA in blood serum samples.

Num.	Found/M	Added/pM	Found/pM	Recovery（%）
1	0	5.0	4.82	96.4
2	0	10.0	9.53	95.3

4. CONCLUSION

Herein, a sensitive electrochemical DNA biosensor based on a ssDNA/ZrO_2/poly(L–cys)/RGO/GCE was reported. The electrochemical response change of MB ("signal-off") was selected as the detection signal to report the target DNA. The experimental results indicate that the biosensor has good selectivity and high sensitivity for target DNA. Additionally, the biosensor is reproducible, stable, and regenerative, and it was used to successfully detect target DNA in blood serum samples.

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