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An Electrochemical DNA Biosensor Based on Au-reduced Graphene Oxide Nanocomposite for Transgenic Event Bt63 Detection

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A simple and reliable electrochemical biosensor based on an *in situ* synthetic Au-reduced graphene oxide (Au-RGO) nanocomposite was constructed in this work, which is considered to be a potential sensing platform for sensitive and selective Bt63 rice detection. UV-vis spectroscopy, transmission electron microscopy (TEM), and cyclic voltammograms of the surface characterization indicated that RGO was prepared successfully and Au nanoparticles were well dispersed on its surface with an average size of 20 nm. The square-wave voltammetry (SWV) response of the electrochemical marker methylene blue (MB) was chosen to monitor the probe immobilization and target hybridization event. Under the optimum conditions, the peak values of MB were linear with the logarithm of the target DNA concentrations being from 1.0×10^{-9} to 1.0×10^{-14} M; the detection limit was 3.36×10^{-15} M. Studies also demonstrated that the DNA biosensor had high reproducibility and stability. At last, we used this biosensor to detect several rice samples; it showed good sensitivity and selectivity. Thus the applicability of the method as an effective tool for genetically modified organism (GMO) quantification was confirmed by its accurate and sensitive results in Bt63 rice screening.

Keywords Graphene, Au nanoparticles, screen-printed gold electrode, SWV, Bt63 rice

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Introduction

Considering its irreplaceable position in feeding the world's largest population, rice will continue to be widely cultivated in the following decades.[1,2] Also wide pesticide application and yield losses of rice caused by the stem borer is no longer a problem, thanks to the development of transgenic insect-resistant rice technology.[3] Bt63 is created by particle bombarding two plasmids into Minghui 63, which are pFHBT1 containing a hybrid Cry1Ab/Ac gene and pGL2RC7 harboring a Chitinase gene (RC7) and a selectable marker gene (Hph).[4] Although it shows great potential to resist certain insect pests for increasing the rice output, related government agencies have not approved Bt63 for commercial growth or food use until now.[3] However, the presence of unauthorized Bt63 has been discovered in raw and finished rice either in the Chinese market or in exports to other countries, such as rice sticks, rice vermicelli, rice noodles, rice in red-bean paste sweet dumpling and so on from 2005 to 2013.[5-7]

In order to screen the presence of Bt63, some reliable detection methods are urgently needed. The leading-edge technology DNA sensors focusing on a rapid, portable and inexpensive way of testing, has achieved great progress in recent years. Recently, various nanomaterials and metal nanoparticles have been broadly applied in constructing DNA sensors due to their unique merits. Among them, a graphene-based material is an ideal candidate, and have been widely used for electrochemical biosensors.[8-11] Gold nanoparticles (AuNP) have also been widely of concern recently because of their unique optical and electronic properties.[12-15] The defects and oxygen-containing groups on the surfaces of reduced graphene oxide (RGO), due to incomplete reduction from GO, make it a perfect carrier to combine AuNPs.[16] Since the Au-RGO nanocomposite has unique properties for increasing the sensing area and enhancing the electron- transfer rate, increasing efforts have been devoted to incorporate AuNPs into the RGO matrix to improve both the sensitivity and selectivity of biosensors.[17-20]

However, the methods used before were either complicated or time-consuming. Here, we describe an easy and rapid approach for preparing an Au-RGO nanocomposite-based sensing platform for genetically modified Bt63 event-specific sequences detection. Compared with an unmodified electrode, the Au-RGO nanocomposite noticeably improved the electrode sensing area, and combined more probe sequences, which improved the sensitivity of the biosensor greatly. The biosensor also showed good reproducibility, stability and selectivity for Bt63 detection. Square-wave voltammetry was used to detect the probe immobilization and double-strain hybridization event through the MB redox signal, as Scheme 1 shows. This research was aimed to establish an electrochemical method for the event-specific screening of Bt63 both qualitatively and quantitatively. To our knowledge, there were no relevant studies before.

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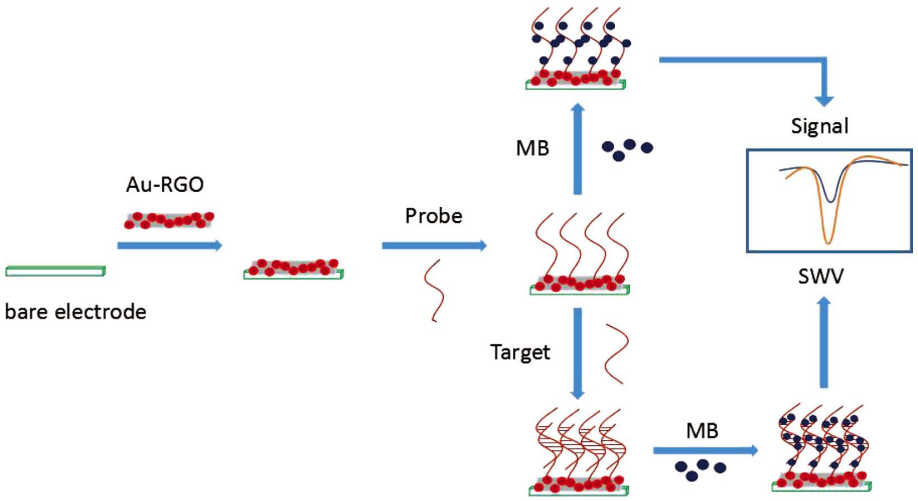

Scheme 1 Illustration of the Bt63 biosensor fabrication and detection procedure.

Experimental

Chemicals and materials

Graphite powder and chloroauric acid ($HAuCl_4$) were purchased from Aladdin Reagent Co., Ltd. (Shanghai, China). Tris(2-carboxyethyl) phosphine (TCEP) and methylene blue (MB) were purchased from Sigma-Aldrich (Shanghai, China); hydrazine hydrate (N_2H_4) and 6-mercapto-1-hexanol (MCH) were purchased from Tianjin Fuchen Chemical Regents Factory (Tianjin, China).

The synthetic oligonucleotide sequences were all purchased from Sangon (Sangon, Shanghai, China), and the sequences are listed below:

Probe sequences: 5′-SH-$(CH_2)_6$-ATCTGCCCCAGCACTCGTCCG-3′

Complementary sequences: 5′-CGGACGAGTGCTGGGGCAGAT-3′

The PCR amplification primers are also reported as follows:

Primer 1: AGAGACTGGTGATTTCAGCGGG

Primer 2: GCGTCCAGAAGGAAAAGGAATA

The primers and probes used in our work were chosen according to Ref. 21. The referential transgenic and non-transgenic rice samples were kindly provided by Chinese Academy of Inspection and Quarantine.

All related chemicals were of analytical grade, and all solutions were prepared with ultrapure water.

Apparatus

UV-vis spectroscopy was acquired and analyzed by NanoDrop 2000 (Thermo Scientific, USA). Transmission electron microscopy (TEM) was performed with a Hitachi HT7700 microscope (Hitachi, Japan). Electrochemical measurements were carried out using a USB powered portable potentiostat (BDTminiSTAT100), which was recommended as being detailed in the research before.[22] The planar screen-printed electrode (SPE) was purchased from BioDevice Technology Co., Ltd. (Japan), which consisted of two gold electrodes as the working and counter electrodes, respectively; an Ag/AgCl electrode was used as the reference electrode.

Synthesis of reduced graphene oxide (RGO)

According to Kovtyukhov's approaches,[23] GO was pre-oxidized from graphite powder and then synthesized according to the modified Hummer's method.[8]

After 100 mg of GO was sonicated sufficiently in 100 mL of deionized water for several hours, 0.5 mL of the reducing agent N_2H_4 was added and heated in 95°C for 2 h. In order to remove any excess reducing agent, the reduced product was centrifuged at 12000 rpm for 5 min and washed by water several times. Finally, the collected precipitate was freeze-dried and redispersed in water by an ultrasonic process for Au-RGO preparation.[24]

Preparation of the Au-RGO nanocomposite

After 10 mL of RGO sheet solution was treated adequately with ultrasonic to ensure a good dispersity, 150 µL of a 20 mM $HAuCl_4$ aqueous solution was added. Then, the ultrasonic procedure lasted for 10 min at room temperature. After the reaction, the products collected by centrifugation were washed for several times with pure water.[25]

Fabrication of Au-RGO nanocomposite assembled electrode

In order to prepare an Au-RGO nanocomposite assembled electrode, the gold electrode was cleaned by cyclic voltammetry 15 times in a 0.1 M H_2SO_4 solution, washed by deionized water and dried naturally. Subsequently, 6 µL of an Au-RGO solution was dropped onto the bare electrode surface and dried naturally at room temperature for following studies.

DNA extraction and amplification

DNA was extracted from different rice samples and amplified with a GMO Crop Extraction & Amplification Kit (TIANGEN BIOTECH CO., China), according to the manufacturer protocol. About 200 ng of DNA was applied as a template for PCR. Its 100 µL stock solutions in a TE buffer (pH 7.4) were stored in a freezer.

Biomodification of the sensor surface

Before probe immobilization, as a precaution step, TCEP was used for 1 h to separate the -S–S- bonds of oligonucleotide dimers. After that, a screen-printed electrode was inserted into 20 µL of a thiolated oligonucleotide solution (0.1 µM in PBS buffer). Chemisorption proceeded for 2 h at 35°C. After that, the electrode was washed with PBS (pH 7.4) buffer.

After immobilization, a 1 mM aqueous solution of MCH was used as a post-treatment step for 1 h, and electrode was then rinsed with ultrapure water.

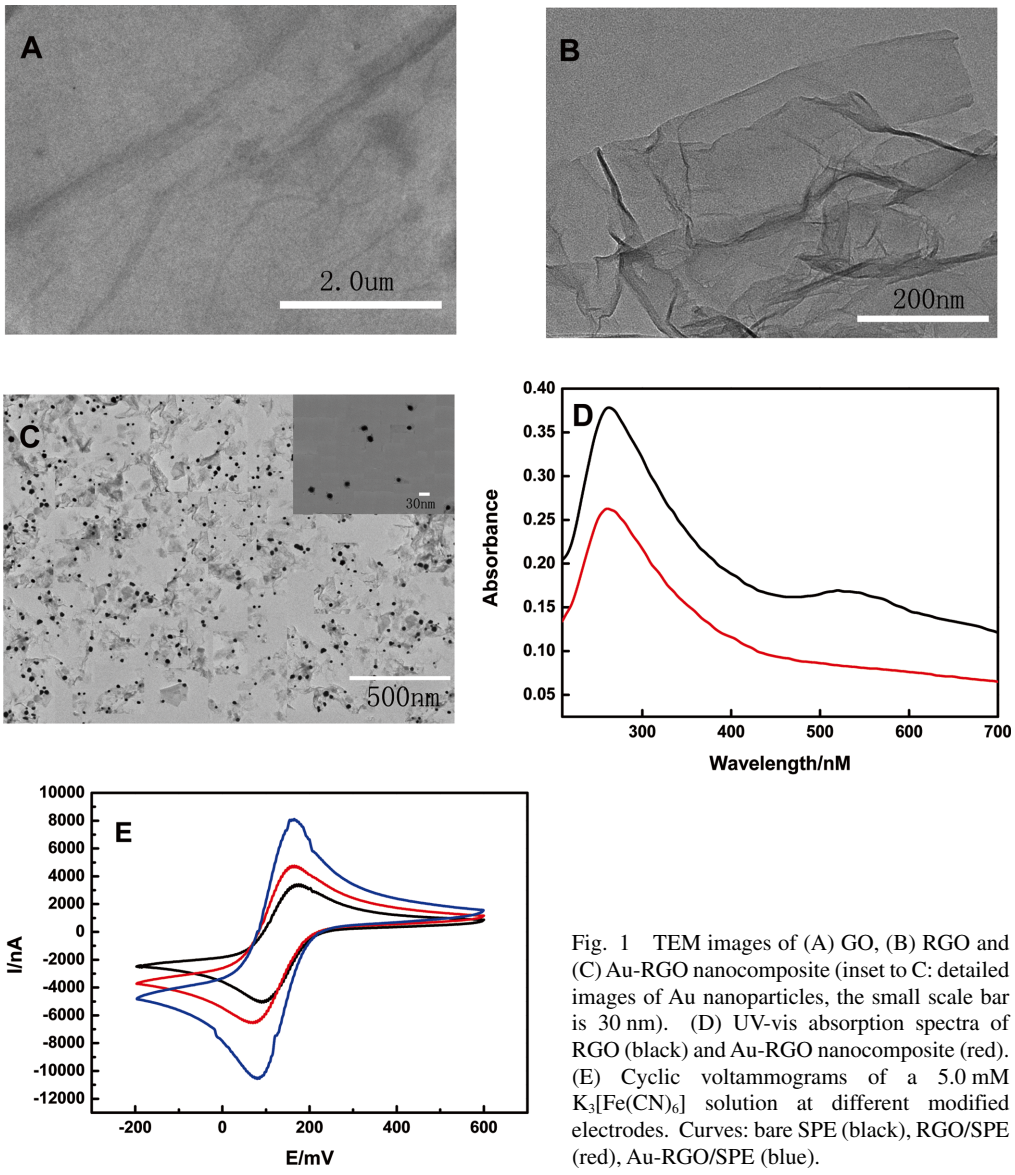

Fig. 1 TEM images of (A) GO, (B) RGO and (C) Au-RGO nanocomposite (inset to C: detailed images of Au nanoparticles, the small scale bar is 30 nm). (D) UV-vis absorption spectra of RGO (black) and Au-RGO nanocomposite (red). (E) Cyclic voltammograms of a 5.0 mM $K_3[Fe(CN)_6]$ solution at different modified electrodes. Curves: bare SPE (black), RGO/SPE (red), Au-RGO/SPE (blue).

Hybridization

Hybridization with synthetic oligonucleotides. The probe-modified SPE was inserted into a 500-μL Eppendorf tube, which contained a 20-μL target sequence solution (buffered with TE; pH 7.4) for 1 h at 40°C. After hybridization, the DNA modified electrode was washed with PBS (pH 7.4) and ultrapure water to remove non-hybridized nucleic acid.

Hybridization with PCR-amplified samples. To investigate the real samples hybridization, the process was as follows: DNA fragments after amplifying were diluted 20 times with PBS (pH 7.4). The double-stranded DNA was first treated for 5 min by being thermally denatured at 95°C, and then freezed in ice for more 3 min. Then, the hybridization procedure was processed the same as before.

Electrochemical measurement

Probe immobilization and DNA hybridization were monitored by the change of the MB response. Before an electrochemical measurement, SPE was immersed into stirring PBS (pH 7.4) with 20 mM MB for 20 min. Then, the electrode was washed in a stirred blank PBS buffer (pH 7.4) without MB for 5 min, and rinsed with ultrapure water to remove any unbound MB compounds.

The SWV measurement conditions were as follows: frequency, 10 Hz; amplitude, 5 mV; scan rate, 50 mV s^{-1}; step potential, 5 mV. The current responses were recorded while scanning in the range of 0 to –700 mV.

The average values of three measurements were calculated, and LOD was defined as the ratio between three-times the standard derivation of the blank group and the linear slope in our work.

Results and Discussion

Characterizations of the GO, RGO and Au-RGO nanocomposite, and electrodes

In order to guarantee the successful preparation of modified materials, we used UV-vis spectroscopy and TEM to investigate the characterizations during the experiment procedure. As shown in Fig. 1A, GO was in a flaky shape, and slight wrinkles could be seen on the surfaces. The result corresponds to a report before.[26] RGO nanomaterials were observed on copper meshes. From the TEM, we could see silk waves and wrinkles

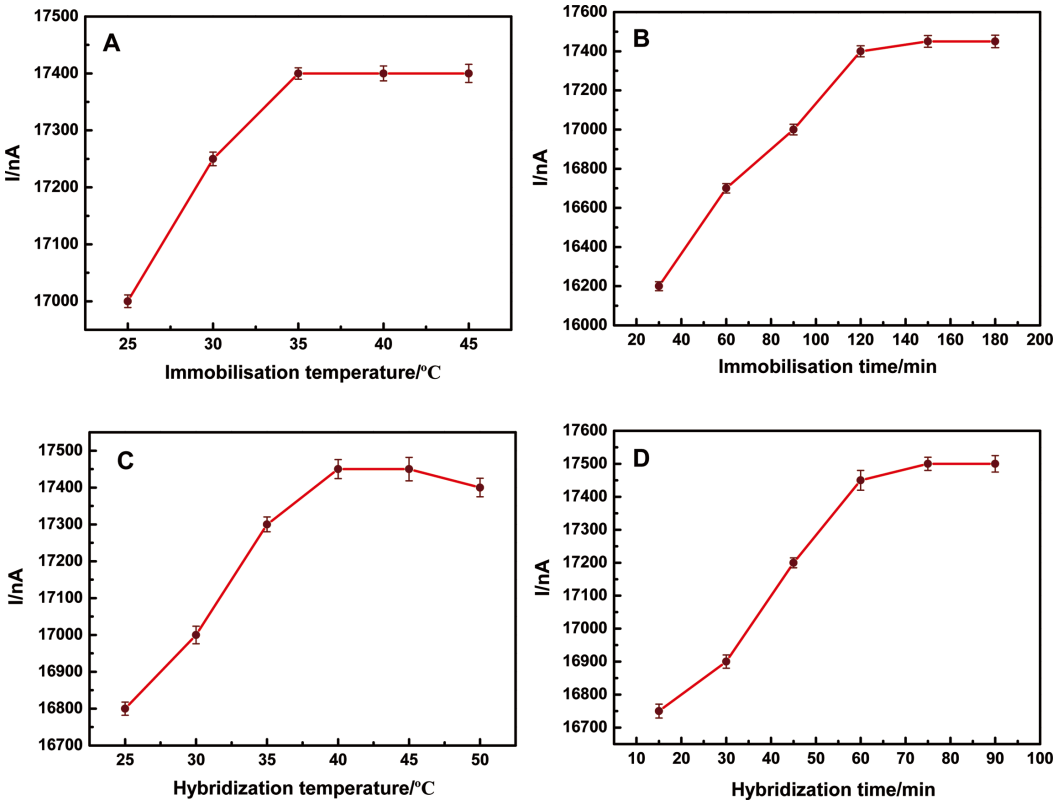

Fig. 2 Optimization of the experimental conditions. (A) and (B) Effect of the probe immobilization temperature and time on the SWV responses of MB. (C) and (D) Effect of the DNA hybridization temperature and time on the SWV responses of MB (complementary DNA = 1.0×10^{-11} M, $C_{MB} = 2.0 \times 10^{-5}$ M).

on the surface of the RGO. They folded and intertwined with each other to ensure their structural stability (see Fig. 1B). Figure 1C shows that the Au nanoparticles were dispersed on the RGO sheets surface evenly with a dark dots shape (Fig. 1C). The magnified image (inset of Fig. 1C) demonstrates that the Au nanoparticles almost had a round shape with an average diameter of around 20 nm. Microscopic characterization indicates the successful reduction of GO and binding with gold nanoparticles, which is consistent with the UV-vis curves in Fig. 1D (RGO displays a typical absorption peak at 260 nm, and a new Au nanostructures absorption band appeared at 525 nm after an ultrasonic treatment).

Electrochemical cyclic voltammetry was applied to investigate different characteristics of the biosensor in a 5.0 mM $[Fe(CN)_6]^{3-/4-}$ solution. The peak current allows a clear rise when the gold electrode was covered by RGO (red line), in contrast to the bare electrode (black line) due to its good electron-transport properties, as shown in Fig. 1E. After an Au-RGO nanocomposite was assembled, the current response saw a significant rise (blue line) for more than twice as much as response on a bare electrode, which shows that Au-RGO is an ideal conductive material, and could highly increase the electron-transfer rate of $[Fe(CN)_6]^{3-/4-}$.

Optimization of the hybridization time and temperature

Different conditions controlled in the experiment could greatly affect the electrochemical response of the sensor. It was assumed that probe immobilization and hybridization processes were the most important reaction in this experiment. Therefore, the effects of time and temperature in these two procedures were investigated. Figure 2A shows that the electrochemical response of MB gradually increased from 25 to 35°C, and reached a plateau after 35°C. The results in Fig. 2B demonstrate that the probe self-assembly on the electrode surface reached a plateau after about a 120-min treatment. As can be seen from Fig. 2C, by changing the hybridization temperature from 25 to 50°C, the electrochemical response of MB was gradually increased, and reached its highest at 40°C; it then decreased when the temperature became higher due to the double-stranded DNA denatured in the high temperature, preventing the aggregation of MB on the surface of electrode. The peak response was enhanced significantly from 15 to 60 min, but remained stable after a 1 h reaction, as shown in Fig. 2D, which indicated that the hybridization process was nearly completed within 60 min. According to the experimental results, 35°C and 2 h were chosen for the immobolisation process, and 40°C and 60 min were selected as the optimal conditions for the hybridization reaction on the electrode.

Performance of synthetic target oligonucleotides

Under the optimal conditions, in order to investigate the performance of this biosensor in detecting synthetic targets, we exposed electrodes to 1.0×10^{-14} - 1.0×10^{-9} M complementary target DNA with 60 min DNA hybridization at 40°C to measure the linear response range of the electrochemical biosensor. We can see that the electrochemical signal of MB greatly increased after hybridizing with complementary DNA, which is similar to some papers published before.[27-29] Also, the signal increased gradually with the increasing concentrations of complementary DNA in Fig. 3A. The response values of MB were linear with

the logarithm of the target DNA concentrations from 1.0×10^{-9} to 1.0×10^{-14} M with a detection limit of 3.36×10^{-15} M, as shown in Fig. 3B. The linear-regression equation was $\Delta I = 2556.102x + 37216.11 \times \log C$, and the correlation coefficient was 0.994. By comparing with other similar biosensors reported before in Table 1, we can see that the biosensor introduced in our work showed a wider screening range of DNA and a lower detection limit.

Reproducibility and stability of this biosensor

In this experiment, we also chose six modified electrodes as a parallel group to study the reproducibility and stability of the biosensor. Each electrode was treated under the same conditions, and they were applied to detect 1.0×10^{-11} M complementary DNA each day for one week independently. As shown in Fig. 4, the RSD for the six electrodes on the first day was 0.4%, and there were no obvious response changes for each electrode during the following 7 days, which indicated that this biosensor has excellent reproducibility and stability.

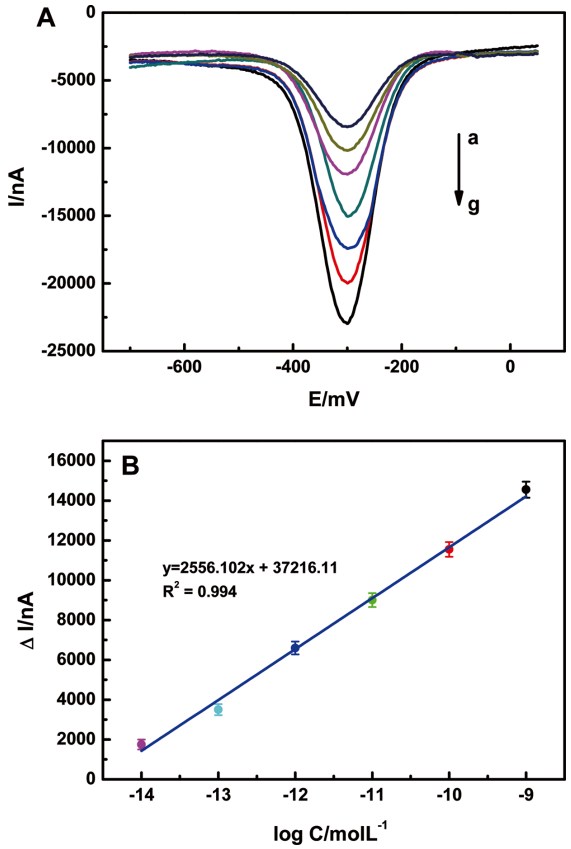

Fig. 3 (A) SWV signals of the probe hybridized with various concentrations of complementary DNA on the surface of electrodes: (a) 0 M; (b) 1.0×10^{-14} M; (c) 1.0×10^{-13} M; (d) 1.0×10^{-12} M; (e) 1.0×10^{-11} M; (f) 1.0×10^{-10} M; (g) 1.0×10^{-9} M. (B) Logarithmic plot of peak current of MB (ΔI) *versus* the concentration of complementary DNA.

Detection with real rice samples

All aforementioned experiments were performed using 100% GMO DNA. According to European Regulation EC 1830/2003, whether in food or a feed product, any authorized transgene ingredient exceeding 0.9% (w/w) demands an identification label.[34] In order to verify the selectivity of this screening tool, and whether it can meet this regulatory threshold requirement, we investigated six rice flour samples, which contained 100%, 0.9% and 0% (w/w) of GM rice in near isogenic samples, "Minghui 63", and three rice samples bought from the market. As Fig. 5(A) demonstrates, the peak current of 100% Bt63 rice was much larger than other kinds of rice. Besides, the peak difference of the threshold 0.9% rice was significantly higher than that of the control group and the peak current of three samples was almost the same as the negative control group, which were supported by the results of gel electrophoresis. The results indicate that this biosensor has good selectivity, and could be used to detect the real rice products.

Conclusions

In this work, a simple and effective method was reported for preparing an Au-RGO nanocomposite assembled electrochemical biosensor. This biosensor showed high sensitivity towards Bt63 rice analysis with a wide detection range from 1.0×10^{-9} to 1.0×10^{-14} M and a low detection limit of 3.36×10^{-15} M. It also had excellent reproducibility and stability, and could be used for transgenic rice sample analysis successfully. Thus,

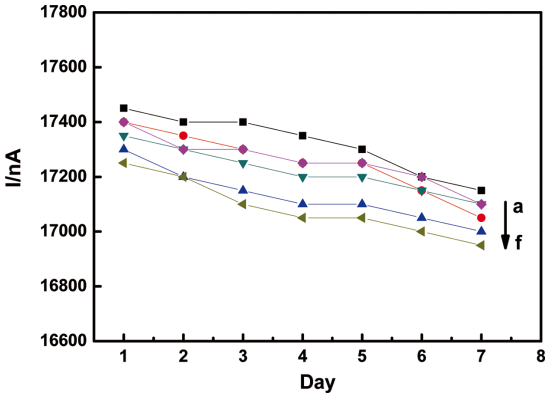

Fig. 4 Signal responses of six electrodes (a – f) obtained within one week.

Table 1 Compared with other different electrochemical DNA biosensors reported before

Modification type	Detection method	Linear range/M	Detection limit/M	Reference
Au NRs-GO	DPV with MB as indicator	1.0×10^{-14} – 1.0×10^{-9}	3.5×10^{-15}	29
Au NPs/rGO	DPV with Adriamycin as indicator	1.0×10^{-13} – 1.0×10^{-8}	3.5×10^{-14}	30
NanoPANI-ZrO_2/tyrosine	EIS (label free)	1.0×10^{-13} – 1.0×10^{-6}	2.68×10^{-14}	31
PDDA/PDC-SWNTs	DPV with MB as indicator	1.0×10^{-11} – 1.0×10^{-6}	2.6×10^{-12}	32
Nanogold/nanoPANI-chitosan	EIS (label free)	1.0×10^{-12} – 1.0×10^{-6}	3.1×10^{-13}	33
Au-RGO	SWV with MB as indicator	1.0×10^{-14} – 1.0×10^{-9}	3.36×10^{-15}	This work

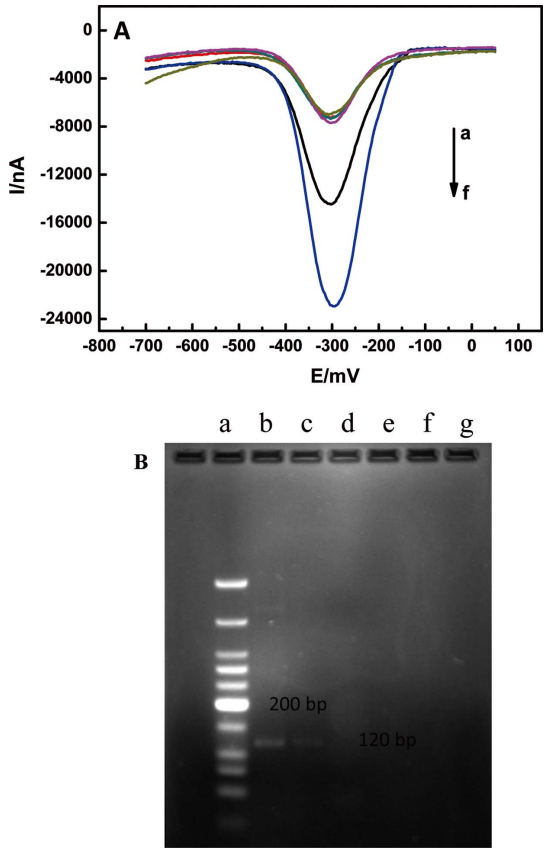

Fig. 5 (A) SWV curves of the biosensor hybridized with six rice samples: (a) negative non-GMO rice; (b), (c), (d) three rice samples chosen from the market randomly; (e), (f) 0.9 and 100% (w/w) positive rice. (B) Agarose gel electrophoresis of different kinds of rice (m/m): (a) marker; (b) 100%; (c) 0.9%; (d) 0%; (e) sample 1; (f) sample 2; (g) sample 3.

this biosensor was considered to have a bright prospect in field analysis, and could be used to monitor the Bt63 event on the market.

Acknowledgements

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