



Investigation of the effect of phytohormone on the expression of microRNA-159a in *Arabidopsis thaliana* seedlings based on mimic enzyme catalysis systematic electrochemical biosensor

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ABSTRACT

MicroRNAs (miRNAs) play very important roles in plant growth and development as well as phytohormones. More importantly, microRNAs were recently found to be a new growth regulator involved in plant hormone signaling. Therefore, for investigating the expression change of microRNAs in plants exposed to phytohormones and understanding the effect of phytohormones on microRNAs expression, we developed a simple, sensitive, and label-free method for microRNAs biosensing based on mimic enzyme catalysis signal amplification, where carboxylic graphene–hemin hybrid nanosheets was synthesized and used to catalyze the oxidation reaction of hydroquinone in the presence of H_2O_2 due to the intrinsic peroxidase-like activity of hemin on the carboxylic graphene surface. The electrochemical reduction current of the oxidative product of benzoquinone was depended on the hybridization amount of microRNAs and used to monitor the microRNAs hybridization event. Under optimal detection conditions, the current response was proportional to the logarithm concentration of microRNA-159a from 0.5 pM to 1.0 nM with the detection limit of 0.17 pM ($S/N=3$). The fabricated biosensor showed highly reproducible (Relative standard deviation (RSD) was 3.53% for 10 biosensors fabricated independently) and detection selectivity (Even discriminating single-base mismatched microRNA sequence). We also found that abscisic acid, a kind of phytohormone, had greatly influence on microRNA-159a expression in *Arabidopsis thaliana* seedlings. With increasing abscisic acid concentration and prolonging incubation time, both the expression level of microRNA-159a increased. This graphene–hemin-based approach provides a novel avenue to detect microRNA with high sensitivity and selectivity while avoiding laborious label, disadvantages of bio-enzymes and complex operations for microRNAs separation and enrichment, which might be attractive for genetic analysis and clinic biomedical application.

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1. Introduction

Phytohormones, such as auxins, gibberellins, cytokinins, abscisic acid (ABA), ethylene, jasmonic acid and brassinosteroids, are identified as key regulators in plant growth and development. Almost every aspect of plant growth and development is under hormonal control to some degree (Gray, 2004; Liu and Chen, 2009). Recently researches illustrated that phytohormones not only act to control many aspects of plant growth and development, but also response to the environment stresses (Klee, 2003). Their signaling pathways can be effectively controlled by modulation of positive and negative regulators during plant growth and development (Huq, 2006). MicroRNAs (miRNAs) are a large family of about 21–22 nucleotide non-coding RNAs, which play very important roles in posttranscriptional gene regulation through

degrading target mRNAs or repressing targeted gene translation in plants. More importantly, microRNAs were recently found to be a new growth regulator involved in plant hormone signaling (Liu et al., 2009). Gibberellic acid has been shown to modulate microRNA-159 levels during floral development (Achard et al., 2004), and auxin induce microRNA-164 to clear *NAC1* mRNA for resetting auxin signaling (Guo et al., 2005). Therefore, the expression level change of microRNA in plant could give expression to the effect of phytohormones on plant growth and development. In order to achieve this purpose, a sensitive and specific method for microRNA detection is necessary.

The traditional and standard detection techniques for microRNAs are northern blotting (Pall et al., 2007), microarray analysis (Nelson et al., 2004), and real-time quantitative polymerase chain reaction (qRT-PCR) (Peltier and Latham, 2008). Though these techniques have been widely used, there are some limitations, such as time-consuming, poor reproducibility, expensive instruments, long and complicated pretreatment process. In addition, these assay technologies require

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well-trained operators, which limit their application in clinical diagnosis. With advances in technology, several new methods have been applied to detect microRNA, such as colorimetry (Wen et al., 2012), fluorescence (Dong et al., 2012b; Tu et al., 2013; Yang et al., 2012), surface Plasmon resonance (SPR) (Fang et al., 2006), and electrochemical biosensor (Cai et al., 2013; Dong et al., 2012a; Wu et al., 2013). Among these methods, electrochemical biosensors, especially enzyme-based biosensors, have been attracted more and more attentions due to the advantages of simple operation, time-saving, inexpensive and pony-size instrument, high sensitivity and good reliability. Pählmann and Sprinzl (2010) established a rapid, selective, and sensitive gap hybridization assay for detection of microRNAs based on four components DNA/RNA hybridization and electrochemical detection using esterase 2-oligodeoxynucleotide conjugates. Gao et al. (2013) employed horseradish peroxidase (HRP) to catalyze polymerization of 3,3'-dimethoxybenzidine in the presence of H_2O_2 to form the insulating polymer film after microRNA hybridization event. The interface electron transfer resistance increased with increasing microRNA concentration and the detection limit was 2.0 fM. Cai et al. (2013) designed a label-free electrochemical sensor based on functional allosteric molecular beacon probe and streptavidin-HRP (SA-HRP). The probe contained a SA aptamer sequence blocked by its hairpin structure. After incubation with target microRNA, the hybridization between the microRNA and probe caused the stem to open and the formation of SA aptamer structure to bind with SA-HRP for oxidizing 3,3',5,5'-tetramethylbenzidine (TMB), and finally generated catalytic current for reducing TMB_{ox} at the electrode surface. In our previous work, we have developed several electrochemical methods for microRNAs detection based HRP catalysis signal amplification in the presence of H_2O_2 and hydroquinone (Yin et al., 2012a, 2012b; Zhou et al., 2012).

Though HRP-based electrochemical biosensor can detect microRNAs with high detection sensitivity, there are several disadvantages which are not conducive to HRP application in microRNAs detection. First, the biological enzymes are expensive. Second, the relative large protein structure of HRP is adverse to signal amplification. Third, as a natural enzyme, the bioactivity of HRP is highly dependent on protein conformation, leading to vulnerability to external conditions (Zhang et al., 2012). Thus, the mimicking of HRP is of great importance and remains a challenging topic.

Hemin (iron protoporphyrin) is the active center of hemin-proteins, which has the peroxidase-like activity similar to the HRP (Zhang and Dasgupta, 1992). So hemin has been widely used as mimic peroxidase and applied in electrochemical biosensors for biomolecule detection (Wang et al., 2013; Zhang et al., 2010). However, because the catalytic activity of hemin is lower than HRP, for obtaining enhanced catalytic activity and high-efficiency signal amplification effect, graphene supported hemin complex has been designed as highly active biomimetic oxidation catalyst through π - π stacking (Guo et al., 2011a; Xue et al., 2012). And this complex has provided excellent opportunities for applications in the fields of artificial enzyme mimetics-based biosensors for detection of cholesterol (Zhang et al., 2012), cancer cells (Song et al., 2011) and glucose (Guo et al., 2011b).

In this work, we prepared carboxylic graphene-hemin hybrid nanosheets by simple wet-chemical strategy through the π - π interaction according to previous report. Compared with previously reported graphene-hemin complex, the carboxylic graphene-hemin complex not only possessed high catalytic activity, but also presented excellent water dispersibility. Based on it, an artificial enzyme mimetics biosensor was fabricated for microRNA detection and investigation of the effect of phytohormone on the expression of microRNA in *Arabidopsis thaliana* seeds using hairpin structure DNA probe and bio bar code functionalized gold nanoparticles (AuNPs). For proving the applicability of the developed

method, the effect of abscisic acid on the microRNA expression in *A. thaliana* seeds was investigated.

2. Experimental

2.1. Reagents and instruments

Carboxylic graphene (CGr, purity > 99.8%, carboxyl ratio ~5.0 wt%, single layer ratio ~80%, thickness 0.8–1.2 nm) was purchased from Nanjing XFNano Materials Tech Co. Ltd. (Nanjing, China). EDC, NHS, tris-(2-carboxyethyl)phosphine hydrochloride (TCEP) and hydroquinone were purchased from Sigma (USA). Mercaptopropionic acid (MPA) was supplied by Alfa Aesar (USA). Chloroauric acid ($HAuCl_4 \cdot 4H_2O$) was obtained from Shanghai DiBo chemical technology Co., Ltd. (Shanghai, China). Diethylenecarbonate (DEPC) and hemin were from Solarbio (China). H_2O_2 (30%) was from Tianjin BASF chemical Co., Ltd. (Tianjin, China). AuNPs were synthesized according to previous report with the average diameter of 13 nm (Liu and Lu, 2006), which were characterized by transmission electron microscopy (TEM). Bio bar code functionalized AuNPs was prepared according to previous report with minor revision (Hu et al., 2008; Yin et al., 2012b) (see Supporting information). DNA and microRNA sequences were synthesized and purified by Sangon (Shanghai, China) and Takara (Dalian, Chian), respectively. Their sequences were as follows. Probe DNA S1: 5'-SH-(CH₂)₆-GGCCGTAGAGTCCCTTCAATCCAAAACATGATACGGCC-3'; microRNA-159a: 5'-UUUGGAUUGAAGGGAGCUCUA-3'; DNA S2: 5'-SH-(CH₂)₆-GGCCGTATCATGTT-3'; DNA S3: 5'-SH-(CH₂)₆-GGCCGTATCATGTT-NH₂-(CH₂)₆-3'; single-bases mismatched microRNA: 5'-UUUGGAUUGGAGGGAGCUCUA-3'; three-bases mismatched microRNA: 5'-UUCGGAUUGGAGGGAGCUAUA-3'; non-complementary microRNA: 5'-UAGCUUAUCAGACUGAUGUUG-3'.

The buffer solutions employed in this work are as follows. Probe immobilization buffer: 10 mM Tris-HCl, 1.0 mM EDTA, 1.0 M NaCl, and 1.0 mM TCEP (pH 7.0), microRNA hybridization buffer: 1 × SSC, DNA hybridization buffer: 10 mM Tris-HCl, 1.0 mM EDTA, and 1.0 M NaCl (pH 7.0), electrochemistry determination solution, 0.1 M PBS (pH 7.4) containing 0.5 mM H_2O_2 and 0.1 mM hydroquinone, washing buffer: 10 mM Tris-HCl buffer (pH 7.4). All reagents were analytically pure grade. All of the solution and redistilled deionized water used were treated with DEPC and autoclaved to protect from RNase degradation.

Electrochemical experiments were performed on a CHI832D electrochemical workstation (CH Instruments Inc., USA) with conventional three-electrode system consisting of GCE, saturated calomel electrode and platinum wire as working, reference and counter electrodes, respectively. The UV-vis absorption spectra were obtained with a UV-2450 UV-vis spectrophotometer (Shimadzu Co., Kyoto, Japan). The transmission electron micrograph (TEM) was obtained using a JEM-1200EX TEM instrument (JEOL, Japan) and Tecnai G2 20 TEM instrument (FEI, USA).

2.2. Preparation of graphene-hemin complex

Graphene-hemin complex was prepared according to previous reports (Guo et al., 2011a). Before assembly of hemin and covalently conjugating on carboxylic graphene surface, carboxylic graphene was first cut into small pieces by ultrasonication (Fig. 1B). Then, 500 μ L carboxylic graphene dispersion (1 mg/mL, dispersed in water) was added into 500 μ L hemin solution (1 mM in methanol). The solution was stirred mildly for 2 h to allow conjugation between graphene and hemin. After centrifugation at 12,000 rpm for 20 min, the obtained precipitate was washed twice with methanol and water to obtain graphene-hemin composite, which was re-dispersed into 500 μ L water. For reacting with -NH₂ at the 3'-end of DNA S3 on bio bar code, the graphene-hemin

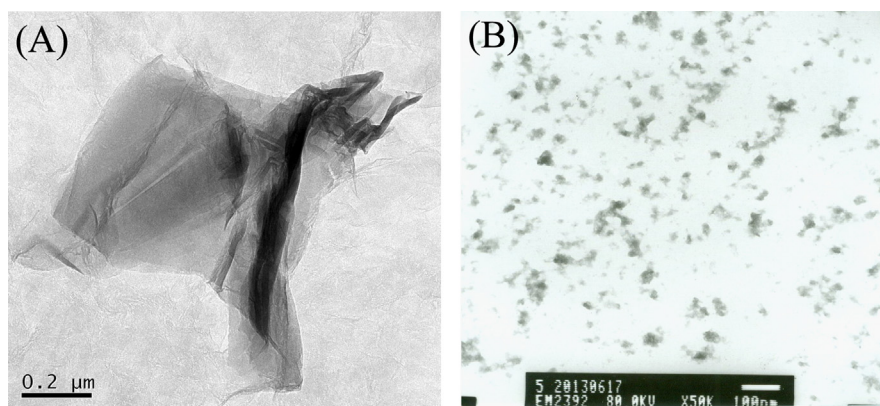
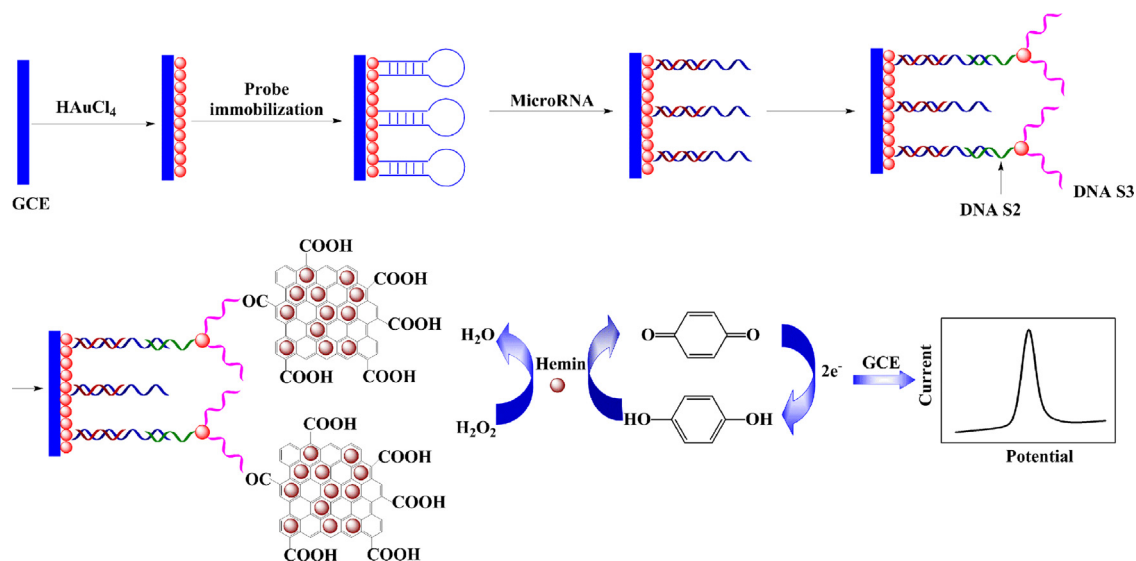


Fig. 1. TEM images of carboxylic graphene before (A) and after (B) ultrasonication.



Scheme 1. Illustration of microRNA detection using mimic enzyme catalysis systematic electrochemical biosensor.

complex was activated with the mixed solution of EDC(25 mM)/NHS(25 mM) for 3 h.

2.3. Biosensor preparation

Prior to modification, a GCE (3 mm in diameter) was successively polished to a mirror finish using 0.3 and 0.05 μm alumina slurry. After the electrode was rinsed thoroughly with double distilled deionized water and dried under nitrogen flow, the GCE was immersed into 3 mM HAuCl_4 solution containing 0.1 M KNO_3 and the AuNPs were electrochemically deposited on GCE surface using single-potential mode at -0.2 V for 250 s. The obtained AuNPs/GCE was washed with double distilled deionized water and dried at room temperature. Then, 10 μL probe immobilization buffer solution containing 5.0×10^{-7} M probe DNA S1 was dripped on AuNPs/GCE surface and incubated for 12 h under humid conditions. The obtained probe modified electrode was noted as ssDNA/AuNPs/GCE. Afterwards, the electrode was further incubated with 10 μL 1.65×10^{-6} M MPA to obtain a well-aligned probe monolayer and remove the unspecific adsorbed probe. After rinsed three times with 10 mM Tris-HCl (pH 7.4), the probe modified electrode was further incubated with 10 μL hybridization buffer containing different concentrations of complementary microRNA-21 for 1.5 h at 37°C in a humidified chamber to complete the hybridization event. After that, the electrode was

rinsed three times with 10 mM Tris-HCl (pH 7.4) to remove the un-hybridized microRNA-159a. The obtained electrode was noted as microRNA/ssDNA/AuNPs/GCE.

Subsequently, the un-hybridized fragment of probe DNA S1 at its 3'-end was further hybridized with DNA S2 on bio bar code for 2 h. (The obtained electrode was noted as barcode/microRNA/ssDNA/AuNPs/GCE.) Then, the electrode was rinsed with 10 mM Tris-HCl (pH 7.4) and incubated with 9 μL carboxylic graphene-hemin complex (activated by EDC/NHS) for 1 h to complete the chemical reaction between the activated $-\text{COOH}$ and $-\text{NH}_2$ at the 3'-end of DNA S3 on bio bar code. Finally, the obtained electrode (graphene-hemin/barcode/microRNA/ssDNA/AuNPs/GCE) was rinsed thoroughly with washing buffer and dried under nitrogen flow. The electrode was stored in 10 mM Tris-HCl (pH 7.4) at 4°C in a refrigerator when not in use.

2.4. Electrochemical detection

For microRNA detection, the fabricated electrochemical immunosensor was first immersed into 10 mL of electrochemical detection solution under magnetic stirring for 7 min. Then, the reduction response of benzoquinone was recorded by differential pulse voltammetry (DPV) using the bare GCE. The parameters of DPV are as follows: initiative potential, 0.5 V, final potential, -0.2 V,

step potential, 0.004 V, amplitude, 0.05 V, pulse width, 0.05 s, pulse period, 0.2 s, quiet time, 2 s.

Electrochemical impedance spectroscopy (EIS) was performed with a CHI660C electrochemistry workstation (Austin, USA) in 5.0 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ (1:1) solution containing 0.1 M KCl at an open circuit potential over a frequency range from 10^{-1} to 10^5 Hz.

3. Results and discussion

3.1. Detection strategy

The detection strategy for microRNA based on the fabricated biosensor is illustrated in Scheme 1. The 5'-thiol modified probe was first assembled on AuNPs modified glassy carbon electrode (GCE) surface through Au-S bond. Then, the stem-loop structure of probe was unfolded when target microRNA was hybridized with probe DNA, which made the un-hybridized 3'-end of probe far away from the electrode surface. Afterwards, the residual single stranded fragment of probe was further hybridized with DNA S2 on bio bar code functionalized AuNPs, which led to the successful immobilization of amino functionalized DNA S3. Under the activation of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS), carboxylic graphene-hemin complex was assembled on the electrode surface through the formation of amido bond between DNA S3 and carboxylic graphene. Carboxylic graphene-hemin complex could effectively catalyze the hydroquinone oxidation in the presence of H_2O_2 . The oxidative product of benzoquinone could be electrochemically reduced and generate a sensitive reduction current response, which was used to monitor the microRNA hybridization event.

3.2. Characterization of carboxylic graphene

The morphology of carboxylic graphene was presented in Fig. 1A. It was clear that the carboxylic graphene had large, transparent and rippled nanosheets structure. After ultrasonic processing, the large carboxylic graphene nanosheets were successively cut into small pieces (Fig. 1B).

3.3. Characterization of graphene-hemin complex and its catalytic activity

The prepared carboxylic graphene-hemin complex was characterized by UV-vis spectra. As seen in Fig. 2A (curve a), the carboxylic graphene showed a strong adsorption peak at 226 nm attributed to the π - π^* transition of aromatic C=C bonds and a weak shoulder peak at 285 nm corresponds to the n - π^* transition of the C=O bond (Li et al., 2008). The spectrum of hemin solution contains two strong peaks at 385 nm and 344 nm attributed to the

Soret band, as well as a weak peak at 603 nm ascribed to the Q-bands (Guo et al., 2011a). The carboxylic graphene-hemin complex exhibited a strong absorption of carboxylic graphene at 225 nm with a weak shoulder peak at 290 nm and a broad and strong hemin absorption peak at 358 nm and a weak adsorption peak at 634 nm (curve c). Compared with the adsorption spectra of hemin, the Q-band of hemin in carboxylic graphene-hemin complex presented a large red shift about 31 nm, which can be attributed to the existence of the π - π interactions between carboxylic graphene and hemin (Guo et al., 2011a; Xu et al., 2009). This clearly confirmed that hemin molecules are attached to carboxylic graphene.

As we know, hemin is the activate site in HRP and exhibits the peroxidase-like activity similar to HRP (Zhang and Dasgupta, 1992). In this work, the catalytic activity of hemin in carboxylic graphene-hemin complex plays crucial role for signal amplification and microRNA detection. Therefore, the catalytic activity of carboxylic graphene-hemin was investigated using TMB as substrate in the presence of H_2O_2 . For comparison, the activity of carboxylic graphene and hemin was also investigated under the same conditions. As seen in Fig. 2B, carboxylic graphene showed no catalytic activity towards the oxidation of TMB by H_2O_2 . However, the carboxylic graphene-hemin complex exhibited satisfactory peroxidase-like activity as well as hemin, showing that the observed peroxidase-like activity could be attributed to hemin on the carboxylic graphene surface.

3.4. Characterization of biosensor

The assembly process of the modified electrode was characterized by EIS. As seen in Fig. 3A, GCE showed an obvious semi-circle in high frequency region, indicating the interface electron transfer resistance of 183.7 Ω (curve a). The semi-circle in high frequency region disappeared and only a straight line was observed for AuNPs/GCE (curve b), indicating a decreased interface electron transfer resistance. This decrease could be attributed to the electrodeposited AuNPs with high conductivity and improved the electron transfer rate of redox probe. Afterwards, the interface electron transfer resistance increased successively after probe immobilization (curve c) and hybridization with microRNA-159a (curve d), which was caused by the electrostatic repulsion between the negatively charged phosphoric acid backbone of oligonucleotide (DNA and microRNA) and the negatively charged $\text{Fe}(\text{CN})_6^{3-/4-}$, inhibiting the diffusion of redox probe. The hybridization reaction between DNA S2 on bio bar code and the 3'-end of hairpin-structure DNA probe S1 resulted in a significant increase on interface electron transfer resistance (curve e). The reason was same to the immobilization of probe DNA S1 and microRNA hybridization. After capturing carboxylic graphene-hemin complex

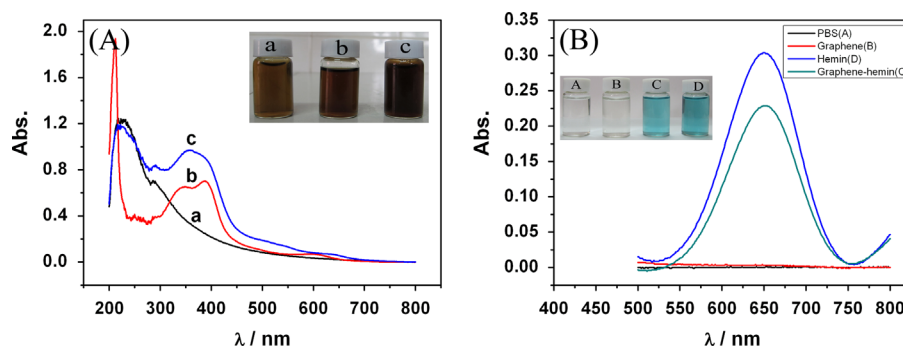


Fig. 2. (A) The UV-vis adsorption and photograph (Insert graph) of carboxylic graphene (a), hemin (b) and carboxylic graphene-hemin complex (c). (B) Typical absorption curves of TMB (1 mM) reaction solutions catalytically oxidized by carboxylic graphene (20 µg/mL), hemin (20 µg/mL) and carboxylic graphene-hemin complex (20 µg/mL) in the presence of 10 mM H_2O_2 at room temperature in phosphate buffer (0.1 M, pH 7.4) after reaction for 5 min. Insert: the corresponding photograph of TMB.

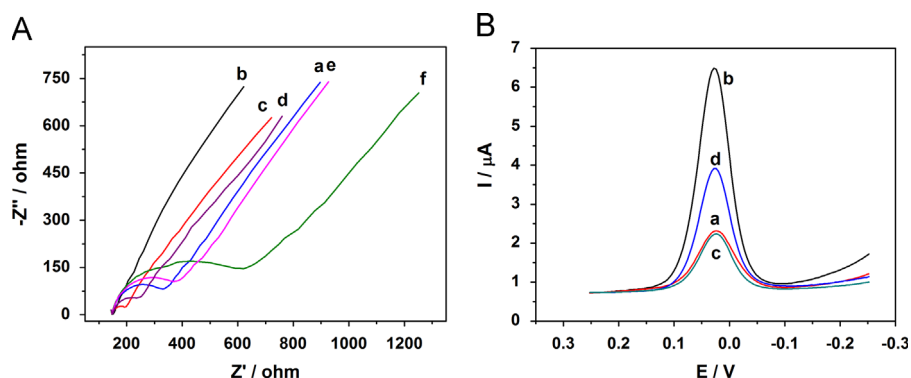


Fig. 3. (A) Nyquist diagrams of EIS recorded from 10^{-1} to 10^5 Hz in $\text{Fe}(\text{CN})_6^{3-/4-}$ (5 mM, 1:1) solution containing 0.1 M KCl at different electrodes. (a) GCE, (b) AuNPs/GCE, (c) probe/AuNPs/GCE, (d) miRNA-probe/AuNPs/GCE, (e) bio-bar-code/miRNA-probe/AuNPs/GCE, (f) graphene-hemin/bio-bar-code/miRNA-probe/AuNPs/GCE. (B) DPV response of 0.1 M PBS (pH 7.4) containing 0.5 mM H_2O_2 and 0.1 mM hydroquinone with (a) and without (b) graphene-hemin/bio-bar-code/miRNA-probe/AuNPs/GCE treatment. Curve c and curve d were the detection solutions treatment with graphene/bio-bar-code/miRNA-probe/AuNPs/GCE and graphene-hemin/miRNA-probe/AuNPs/GCE, respectively.

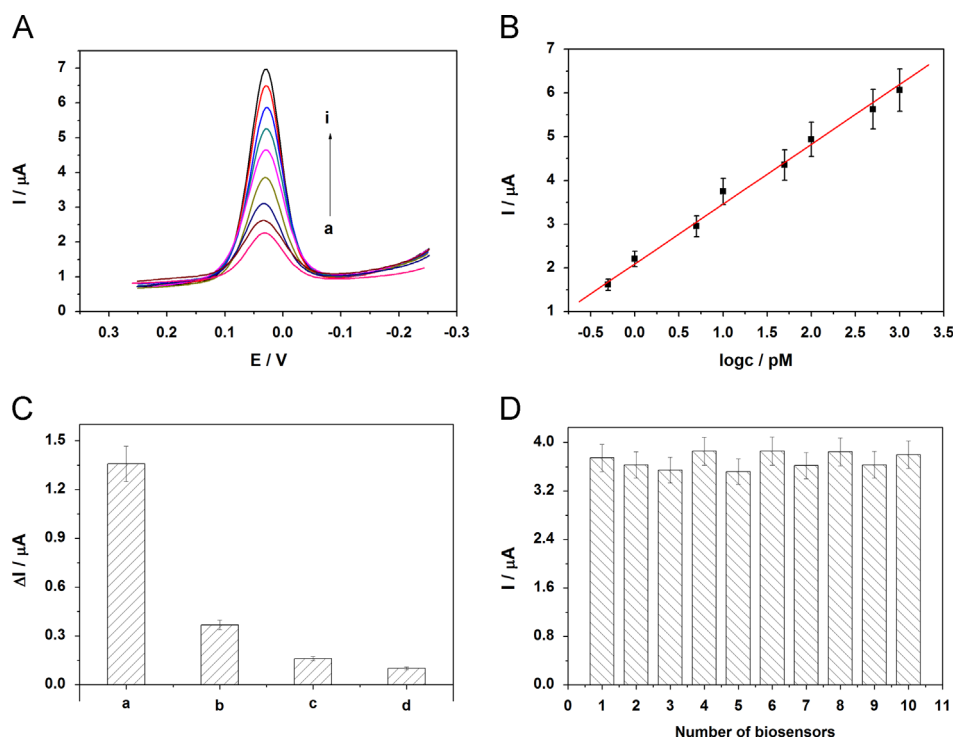


Fig. 4. (A) DPV response of the biosensor in 0.1 M PBS (pH 7.4) containing 0.5 mM H_2O_2 and 0.1 mM hydroquinone at 0, 0.5, 1, 5, 10, 50, 100, 500, 1000 pM target microRNA-159a (from a to i). (B) Plot of the reduction peak current versus the logarithm of target microRNA-159a concentration. (C) The reduction peak current change of biosensor in 0.1 M PBS (pH 7.4) containing 0.5 mM H_2O_2 and 0.1 mM hydroquinone for detection of 1 pM complementary target, single-base mismatched, three-base mismatched and non-complementary microRNA. (D) Reproducibility of 10 biosensors towards 10 pM microRNA-159a.

onto the electrode surface, the interface electron transfer resistance still enhanced (curve f). This increase was caused by two factors. One was the negative charged carboxylic group in graphene surface, which inhibited the diffusion of $\text{Fe}(\text{CN})_6^{3-/4-}$ towards electrode surface. The other was the inhibition of hemin towards the electron transfer of redox probe due to the steric hinderance (Zuo et al., 2007). These results also demonstrated the successful assembly of the biosensor.

3.5. The feasibility for microRNA detection

In order to testify the feasibility of the fabricated electrochemical biosensor for microRNA detection based on mimic enzymatic signal amplification using carboxylic graphene as carrier of mimic enzyme,

the DPV response of electrochemical detection solution before and after mimic enzymatic treatment was investigated. Fig. 3B shows the DPV responses of electrochemical detection solution with (curve b) and without (curve a) mimic enzymatic treatment. As expected, the reduction current increased significantly after the detection solution was treated with the fabricated biosensor, indicating that hemin immobilized on the electrode surface could effectively catalytic the oxidation reaction of hydroquinone by H_2O_2 , and enhanced the electrochemical reduction signal of benzoquinone. Based on the increased electrochemical reduction response, the developed method could be used to detect microRNA concentration. For further confirming the mimic enzymatic effects of hemin, the DPV was also recorded when the electrochemical detection solution was incubated Graphene/barcode/miRNA/ssDNA/AuNPs/GCE. As illustrated as curve c, the

DPV response was same to curve a, indicating that no catalytic effect was achieved without hemin.

For improving the detection sensitivity, the amplification unit of bio bar code was adopted to increase the immobilization amount of carboxylic graphene–hemin complex, and then enhance the current response. In order to prove it, the graphene–hemin/microRNA/ssDNA/AuNPs/GCE was first immersed into electrochemical detection solution, then the DPV response of this detection solution was recorded using the bare GCE as working electrode. As seen in Fig. 2B, the electrochemical reduction signal of benzoquinone decreased obviously when the electrochemical detection solution was incubated with graphene–hemin/microRNA/ssDNA/AuNPs/GCE (curve d). However, the DPV response increased significantly when the electrochemical detection solution was incubated with graphene–hemin/barcode/microRNA/ssDNA/AuNPs/GCE (curve b). Compared with the biosensors used in curve b and curve d, the only difference was located at the modification of bio bar code. Therefore, the increased DPV response could be attributed to the immobilization of bio bar code, which had the ability to amplify the detection signal and increase the detection sensitivity.

3.6. MicroRNA-21 detection performance

Under optimal experimental conditions (see Supporting information), the prepared electrochemical biosensor was examined with different concentrations of microRNA-159a. As shown in Fig. 4A, the reduction current increased with increasing the concentration of microRNA-159a. The reduction peak current was proportional to the logarithm concentration of microRNA-159a from 0.5 pM to 1.0 nM (Fig. 4B). The linear regression equation could be expressed as $I_{pc} (\mu A) = 1.37 \log c (pM) + 2.09$ ($R = 0.9957$) and the detection limit was estimated to be 0.17 pM ($S/N = 3$). The detection limit was higher than our previous work using HRP-based biosensor for microRNA detection (Yin et al., 2012b; Zhou et al., 2012), indicating that the catalytic activity of hemin was lower than HRP. However, this hemin-based biosensor is still a promising assay method due to the lower price of hemin than HRP.

In order to investigate the detection selectivity of the biosensor, the probe was first hybridized with different target microRNA with same concentration of 1 pM, such as complementary microRNA-159a, single-base mismatched, three-base mismatched and non-complementary microRNA sequences. Then, the electrode was further treated with bio bar code and carboxylic graphene–hemin complex. The electrochemical response (I_1) was recorded by DPV technique in 0.1 M PBS (pH 7.4) containing 0.5 mM H_2O_2 and 0.1 mM hydroquinone after 7 min of mimic enzymatic reaction. The obtained reduction peak current of benzoquinone was compared with the reduction current (I_2) originated from the same detection buffer without mimic enzymatic reaction. In Fig. 4C, $\Delta I (\mu A) = I_1 (\mu A) - I_2 (\mu A)$. It was clear that the ΔI for complementary microRNA-159a was 1.358 μA , which was much higher than that obtained at other biosensors where the probe hybridized with single-base mismatched (0.367 μA), three-base mismatched (0.161 μA) and non-complementary (0.101 μA) microRNA sequences. These results indicated the fabricated biosensor has good ability for identification of complementary sequences and mismatch sequences.

The reproducibility of the electrochemical biosensor was estimated by determining 10 pM microRNA-159a with 10 biosensors fabricated independently, and the results are shown in Fig. 4D. Ten biosensors exhibited similar DPV responses and the RSD was 3.53%. It indicated that the fabricated biosensor showed good reproducibility.

3.7. Effect of ABA on microRNA-159a expression in *A. thaliana* seeds

Phytohormones and microRNAs play crucial role in plant growth and development. Recent evidence has also implicated that phytohormones can regulate microRNA gene expression (Achard et al., 2004). In order to prove the applicability of the developed method and understand the dose–effect relationship between microRNA expression level and phytohormone, we investigated the effect of abscisic acid (a kind of phytohormone) on microRNA-159a expression. For achieving this aim, the germinated *A. thaliana* seeds were treated with abscisic acid under different times and concentrations. Then, the total RNA was isolated (see Supporting Information). As seen in Fig. 5A, the expression level of microRNA-159a increased quickly with extending treatment time of 1 $\mu g/mL$ abscisic acid towards *A. thaliana* seedlings from 0.5 to 3.5 h compared with the untreated control seedlings. When *A. thaliana* seedlings were treated for 3.5 h, the expression level of microRNA-159a increased 2.756 times. With further prolonging treatment time to 4 h, the increase of the expression level was inconspicuous. Fig. 5B presents the effect of abscisic acid concentration on microRNA-159a expression with treatment time of 4 h. The expression level increased rapidly with increasing abscisic acid concentration from 0.2 to 1.0 $\mu g/mL$, and then leveled off. According to these results, one can conclude that the concentration and incubation time of abscisic acid could greatly influence the expression level of microRNA-159a, which

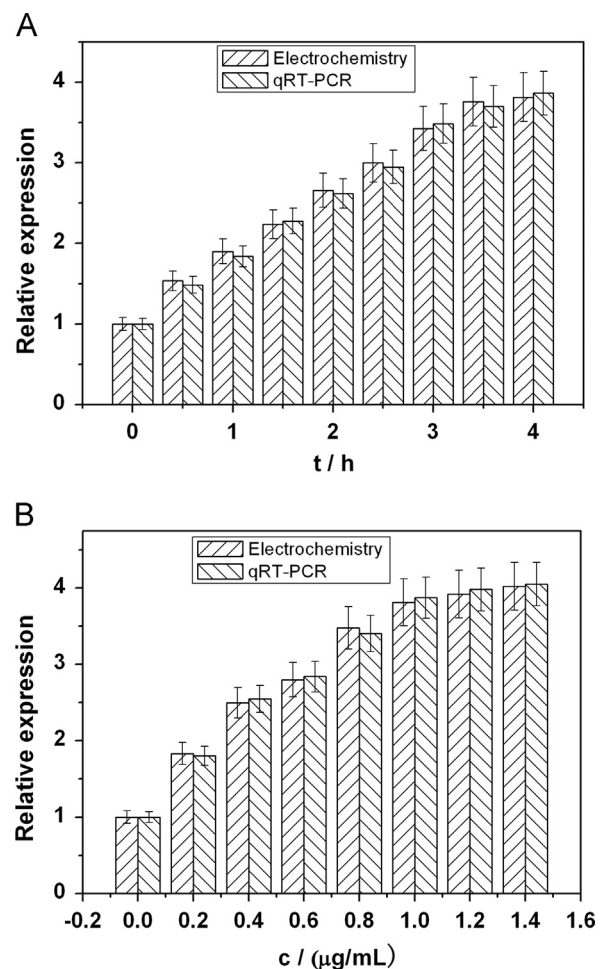


Fig. 5. The expression change of microRNA-159a extracted from *Arabidopsis thaliana* seedlings treated with abscisic acid under different time (A) and concentrations (B).

might provide useful information on understanding the regulation of plant growth and development by phytohormones and microRNAs. For confirming the accuracy of the fabricated biosensor, the expression level of microRNA-159a in *A. thaliana* seedlings was also detected by qRT-PCR, and the results were in accordance with them obtained by our method, which indicated that the developed might be applied in actual samples for microRNA detection.

4. Conclusion

In this work, a simple, sensitive, selective, and label-free microRNA biosensing strategy based on hairpin structure probe and the mimic enzymatic activity of carboxylic graphene–hemin complex toward hydroquinone oxidation by H_2O_2 was proposed. As far as possible to maximize signal amplification, AuNPs functionalized bio bar codes were selected to capture more carboxylic graphene–hemin complex, which was confirmed by the increased electrochemical reduction current. The high mimic enzymatic property of carboxylic graphene–hemin complex resulted in a sensitive electrochemical microRNAs biosensor, while the inherent selectivity of the hairpin structure probe endowed the biosensor with high base discrimination ability. Without tedious microRNAs extraction, enrichment and tagging procedures, the proposed biosensor is an attractive candidate for the development of a selective and sensitive microRNAs expression profiling platform.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bios.2013.11.026>.

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