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Electrochemical biosensor for microRNA detection based on poly (U) polymerase mediated isothermal signal amplification

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ABSTRACT

MicroRNAs play crucial role in post-transcriptional regulation for gene expression in animals, plants, and viruses. For the better understanding of microRNA and its functions, it is very important to develop effectively analytical method for microRNA detection. Herein, a novel electrochemical biosensor was fabricated for sensitive and selective detection of microRNA based on poly(U) polymerase mediated isothermal signal amplification, where poly(U) polymerase can catalyze the template independent addition of UMP from UTP to the 3' end of RNA. Using this activity, the target microRNA can be successfully labeled with biotin conjugated UMPs at its 3′-end using biotin conjugated UTP (biotin-UTP) as donor. Then, the avidin conjugated alkaline phosphatase can be further captured to the 3′-end of the target microRNA based on the specific interaction between biotin and avidin. Finally, under the catalytic activity of alkaline phosphatase, the substrate of *p*-nitrophenyl phosphate disodium salt hexahydrate can be hydrolyzed to produce 4-nitrophenol. According to the relationship between the electrochemical signal of *p*-nitrophenol and the concentration of microRNA-319a, the content of microRNA-319a can be detected. This signal amplification method is simple and sensitive. The developed method can detect as low as 1.7 fM microRNA and produce precise and accurate linear dynamic range from 10 to 1000 fM. The fabricated biosensor was further applied to detect the expression level change of microRNA-319a in rice seedlings after incubation with five kinds of different phytohormones.

1. Introduction

MicroRNAs are short, endogenous and noncoding RNA, which have been found in animals, plants and viruses, and play crucial roles in gene regulation, cell proliferation, cell apoptosis (Winter et al., 2009). It has been reported that the expression level change of microRNAs are not only associated with various human diseases, especially cancers, but also related to the signal transduction of phytohormone in plant growth and development process (Calin and Croce, 2006; Liu and Chen, 2009). In order to further understand the functions of microRNA in detail, it is necessary to develop sensitive and selective method for microRNA detection.

The traditional methods for microRNAs detection are northern blotting (Pall et al., 2007), microarray (Li and Ruan, 2009) and qRT-PCR (Kroh et al., 2010). Though these methods showed great applicability for microRNA detection, there are still some weaknesses. Northern blotting needs large amount of detection sample and time-consuming detection process. And this method can not quantitative detect microRNA with high sensitivity and specificity. Microarray requires high consumption and complicated detection process. Moreover, the microarray technique also suffers from the low distinguishing ability for microRNA precursor and matured microRNA. For qRT-PCR, the reverse transcription from miRNA to cDNA is rather difficult because of the small size of miRNA, which requires to design sophisticated sequences.

In order to overcome the disadvantages of the above methods, many other analytical techniques, including electrochemistry (Bartosik et al., 2014; Liu et al., 2015; Miao et al., 2015; Wang et al., 2014a; Wu et al., 2013; Yang et al., 2014a; Yu et al., 2014; Zhu et al., 2014), fluorescence (Dong et al., 2014; Ge et al., 2014a; Ho et al., 2014; Xi et al. 2014; Xu et al., 2014), colorimetry (Park and Yeo, 2014; Shen et al., 2013), UV–visible spectroscopy (Deng et al., 2014), photoelectrochemistry (Cao et al., 2014; Wang et al., 2014b; Yin et al., 2014), chemiluminiscence (Bi et al., 2015; Zhang et al., 2015b), electrochemiluminescence (Cheng et al., 2014; Hao et al., 2014; Zhang et al., 2015a), surface plasmon resonance (Ding et al., 2015; Vaisocherová et al., 2015; Zhang et al., 2013), have been

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developed for sensitive detection of microRNA. For obtaining high detection sensitivity, the appropriate and effective signal amplification technique is required. Among various signal amplification strategies, isothermal amplification approaches have attracted more and more attentions in the field of microRNA detection due to high signal amplification capability, such as rolling circle amplification (Ge et al., 2014a; Xu et al., 2014; Zhang et al., 2015a), hybridization chain reaction amplification (Ge et al., 2014b; Liu et al., 2015; Zhang et al., 2014), isothermal exponential amplification reaction (Jia et al., 2010; Yu et al., 2014), ligase chain amplification reaction (Yan et al., 2010; Zhu et al., 2014) and loop-meditated isothermal amplification (Li et al., 2011). All the above amplification strategies require specific nucleic acid sequence and complicated amplification process, which might limit the application of the above mentioned methods. In this regard, it still needs to develop efficient microRNA detection techniques with simple signal amplification strategy.

Recently, several reports have drawn our attentions on DNA detection using a kind of unique *DNA polymerase* named *terminal deoxynucleotidyl transferase* (TdT) (Lin et al., 2015; Wan et al., 2014a), where the TDT is a template independent polymerase that catalyzes the addition of deoxynucleotides to the 3′ hydroxyl terminus of DNA molecules. For instance, Wan et al. introduced TDT into the fabrication of electrochemical DNA biosensor (Wan et al., 2013). After probe DNA hybridized with target DNA, the TDT-mediated extension reaction of target DNA at its 3′-terminal was preformed, where biotin conjugated deoxyadenosine triphosphates (Biotin-dATPs) were added into the mixture of dNTP. Thus, the extension DNA chain was labeled with biotin, which resulted in the further capture of avidin conjugated horseradish peroxidase (HRP) on the electrode surface through the specific interaction between biotin and avidin. Based on the catalysis effect of HRP, the target DNA was detected. Yang et al. reported a template-free signal amplification strategy for highly sensitive and sequence-specific DNA detection based on *in situ* grown DNA nanotail (IGT) to capture the electrochemical signal probe of $[Ru(NH_3)_6]^{3+}$, where the IGT was produced under the catalysis of TDT in the presence of dNTP (Yang et al., 2014b). By introduction of TdT to this sensor design, both the sensitivity and selectivity have been significantly enhanced with the low detection limit of 20 fM for 22 nt DNA and the linear range of 0.1–1000 pM. Hu et al. (2015) developed a novel label-free amplified multifunctional strategy of dendritic electrochemical DNA sensor based on TdT mediated DNA extension and DNA dendritic structure signal amplification. The fabricated biosensor presented excellent detection sensitivity with low detection limit of 1 fM. The signal amplification strategy based on TDT shows several advantages, such as easy operation, multiplex labeling, high detection sensitivity and reaction carried out under isothermal condition.

Inspiring by these investigations, a simple and sensitive electrochemical method was developed for microRNA detection based on poly(U) polymerase mediated isothermal signal amplification, where poly(U) polymerase can catalyze the template independent addition of uridine monophosphate (UMP) from uridine triphosphate (UTP) to the 3′-terminal of RNA (Lehrbach et al., 2009; Rissland and Norbury, 2008). In this work, biotin conjugated UTP (biotin-UTP) was selected as UMP donor to form poly(U)-biotin tail at the 3′-terminal of hybridized microRNA. Based on the biotin-avidin bridge, a large amount of avidin conjugated alkaline phosphatase (avidin-ALP) was further captured to catalyze the hydrolysis reaction of *p*-nitrophenyl phosphate disodium salt hexahydrate (PNPP). Based on the electrochemical signal of *p*-nitrophenol (PNP), the microRNA detection can be achieved. The effect of phytohormone on the expression level of microRNA in rice seedlings was also investigated.

2. Experimental

2.1. 1 Materials and methods

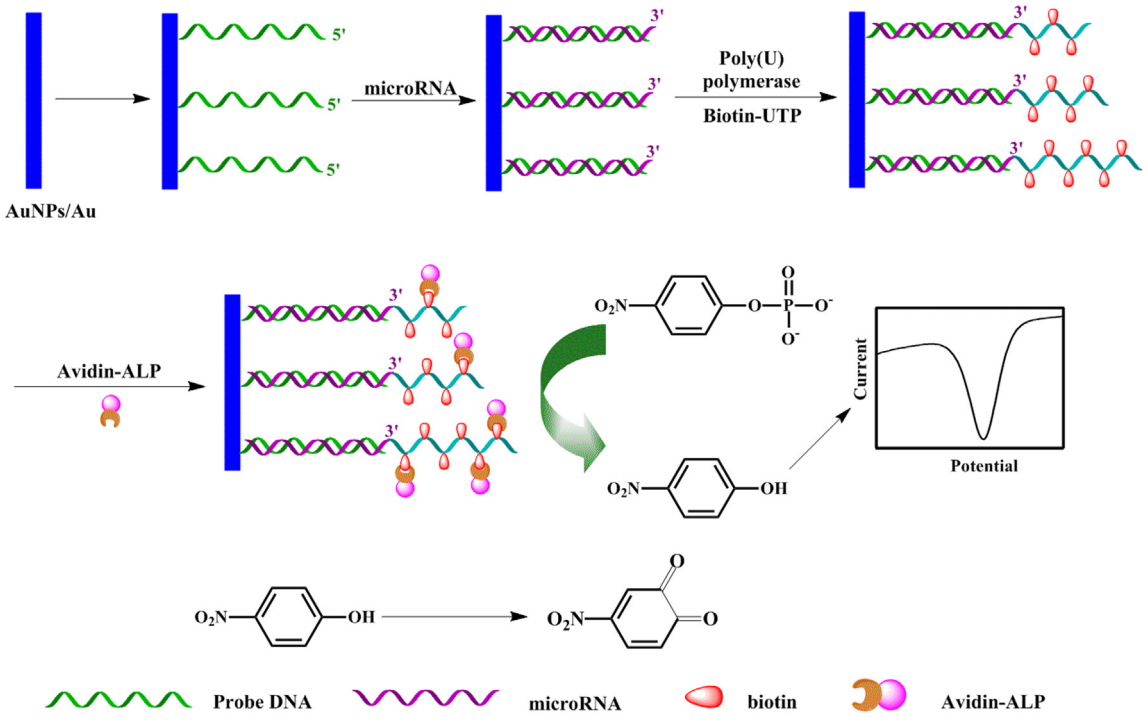
Biotin-UTP was purchased from Biocompare (USA). Gibberellin, abscisic acid, 6-benzyladenine, chloroauric acid and PNPP was obtained from Shanghai Dibo Chemical Technology Co., Ltd. (China). Poly(U) polymerase was provided by New England Biolabs (USA). Diethypyrocarbonate (DEPC) was supplied by Solarbio (China). Tris(hydroxymethyl)aminomethane (Tris), tris(2-carboxyethyl)phosphine hydrochloride, 3-indoleacetic acid and 3-indolepropionic acid were purchased from Aladdin (Shanghai, China). 3-Mercaptopropionic acid (MPA) was obtained from Alfa Aesar (UK). HPLC-purified microRNAs were purchased from Takara Biotechnology Co. Ltd. (Dalian, China). DNA probe and avidin-ALP was supplied by Sangon Biotech (Shanghai) Co.,Ltd. The oligonucleotides sequences were shown as follows. Probe DNA, 5′-SH-$(CH_2)_6$-GGGAGCACCCTTCAGTCCAA-3′; target microRNA-319a, 5′-UUGGACUGAAGGGUGCUCCC-3'; single-base mismatched microRNA, 5′-UUGGACUG***U***AGGGUGCUCCC-3′; three-base mismatched microRNA, 5′-UU***C***GACUGA***U***GGGUGCUC***G***C-3′; non-complementary microRNA, 5′-ACCAGCCAGGUCCAAUCUAA-3′. Biotin labeled microRNA-319a, 5′-biotin-UUGGACUGAAGGGUGCUCCC-3′. The nucleotide mismatches were indicated as italic and bold letters. Synthetic DNA and microRNA sequences were dissolved in TE buffer (pH 8.0) according to the manufacturer's recommendations, and stored at −20 °C and −80 °C, separately. All other reagents were analytically pure grade.

The buffer solutions used in this work were listed as follows. DNA immobilization buffer, 10 mM Tris–HCl, 1.0 mM EDTA, 1.0 M NaCl and 1.0 mM TCEP (pH 7.4). MicroRNA hybridization buffer, 1 × SSC (0.15 M sodium chloride and 15 mM sodium citrate). Poly (U) polymerase reaction buffer, 50 mM NaCl, 10 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM DTT (pH 7.9). Electrochemical detection buffer for differential pulse voltammetry (DPV), 10 mM Tris–HCl containing 0.6 mM PNPP and 0.1 mM $MgCl_2$ (pH 9.8). Washing buffer, 10 mM Tris–HCl containing 50 mM NaCl (pH 7.4). Electrochemical detection buffer for electrochemical impedance spectrum (EIS), 10 mM Tris–HCl containing 5 mM $K_3[Fe(CN)_6]$, 5 mM $K_4[Fe(CN)_6]$ and 0.1 M KCl (pH 7.4).

2.2. Sample treatment and analysis

The rice (it was supplied by Beijing Normal University, The name of rice variety is japonica rice Oryza sativa cv. Hwa-young) was cultivated and treated according to our previous work except that five kinds of plant hormones were used in this work (Wang et al., 2015). In brief, mature rice seeds were soaked and embedded in gauze for about 10 days at room temperature for germination with deionized water. After these seeds germinated, they were transferred to culture dishes and incubated at room temperature for further experiments on the effect of phytohormone on the expression level of microRNA-319a. For investigating the concentration effect, 50 mg/L of gibberellin, abscisic acid, 6-benzyladenine, 3-indoleacetic acid and 3-indolepropionic acid were sprinkled on three groups of the leaves of rice seedlings as water source, respectively. And then these rice seedlings were cultured for 24 h. For control, one group rice seedling was cultured with distilled deionized water as water source. Finally, the seedlings with different treatment were harvested for RNA extraction. The extraction procedure was performed by using RNA extraction kit according to the manufacturer′s recommended protocol (Invitrogen, USA).

In order to analyze the expression level of microRNA-319a in the extracted total RNA and confirm the effect of five kinds of phytohormones on microRNA-319a expression, the probe DNA on



Scheme 1. Schematic illustration of the biosensor fabrication and microRNA detection.

AuNPs/Au surface was hybridized directly with microRNA-319a in total RNA, and then the following experiment process was same with Sections 2.4 and 2.5.

2.3. DNA self-assembly and hybridization with microRNA-319a

Gold disc electrode (named as Au electrode) was used as basal electrode (Bhanjana et al., 2015). Firstly, the AuNPs/Au electrode was prepared by electrochemical deposition according to previous reports (Kumar et al., 2015; Yin et al., 2013). In brief, the Au electrode was immersed into 8 mL solution containing 3 mM $HAuCl_4$ and 0.1 M KNO_3, and then AuNPs were electrochemically deposited on the Au surface under single-potential mode (−0.2 V) for 200 s with magnetic stirring. After dried under N_2 blowing, 5 μL of probe immobilization buffer containing 1.0 μM thiolated DNA probe was dripped on the electrode surface and incubated for 12 h in a humid cell. The modified electrode was noted as DNA/AuNPs/Au and rinsed with washing buffer. Then, the DNA/AuNPs/Au was incubated with 1.0 mM MPA for 1 h to obtain mixed self-assembled monolayer. The electrode was rinsed with washing buffer and dried with N_2. After that, the electrode was incubated with 5 μL of microRNA hybridization buffer containing different concentrations of microRNA-319a for 2 h at 37 °C in a humid cell. The obtained electrode was rinsed with washing buffer for three times and noted as microRNA-DNA/AuNPs/Au.

2.4. Poly(U) polymerase-mediated strand extension reaction and ALP immobilization

Poly(U) polymerase-mediated strand extension reaction was preformed according to previous reports (Hu et al., 2015; Wan et al., 2014b) with some modifications and the specification of the manufacturer. In brief, 5 μL of poly(U) polymerase reaction buffer containing 30 unit/mL poly(U) polymerase and 1.0 μM biotin-UTP was dripped on the surface of microRNA-DNA/AuNPs/Au. The Poly (U) polymerase-mediated strand extension reaction was carried out at 37 °C for 75 min in a humid cell. After being washed with washing buffer for three times, the obtained electrode was dried under N_2 blowing and named as biotin-U/microRNA-DNA/AuNPs/Au. Afterwards, 5 μL of 10 mM Tris–HCl solution (pH 7.4) containing 20 μg/mL avidin-ALP and 50 mM NaCl was dripped on the electrode surface. After incubated for 50 min at the ambient temperature, the electrode was rinsed with washing buffer for three times. The fabricated electrode was noted as ALP/biotin-U/microRNA-DNA/AuNPs/Au.

2.5. Electrochemical detection

Electrochemical experiments were performed at CHI660C electrochemical workstation (Austin, USA) with three electrode system. The fabricated electrode was used as working electrode, the saturated calomel electrode (SCE) was as reference electrode and the platinum wire electrode was as the counter electrode. For differential pulse voltammetry (DPV), the scan potential is ranged from 0.4 to 1.2 V with the parameters of 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. The bismuth-coated carbon electrode (Bi/GCE) was prepared using i-t technique at −1.2 V for 200 s in 0.1 M acetate buffer (pH 4.5) containing 400 μg/L bismuth nitrate (Zhou et al., 2014).

For detecting microRNA-319a, 10 mM Tris–HCl (pH 9.8) containing 1 mM $MgCl_2$ and 1 mM PNPP was used as the detection solution. Firstly, the fabricated electrode was first immersed into the detection solution and incubated for 30 min at 37 °C under magnetic stirring, where the immobilized ALP can catalyze the hydrolysis of PNPP to produce the electrochemical activity molecule of PNP. Then, the DPV for PNP was performed using Bi/GCE as working electrode (Because the oxidation potential of PNP was close to that for AuNPs/Au, thus it is impossible to perform directly the detection using AuNPs/Au. For avoiding the interference, Bi/GCE was used.), saturated calomel electrode as the reference electrode and platinum wire electrode as the counter electrode. qRT-PCR data were supplied by Well-Biology (Changsha, China).

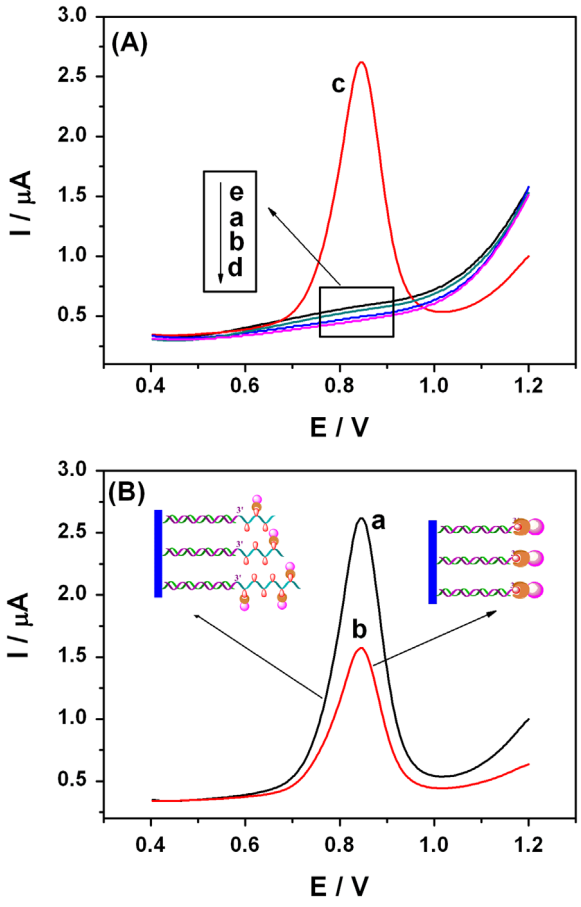


Fig. 1. Differential pulse voltammograms of Bi/GCE in 10 mM Tris–HCl (pH 9.8) containing 1 mM $MgCl_2$ and 1 mM PNPP after the solution was treated with different electrodes for 30 min. (A) (a) microRNA–DNA/AuNPs/Au. (b) Biotin-U/microRNA–DNA/AuNPs/Au. (c) ALP/biotin-U/microRNA–DNA/AuNPs/Au. (d) microRNA–DNA/AuNPs/Au after incubated with 20 μg/mL avidin-ALP for 50 min. (e) DNA/AuNPs/Au after incubate with 30 unit/mL poly(U) polymerase and 1.0 μM biotin-UTP for 75 min, and then incubated with 20 μg/mL avidin-ALP for 50 min. (B) (a) ALP/biotin-U/microRNA–DNA/AuNPs/Au, (b) ALP/microRNA–DNA/AuNPs/Au. microRNA-319a concentration is 10 nM.

3. Results and discussion

3.1. Detection strategy

In this work, a simple and sensitive electrochemical biosensor was fabricated for microRNA detection based on poly (U) polymerase mediated microRNA extension and ALP catalysis signal amplification. As shown in Scheme 1, the probe DNA was firstly assembled on AuNPs/Au electrode surface through the Au–S bond, where the –SH was modified at the 3'-terminal of probe DNA. Then, the complementary microRNA was hybridized with probe DNA with its 3'-terminal away from the electrode surface. Afterwards, microRNA extension event was triggered under the catalysis effect of poly(U) polymerase in the presence of UMP donor of biotin-UTP. As a result, the long chain UMP conjugated with biotin was formed at the 3′-terminal of microRNA, which can further capture avidin-ALP on the electrode surface through the specific interaction between biotin and avidin. Finally, under the catalysis effect of ALP on its substrate of PNPP, the electrochemical activity molecule of PNP can be produced, which can generate an obviously electrochemical oxidation response. In a certain concentration range, for more microRNA hybridization, more ALP can be modified on the electrode surface, which can lead to a much strong electrochemical response. Based on the relationship of microRNA concentration and electrochemical signal, the sensitive microRNA detection can be achieved. The electrode fabrication process was characterized by EIS (Fig. S1, see Supplementary materials).

3.2. Detection feasibility assay

For verifying the detection feasibility of our proposed method, the differential pulse voltammograms of Bi/GCE in the detection solution was compared after the detection solution was incubated with different electrodes. As illustrated in Fig. 1A, no electrochemical response was observed for microRNA-DNA/AuNPs/Au (curve a) and Biotin-U/microRNA-DNA/AuNPs/Au (curve b), which can be attributed to the absence of ALP for catalyzing the hydrolysis reaction of PNPP to generate the electrochemical activity molecule of PNP. As expected, after ALP was modified on the electrode surface through the specific interaction between biotin and avidin, a significant electrochemical oxidation peak was obtained (curve c), which can be ascribed to the oxidation of PNP, the hydrolysis product of PNPP catalyzed by ALP in the detection solution. In our detection strategy, poly(U) polymerase-mediated strand extension reaction is a key step, because avidin-ALP can be captured on the electrode surface to catalyze the hydrolysis reaction of PNPP only based on poly(U) polymerase-mediated strand extension reaction. For proving it, a control experiment was performed. That is, microRNA-DNA/AuNPs/Au was directly incubated with 20 μg/mL avidin-ALP for 50 min. As presented in Fig. 1A, curve d, no electrochemical response was generated due to the absence of poly(U) polymerase-mediated strand extension reaction. Also, the poly(U) polymerase-mediated strand extension reaction was triggered by the hybridization event of microRNA with probe DNA. In order to testify it, another control experiment was carried out. DNA/AuNPs/Au was firstly incubated with the mixture solution containing 30 unit/mL poly(U) polymerase and 1.0 μM biotin-UTP for 75 min, and then further incubated with 20 μg/mL avidin-ALP for 50 min. As we anticipated, no electrochemical response was obtained in the selected potential range (curve e), which strong proved the fact that biotinylated uracil and avidin-ALP can only be modified on the electrode surface after the hybridization event. Based on the above investigations, one can conclude that the developed method can be used to detect microRNA-319a.

In order to evaluate the availability of poly(U) polymerase-mediated strand extension reaction and ALP catalytic signal amplification, another control experiment was carried out without poly(U) polymerase-mediated strand extension reaction, where the probe DNA on DNA/AuNPs/Au was hybridized with 10 nM biotin labeled microRNA-319a for 120 min and then incubated with 20 μg/mL avidin-ALP for 50 min. The electrode was noted as ALP/microRNA-DNA/AuNPs/Au. Then the DPV response was recorded using Bi/GCE as working electrode after the detection solution was incubated with different electrodes for 30 min at 37 °C. As seen in Fig. 1B, the electrochemical response for ALP/biotin-U/microRNA-DNA/AuNPs/Au is higher than that obtained at ALP/microRNA-DNA/AuNPs/Au. However, the current obtained with the poly(U) polymerase mediated isothermal signal amplification was two-fold than control. Even so, in this work, after the amplified detection strategy was applied, the current response was increased, which should be attributed to the use of poly (U) polymerase mediated isothermal signal amplification. That is to say, under the catalytic effect of poly(U) polymerase, the chain of microRNA was really extended, and some biotin-UMP molecules were added at the end of 3′-end of microRNA. Though the extended chain was not so long, it also captured more ALP to catalyze the signal amplification than control experiment as shown in Fig. 1B. Then, ALP can catalyze the hydrolysis reaction of PNPP to

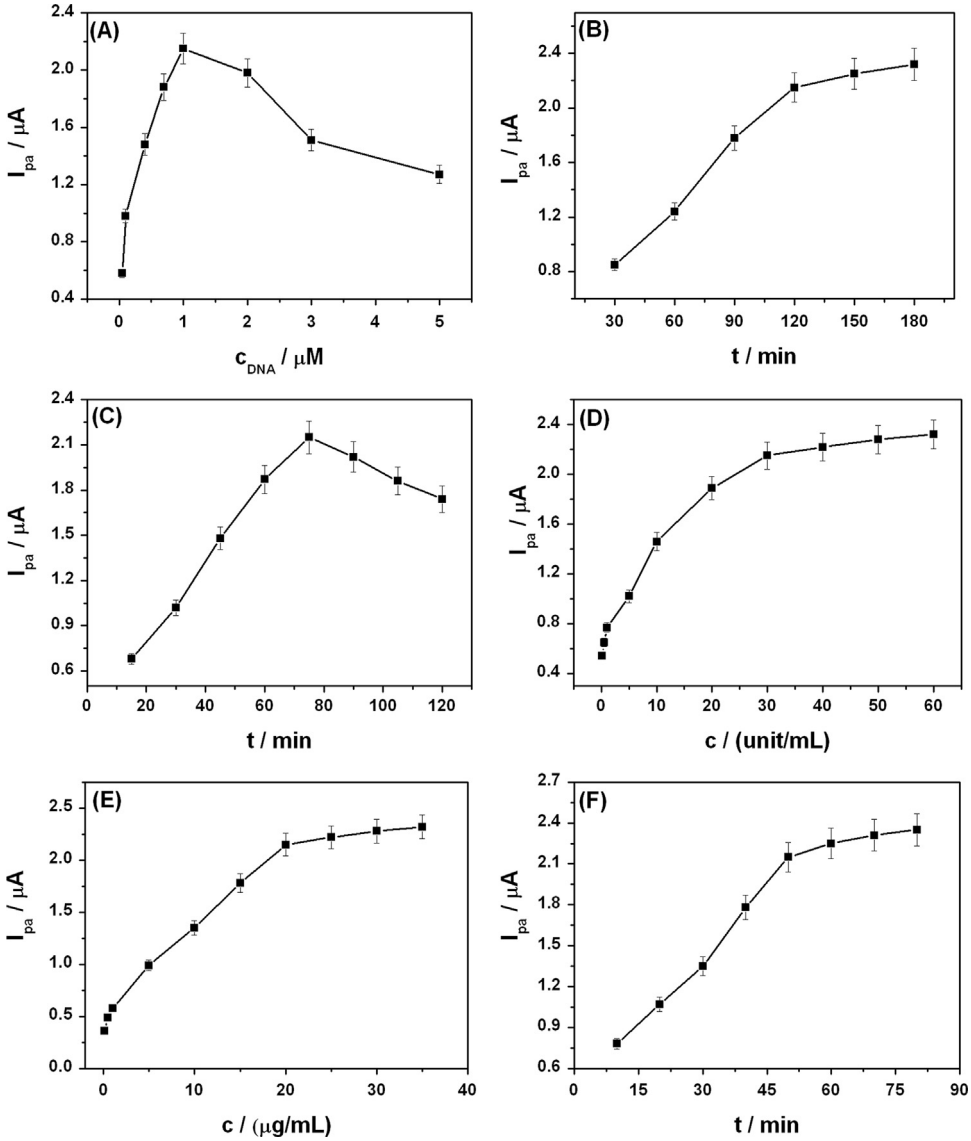


Fig. 2. The effect of probe DNA concentration (A), microRNA-319a hybridization time (B), reaction time for poly(U) polymerase-mediated single-stranded extension (C), poly (U) polymerase concentration (D), avidin-ALP concentration (E) and avidin-ALP immobilization time (F) on the electrochemical response of the detection solution.

produce more PNP, and as a result, an increased electrochemical oxidation signal of PNP can be achieved. According to this result, one can conclude that the poly(U) polymerase-mediated strand extension reaction can effective improve the detection sensitivity.

3.3. Optimum of experimental conditions

To improve the detection sensitivity, several parameters were optimized, such as probe DNA concentration, microRNA-319a hybridization time, reaction time for poly(U) polymerase-mediated single-stranded extension, poly(U) polymerase concentration, avidin-ALP concentration and avidin-ALP immobilization time.

Probe DNA hybridization efficiency is an important parameter for microRNA detection, which was influenced by the probe DNA density on the electrode surface. For obtaining high detection sensitivity, the concentration of probe DNA was optimized. As shown in Fig. 2A, the electrochemical response enhanced with increasing the probe DNA concentration from 0.05 to 1 μM, and then the electrochemical response decreased when further increasing probe DNA concentration to 5 μM. With increasing the probe DNA concentration, the self-assembled probe DNA on the electrode surface also increased, which can increase the hybridization efficiency and capture more target microRNA. However, when the probe DNA concentration is too high, the probe density on the electrode surface is also increased, which can decrease the hybridization efficiency due to the steric hindrance effect. Thus, 1 μM DNA probe was used in this work.

MicroRNA hybridization time can also influence the hybridization efficiency and the immobilization amount of target microRNA, which can further influence the detection sensitivity. As illustrated in Fig. 2B, the electrochemical response increased quickly with extending the hybridization time from 30 to 120 min. Then the oxidation peak current of PNP tended to level off with further prolonging the hybridization time. The immobilization amount of target microRNA can be increased with long hybridization time, which can trigger more poly(U) polymerase-mediated single-stranded extension reaction to capture more ALP and result in high electrochemical response. However, longer hybridization time can lead to the saturated hybridization event, which can make a slight enhancement on the electrochemical response. Therefore, considering the detection efficiency, 120 min was chosen.

Fig. 2C presented the effect of reaction time for poly (U) polymerase-mediated single-stranded extension on the electrochemical response of the detection solution. The

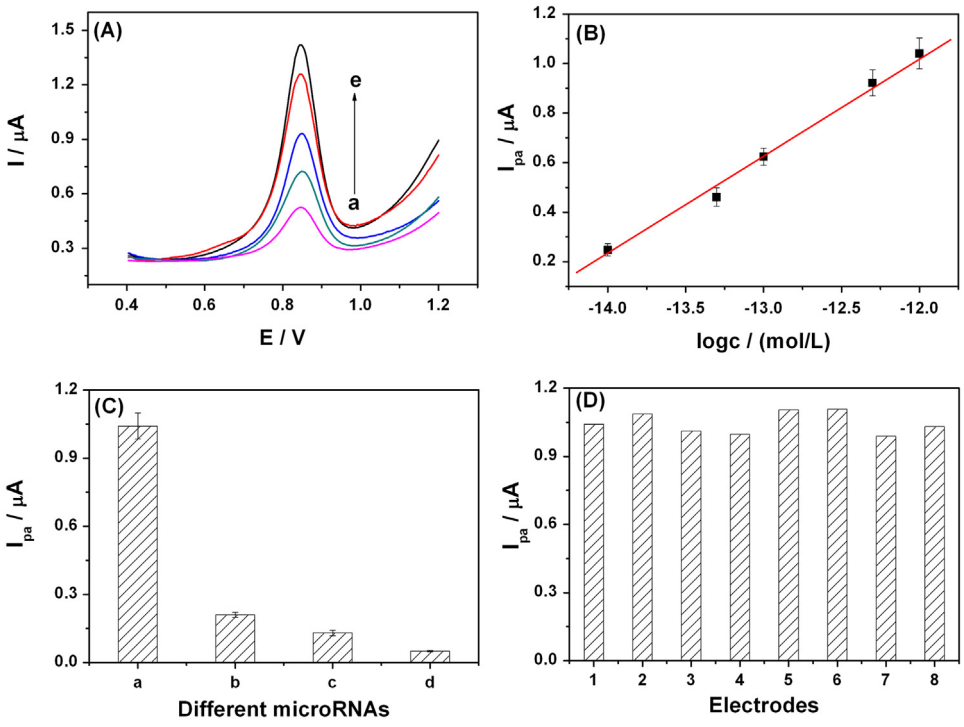


Fig. 3. (A) Differential pulse voltammograms of the fabricated biosensors with different concentrations of microRNA-319a. a–e, 10, 50, 100, 500, 1000 fM. (B) Calibration curve. (C) The histogram for the electrochemical response of the biosensor after the probe was hybridized with different microRNAs. a, complementary microRNA-319a, b, single-base mismatched microRNA, c, three-base mismatched microRNA, d, non-complementary microRNA. (D) The electrochemical response of independently fabricated eight biosensors.

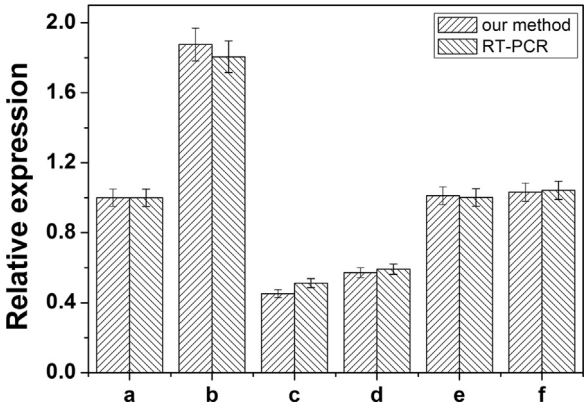


Fig. 4. The effect of gibberellin (b), abscisic acid (c), 6-benzyladenine (d), 3-indoleacetic acid (e) and 3-indolepropionic acid (f) on the expression of microRNA-319a in the leaves of rice seedlings. (a) is the control. The concentration of phytohormone is 50 mg/L.

electrochemical response firstly increased with extending the reaction time from 15 to 75 min, and then the reversed decrease was observed when the reaction time was further increased. With long reaction time, the long single-stranded RNA can be obtained at the end of target microRNA-319a, which can capture more ALP to catalyze the hydrolysis of PNPP and generate a stronger electrochemical response of PNP. However, the longer single-stranded of RNA can also increase the steric hindrance effect and decrease the capture efficiency of ALP, which can result in a decreased electrochemical signal. So 75 min was used as the optimal experiment condition. We also optimized the concentration of poly (U) polymerase. As illustrated in Fig. 2D, the electrochemical response increased greatly with changing the concentration of poly (U) polymerase from 0.1 to 30 unit/mL, then the current tended to level off. Therefore 30 unit/mL poly(U) polymerase was selected.

The amount of ALP can also influence the electrochemical response and further impact the detection sensitivity. Thus the concentration and immobilization time of ALP was optimized. The results were shown in Fig. 2E and F. According to the results, 20 μg/mL and 45 min was chosen as the optimal ALP concentration and ALP immobilization time, respectively.

3.4. Detection performances

Under the optimal experiment conditions, the electrochemical response of PNP increased with varying microRNA-319a concentration (Fig. 3A). As shown in Fig. 3B, the electrochemical oxidation current of PNP was proportional to the logarithm value of microRNA concentration in the range of 10 to 1000 fM. The linear regression equation could be expressed as I_{pa} (μA)= 0.39log c (fM)+5.72 (R=0.9957) and the detection limit was calculated to be 1.7 fM (S/N=3).

In order to examine the detection selectivity of the proposed method for microRNA assay, a comparative experiment was performed between complementary target microRNA-319a and mismatched microRNA. Fig. 3C exhibited the comparison of the electrochemical responses of these different microRNAs with the concentration of 1 pM. As expected, the complementary target microRNA-319a generated a significant stronger electrochemical response than that obtained at other three kinds of microRNA. In brief, the electrochemical oxidation current for complementary microRNA was 4.96, 8.01 and 20.82 times to single-base mismatched, three-base mismatched non-complementary microRNA. The significant difference for electrochemical response between complementary and mismatched microRNA indicated that our strategy possessed acceptable detection specificity.

The reproducibility of the proposed biosensor was also assessed. As seen in Fig. 3D, the relative standard deviation (RSD) of the electrochemical response was 4.62% for eight biosensors, where these biosensors were fabricated independently with the

same process. The RSD value indicated the good detection reproducibility.

3.5. sample assay

In order to evaluate the applicability of our proposed method, we investigated the effect of different phytohormones on the expression of microRNA-319a in the leaves of rice seedlings. As shown in Fig. 4, different phytohormones presented different effects towards the expression of microRNA-319a in the leaves of rice seedlings. Compared with the control, gibberellin improved the expression level of microRNA-319a, abscisic acid and 6-benzyladenine inhibited the expression level of microRNA-319a, 3-indoleacetic acid and 3-indolepropionic acid only showed little effect on the expression level of microRNA-319a. According to the above results, we can conclude that the expression of microRNA-319a in the leaves of rice seedlings was dysregulated under the treatment of gibberellin, abscisic acid and 6-benzyladenine. It has been reported that many microRNAs have been involved in phytohormone signaling (Liu and Chen, 2009). In order to investigate the function of microRNAs in phytohormone signaling, an accurate detection method is necessary. For proving the accuracy of our results, the effect of phytohormones on the expression level of microRNA-319a was also detected by qRT-PCR. As illustrated in Fig. 4, the error for the two methods was lower than 5%, indicating the veracity of our method. Therefore, based on the above results, one can concluded that our method can be applied to investigate the correlations between the expression patterns of miRNAs and phytohormone signaling, even the function of microRNAs in phytohormone signaling.

4. Conclusions

In summary, this paper reported a simple, sensitive and label-free microRNA biosensing platform based on poly(U) polymerase-mediated single-stranded RNA extension reaction and ALP catalytic signal amplification. Through the two signal amplifications, the detection sensitivity was improved greatly with low detection limit of 1.7 fM. The applicability of this simple method was also testified by investigating the effect of phytohormones on the expression level of microRNA-319a in rice seedlings. We believe that the proposed method has a great potential in the filed of microRNA detection assay and the role of microRNA in phytohormone signaling.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bios.2015.12.009.

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