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Signal-on electrochemiluminescence biosensor for microRNA-319a detection based on two-stage isothermal strand-displacement polymerase reaction

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ABSTRACT

MicroRNAs play crucial role in regulating gene expression in organism, thus it is very necessary to exploit an efficient method for the sensitive and specific detection of microRNA. Herein, a signal-on electrochemiluminescence biosensor was fabricated for microRNA-319a detection based on two-stage isothermal strand-displacement polymerase reaction (ISDPR). In the presence of target microRNA, amounts of trigger DNA could be generated by the first ISDPR. Then, the trigger DNA and the primer hybridized simultaneously with the hairpin probe to open the stem of the probe, and then the ECL signal will be emitted. In the presence of phi29 DNA polymerase and dNTPs, the trigger DNA could be displaced to initiate a new cycle which was the second ISDPR. Due to the two-stage amplification, this method presented excellent detection sensitivity with a low detection limit of 0.14 fM. Moreover, the applicability of the developed method was demonstrated by detecting the change of microRNA-319a content in the leaves of rice seedlings after the rice seeds were incubated with chemical mutagen of ethyl methanesulfonate.

1. Introduction

MicroRNAs are a sort of small noncoding and endogenous RNAs with the length of about 22 nucleotides, which exist in animals, plants and viruses, and play crucial role in gene regulation, cell development, differentiation and apoptosis (Rigoutsos and Furnari, 2010; Winter et al., 2009). Due to the small dimension, low content, and high sequence similarity, there is a certain challenge to its quantitative detection of microRNAs. Therefore, it is essential to develop sensitive, dependable and handy analytical method for microRNA detection (Wang et al., 2014). Up to now, various methods have been developed for microRNA detection, such as electrochemistry (Liu et al., 2017a), photoelectrochemistry (Yin et al., 2016), electrochemiluminescence (ECL) (Chen et al., 2016) and fluorescence (Yin et al., 2017). Compared with other methods, ECL has attracted particular attentions due to its fast response speed, high sensitivity and easy operation (Cui et al., 2017; Feng et al., 2016), which provided a new chance for bioanalysis applications.

ECL material is one of the major parameters for ECL biosensor, which will greatly influence its detection performance. The ECL properties of Ru complex have caused great concern due to their high ECL efficiency, good biocompatibility and stability. Typically, tripropylamine (TPrA) is usually used as co-reagent which could significantly improve the ECL signal intensity of Ru complex, and the ECL signal intensity is proportional to the Ru complex concentration in the case of excess TPrA (Zhou et al., 2014). It suggests that the Ru complex/TPrA system could be applied to the quantitative bioanalysis. Significantly, it has been reported that the ECL signal of Ru complex/TPrA system could be effectively quenched by graphene oxide (GO) due to the poor conductivity and its oxidative functional groups. In addition, GO is an efficient carrier due to its excellent mechanical strength and lager specific surface area (Liu et al., 2017b). For example, Huang et al. developed an ECL sensor for ultrasensitive nucleic acid detection where GO was acted as quencher and exhibited excellent performance (Huang et al., 2015) (More details were shown in Supplementary material). Nevertheless, this method is not sensitive enough for miRNAs detection as miRNAs are trace in cells. This problem can be solved by amplifying the detection signal. When combined with appropriate signal amplification strategy, this method can be applied for the ultrasensitive detection of miRNA.

Various signal amplification strategies, especially DNA isothermal amplification technologies, have been widely applied in the field of biosensors for improve the detection sensitivity, including hybridization chain reaction (HCR) (Schwarzkopf and Pierce, 2016; Xie et al.,

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2016), rolling circle amplification (RCA) (Goo and Kim, 2016; Kim et al., 2017), isothermal strand-displacement polymerase reaction (ISDPR) (Wang et al., 2017; Zhang et al., 2017), etc. Among these signal amplification strategies, ISDPR attracted great attention since it does not require specific recognition sites, specially designed circular templates and repeated thermal cycling (Wang et al., 2015). In ISDPR, the hybridization between the template and target can induce polymerization reaction which could produce amounts of trigger DNA to replace the target strand. The released target strand combines with another template to initiate next polymerization reaction, resulting in the recovery of the target DNA and the amplification of the signal.

In traditional ISDPR, the thermodynamic properties of the template and primer are unstable which would affect the efficiency of the reaction. To solve this problem, many studies have been carried out by using the coaxial stacking hybridization to improve the stability in recent years. Coaxial stacking hybridization means that two or more paratactic terminal nucleotides hybridized with a longer complementary strand and form a compact head-to-tail structure which would obtain the excellent stability (Arata et al., 2012). Liu et al. (2016) investigated the effect of invading stacking primers (IS-Primer) on the ISDPR efficiency, and found that the IS-Primer could enhance the specificity and sensitivity of the ISDPR (More details were shown in Supplementary material). In the presence of primer, not only the thermodynamic stability of the nucleic acid hybridization was increased, but also the untying efficiency of the hairpin probe was improved.

In this work, we fabricated a signal-on ECL biosensor based on two-stage isothermal strand-displacement polymerase reaction for microRNA-319a detection. The signal was amplified twice by ISDPR. Firstly, target microRNA hybridized with template DNA, and induced polymerization reaction which produced amounts of trigger DNA for the following amplification reaction. This is the first isothermal strand-displacement polymerase reaction. Then, the trigger DNA along with the primer hybridized with the hairpin probe to open the stem of the probe, and then the ECL signal will be emitted. Simultaneously, in the presence of phi29 DNA polymerase and dNTPs, the trigger DNA would be displaced to initiate a new cycle, and this is the second isothermal strand-displacement polymerase reaction. Due to the two-stage amplification, this ECL biosensor exhibited an ultrasensitive detection performance for the target microRNA.

2. Experimental section

2.1. Materials and reagents

See Supplementary material.

2.2. Synthesis of Ru-hp-DNA complex

Ru-hp-DNA complex was prepared according to previous report (Huang et al., 2015). 4.0 mg bis(isothiocyanato)bis(2,2′-bipyridyl-4,4′-dicarboxylato)ruthenium(II) was dispersed in 1 mL of 0.1 M PBS (pH 7.4), and then 100 μL of the Ru complex dispersion was activated by 100 μL of 0.1 M PBS containing NHS (0.5 mg/mL) and EDC (0.5 mg/mL) for 4 h. After that, the Ru complex dispersion was added to the hairpin probe solution, and then shocked at 25 °C for 2 h in a dark place to immobilize the Ru complex on the hairpin probe. After that, 100 μL of 3 M sodium acetate solution and 2 mL of ice anhydrous ethanol were added to the reaction system and standed at 4 °C for 12 h. Subsequently, the obtained mixture was centrifuged with 12,000 rpm for 30 min and the precipitates were collected and washed with 70% of the ice anhydrous ethanol for three times. After dried in vacuum freeze dryer, the precipitates (Ru complex modified hairpin probe) were dispersed in 100 μL of 0.1 M PBS (pH 7.4).

2.3. Preparation of AuNPs/GO/GCE

Glassy carbon electrode (GCE) was firstly polished to mirror-like surface using 0.3 μm alumina slurry, and then sonicated with double-distilled deionized water and anhydrous alcohol for 3 min, respectively. After dried under N_2 blowing, 10 μL of 2 mg/mL graphene oxide dispersion was dripped on the bare GCE surface and dried under the irradiation of infrared lamp. Finally, 10 μL gold nanoparticles (AuNPs) were dripped on electrode surface and dried under irradiation of infrared lamp, where AuNPs were synthesized according to previous report (Liu and Lu, 2006). The obtained electrode was noted as AuNPs/GO/GCE.

2.4. Probe immobilization

Prior to immobilization, 100 μL of 10 μM hairpin probe was mixed with 1 μL of 100 mM TCEP, and incubated for 1 h at room temperature (Qian et al., 2015). Then, the mixed solution was annealed by incubating at 95 °C for five min and cooled down to room temperature naturally to form the hairpin structure.

Afterwards, the 10 μL of the Ru-hp-DNA solution and probe immobilization buffer was mixed, and then it was dropped on AuNPs/GO/GCE surface and incubated for 12 h in a moist environment at 37 °C. The obtained electrode was then washed with washing buffer to remove the unconjugated probe, and it was noted as hp-DNA/AuNPs/GO/GCE. Then, 10 μL of 0.1 M PBS containing 1.0 mM MPA was dropped on the hp-DNA/AuNPs/GO/GCE surface and incubated for 1 h to block the electrode, followed by rinsing with washing buffer.

2.5. Isothermal strand-displacement polymerase reaction

20 μL of 1 μM template DNA, 20 μL of different concentrations of microRNA-319a and 10 μL of 5 × microRNA hybridization buffer were first mixed. Then, the mixed solution was heated to 95 °C for five min and cooled down to room temperature naturally and incubated for 2 h to make the complete hybridization of DNA and microRNA.

After the addition of a 50 μL solution consisting 250 μM dNTPs, 100 U/mL Nt.*Bsm*AI, 100 U/mL phi29 DNA polymerase and 1 × reaction buffer (50 mM Tris-HCL, 10 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 4 mM DTT, 200 μg/mL BSA, 50 mM KCl. pH 7.5), the mixture was incubated for 2.5 h at 37 °C to generate trigger DNA. Then, the reaction was terminated with incubation at 65 °C for 10 min.

2.6. Fabrication of the ECL biosensor

10 μL of DNA hybridization buffer containing products of ISDPR and primer (10 μM) was dropped onto the surface of hp-DNA/AuNPs/GO/GCE and incubated for 2 h at 37 °C. Then, the electrode was rinsed with washing buffer and noted as dsDNA/AuNPs/GO/GCE. Subsequently, the electrode was incubated with 10 μL of polymerase reaction mixture including 100 U/mL phi29 DNA polymerase, 250 μM dNTPs and 1 × phi29 reaction buffer (50 mM Tris-HCL, 10 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 4 mM DTT, 200 μg/mL BSA, pH 7.5) for 2.5 h at 37 °C, and then rinsed with washing buffer.

2.7. ECL detection

The ECL detection was performed at a computerized MPI-A electrochemiluminescence analyzer (Xi'An Remax Electronic Science Technology. Co. Ltd. Xi'An, China) with a photomultiplier tube voltage of 900 V. A conventional three-electrode system was used in the experiment, with a modified GCE as the working electrode, a platinum wire as counter electrode, and Ag/AgCl (saturated KCl) as the reference electrode. ECL measurements were carried out under scanning from 0.2 V to 1.25 V at a scanning rate of 100 mV/s in ECL detection buffer.

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