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# Dual signal amplification strategy for enzyme-free electrochemical detection of microRNAs

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ABSTRACT

The combination of catalyzed hairpin assembly reaction (CHA) and hybridization chain reaction (HCR) is introduced to develop an electrochemical biosensor without the assistance of enzyme for highly sensitive microRNA (miRNA) detection. Firstly, the hairpin-shaped capture probe H1 immobilized on the electrode surface was opened by target. In the presence of another hairpin probe H2, hybridization of H1 to H2 resulted in the release of target from H1-target complex by strand-displacement reaction. The released target further hybridized with the remaining capture probe H1. After the target recycling process, H1–H2 complex was achieved with an exposed stem of H2. Then, the exposed stem of H2 served as initiator to trigger HCR event, yielding long double strands (dsDNA) molecule. Ultimately, numerous methylene blue (MB) as redox probes intercalated into the minor groove of the long dsDNA polymers to achieve amplified electrochemical signal. The proposed miRNA biosensor achieved a linear range from 10 fM to 1 nM with a wide dynamic range of six orders of magnitude.



## 1. Introduction

MicroRNAs (miRNA) are highly conserved, endogenous, small (typically 18–24 nucleotides long), non-coding RNAs that can modulate gene expression in plants and animals through messenger RNA degradation or translation repression [1–3]. Evidence is accumulating that differential expression of miRNAs can reflect and predict disease progression, particularly most human malignancies and disorders [4]. MiRNA-155, chosen as the subject in this work, is over expressed in several types of neoplastic diseases [5,6], such as malignancies of the hematopoietic system (*i.e.* chronic lymphocytic leukemia) and solid tumors (*i.e.* colon, cervical, lung, breast, pancreatic and thyroid cancer) [7]. Therefore, miRNA-155 may be served as a biomarker to prognose cancer progression.

For miRNA detection, various new techniques using different signal readout assays are reported recently, including fluorescence [8], colorimetry [9], surface plasmon resonance (SPR) [10] and electrochemistry [11]. Electrochemical biosensors have attracted increasing attentions due to its advantages such as simplicity, portability, sensitivity, fast response and low-cost [12]. To enhance the detection sensitivity, many nucleases [13–15] have been popularly employed to achieve target recycling. However, nuclease-assisted target recycling makes the experimental process complicated and detection cost high. Therefore, developing enzyme-free electrochemical miRNA biosensor [16,17] with high sensitivity is desirable. To achieve not only target recycling but also signal amplification without enzyme assisting, catalyzed hairpin assembly reaction (CHA) and hybridization chain reaction (HCR) are available and attractive. CHA can accomplish target recycling-oriented amplification only based on strand displacement and hybridization, which is relatively convenient [18,19]. HCR event can produce long nicked double-strands (dsDNA) molecule, only relying on a pair of hairpins with overlapping partial complementarities [20,21]. To date, a few works were related to enzyme-free miRNA biosensor based on these two amplification strategies.

Herein, we combined CHA and HCR strategies to realize target recycling and signal amplification simultaneously for sensitive detection of miRNA-155. In this assay, hairpin probe H1 was opened in the presence of target firstly and then assembled with H2 to displace target from H1-target complex for target recycling. Each newly emerging DNA fragment of H2 after hairpin assembly could serve as an initiator to trigger a HCR event, resulting in the polymerization of oligonucleotides into a long double strands (dsDNA) molecule. A mass of redox probes, methylene blue (MB), could be intercalated into the minor groove of the long dsDNA polymers to achieve amplified electrochemical signal [22,23]. Through this procedure, we could quantitatively detect target according to the correlation between target concentration and

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electrochemical signal of MB. With conducting gold nanoparticles-graphene nanohybrid films (Au-Gra) as substrate, analyte-induced autonomous cross-opening of hairpins for target recycling and the HCR-mediated signal amplification protocol, the sensitivity of our proposed method for electrochemical detection of miRNA-155 could be enormously enhanced. Possessing the advantages of portability and affordability of electrochemical biosensor, this strategy for the determination of miRNA might meet the requirement of early diagnosis of human cancers.

## 2. Experimental

### 2.1. Reagents and material

Graphene oxide sheets (GO) were obtained in Pioneer Nanotechnology Co. (Nanjing, China). Gold chloride ($HAuCl_4$), L-ascorbic acid (L-AA), bovine serum albumin (BSA, 96–99%), methylene blue (MB) and Tris–HCl were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ethylenediaminetetraacetic acid (EDTA), $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ were purchased from Kelong Chemical Inc. (Chengdu, China). The serum specimens were obtained from the Ninth People's Hospital of Chongqing, China.

All oligonucleotides were designed according to previously reported methods [24–26] and were synthesized by TaKaRa Bio Inc. (Dalian, China). The sequences were listed in Table 1. In the hairpin sequences, loops were italicized and sticky ends were underlined.

The following buffer solutions were prepared in our laboratory. 1× TE buffer consisting 10 mM Tris–HCl and 1 mM EDTA (pH 8.0) was used for dissolving all synthetic oligonucleotides. Probe immobilization buffer (IB) was made of 10 mM Tris–HCl, 1 mM EDTA and 0.1 M NaCl (pH 7.4). Hybrization buffer (HB) containing 10 mM Tris–HCl, 1 M EDTA, 0.2 mM NaCl and 10 mM $MgCl_2$ (pH 8.0). 0.1 M sodium phosphate buffer (PBS) containing 10 mM KCl and 2 mM $MgCl_2$ (pH 7.0) was used as differential pulse voltammograms (DPV) detection buffer solution. 0.1 M PBS containing 5 mM $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ (pH 7.0) was employed for cyclic voltammetry (CV) investigation. Double distilled water was used throughout this study. All other chemicals were of reagent grade and used as received.

### 2.2. Apparatus

An electrochemical analyzer (CHI 660D electrochemical workstation) was used for the CV and DPV experiments. And the conventional three-compartment electrochemical system comprised a platinum wire auxiliary electrode, a saturated calomel reference electrode (SCE) and the modified glassy carbon electrode (GCE, Φ = 4 mm) as working electrode. The scanning electron micrographs were taken with scanning electron microscope (SEM, S-4800, Hitachi, Tokyo, Japan). The pH measurements were finished with a pH meter (MP 230, Mettler-Toledo, Switzerland).

### 2.3. Electrochemical measurements

Electrochemical measurements were performed in a conventional electrochemical cell containing a three-electrode arrangement. The electrochemical characteristics of the stepwise modified process were investigated by CV scanning from −0.2 to 0.6 V (*vs.* SCE) in 2 mL 0.1 M PBS containing 5 mM $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ (pH 7.0) with a scan rate of 50 mV $s^{-1}$. DPVs were carried out in 2 mL 0.1 M PBS containing 10 mM KCl and 2 mM $MgCl_2$ (pH 7.0) to investigate the performance of the sensor. The DPV parameters applied were: 50 mV modulation amplitude, 0.05 ms pulse width, 0.2 s pulse period and potential range from −0.5 to 0.1 V.

### 2.4. Preparation of Au-Gra

Au-Gra was prepared successfully according to a simple method reported previously with slight modifications [27]. Firstly, GO were dissolved in water by ultrasonication. Subsequently, under successively stirring, 10 mg L-AA was added into 10 mL of an aqueous dispersion of GO (0.1 mg $mL^{-1}$). After 12 h of stirring at room temperature, 2 mL 1% $HAuCl_4$ solution was added to the above mixture and stirred for another 8 h. Following that, products of Au-Gra were obtained by centrifugation and were washed extensively with double distilled water in order to remove excessive GO and L-AA. Finally, products of Au-Gra were dispersed in double distilled water.

### 2.5. Fabrication of the modified electrodes

Prior to use, all the hairpin oligonucleotides were separately denatured at 95 °C for 2 min, followed by slowly cooling to room temperature for 1 h to form stem-loop structures [28]. Besides, the pretreatment of GCE was necessary. To obtain a mirror-like surface, the GCE with a diameter of 4 mm was polished carefully with 0.3 μm and 0.05 μm alumina slurries, followed by ultrasonic cleaning with water, ethanol and water sequentially for several minutes. After that, the GCE was allowed to dry at room temperature.

The schematic illustration of the stepwise fabrication was depicted in Scheme 1. First of all, 10 μL Au-Gra was coated on the pretreated electrode surface. Then, the modified electrode was incubated with 5 mg $mL^{-1}$ BSA in 10 mM PBS (pH 7.4) at open-circuit potential for 15 min at room temperature, which aimed at better controlling the spatial arrangement of the probe molecules and backfilling the unoccupied space [29–31]. Subsequently, 10 μL of 2.5 μM SH-CP solution was cast onto the BSA-modified electrode and incubated for 2 h at room temperature. After washing, the electrode was immersed into 20 μL mixture solution containing H1 (1 μM) and H2 (1.25 μM) with various concentrations of target (miRNA-155) at 37 °C for 2 h. Following that, the modified electrode was rinsed with distilled water and soaked in a mixture of 20 μL H3 (1 μM), H4 (1 μM) and MB (1 mM) at 37 °C for 2 h to trigger the HCR. Finally, the sensor was extensively rinsed and subjected to electrochemical measurements.

## 3. Results and discussion

### 3.1. Characteristics of Au-Gra nanomaterial

SEM images were taken to observe the morphological features of the as synthesized Au-Gra. As shown in Fig. 1A, a thin layer film of graphene presented the typical crumpled and wrinkled configuration. In addition, the coverage of AuNPs (pointed out by some arrows) on the graphene could be observed in Fig. 1B by comparison with Fig. 1A, implying AuNPs were integrated successfully with the graphene nanosheets by one step of reducing GO and $HAuCl_4$ *via* L-AA under gentle conditions.

### 3.2. Sensing mechanism of the biosensor

Seen from Scheme 1, Au-Gra was used as substrate to load SH-CP *via* Au-S bond firstly. Subsequently, BSA-based DNA capture probe carrier platform was designed to better control the spatial arrangement of the DNA probe and probe-target interactions on surfaces. This platform also could backfill the unoccupied space [29]. Two stable hairpins (H1 and H2) which could coexist in solution were employed for the target recycling-oriented amplification. Seen from Scheme 2, the H1 composed of five domains (0′, 1′, 2, 3, 4) and the H2 composed of four domains (2, 3, 4, 5). Numbers marked with a prime symbol (′) are complementary