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An electrochemiluminescent microRNA biosensor based on hybridization chain reaction coupled with hemin as the signal enhancer

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In this study, a new universal biosensor based on luminol anodic electrochemiluminescence (ECL) for the detection of microRNA-155 was constructed by using hydrogen peroxide (H_2O_2) as co-reactant and hemin as catalyzer for signal amplification. The bare glassy carbon electrode (GCE) was firstly electrodeposited with Au nanoparticles (AuNPs). Then helper DNA which was partly complementary with the hairpin DNA chains was assembled on the prepared GCE. Target microRNA-155 and the hairpin hybridization chains could create the formation of extended double stranded DNA (dsDNA) polymers through displacement of hybridization chains and the hybridization chain reaction (HCR). The HCR-generated dsDNA polymers give rise to the intercalation of numerous hemin which could catalyze the oxidation of H_2O_2, leading to remarkably amplified ECL signal output. The proposed biosensor showed a wide linear range from 5 fM to 50 pM with a relatively low detection limit of 1.67 fM for microRNA-155 detection. With excellent selectivity, good stability and high sensitivity, the proposed biosensor is promising in developing a high-through assay of microRNA-155.

Introduction

MicroRNA (miRNA) is a sort of small molecule, similar to the small interfering RNA (siRNA), which is encoded by the higher eukaryotic genomes. The structure of miRNA is much different from DNA. They are short, non-coding, endogenous, single-stranded that involve in translational and transcriptional regulation of gene expression in animals and plants[1-4]. Recently, miRNAs emerge as crucial regulators of "fine-tuning" gene expression in the regulation of development[5] and some miRNAs have key roles in the response to physiologic stresses[6]. In this work, we chose miRNA-155 as the target RNA, which has been implicated in the innate and adaptative immune response[7] as well as in the analysis of biochemical manipulations[8]. Therefore, miRNA-155 is reasonable and effective to be treated as a biomarker in certain types of cancer[9-10]. A simple technique for real-time imaging and repetitive monitoring of the miRNA-155 detection hold great potential in clinic research.

Northern blot analysis and its variants was usually regarded as a gold standard method in early miRNA profiling studies[11-12], however it is time and labor consuming, low sensitivity and demands a large amount of total RNA sample[13]. Recently, different signal read out methods for detection miRNAs have been developed based on electrochemical biosensor[14-15], fluorescence[16-17] and surface plasmon resonance[18]. With high detection sensitivity, simplified operation and low cost, ECL for the detection of miRNA has received remarkable attention and has been of great interest for applications in biological analysis[19-21]. For example, Liu et al.[22] described a biosensor based on long-range self-assembled DNA nanostructures as carrier and $Ru(phen)_3^{2+}$ as probe to intercalate into the the DNA concatamers for miRNA detection. Moreover, Cheng et al.[23] designed an ECL resonance energy transfer system based on CdTe nanocrystals and Au nanoclusters with the aid of ligase for detection of miRNA. However, little researchers have focused on the study of luminol-based ECL biosensor for the detection of miRNA.

In ECL systems, luminol is one of the most extensively used luminophores[24-25] and luminol itself can be directly oxidized at the surface of the electrode to produce light. The ECL intensity of luminol could be enhanced by H_2O_2 which was the co-reactant of luminol in alkaline solution[26-27]. The mechanism of H_2O_2 can be used as the co-reactant is that the decomposition of H_2O_2 could produce amouts of reactive oxygen species (ROSs) such as hydroxyl radical and superoxide anion, so that H_2O_2 could amplify the ECL signal of luminol. Hemin could act as an HRP-mimicking DNAzyme that could exhibit strong catalytic and electrocatalytic activity towards H_2O_2[28-29]. In our previous work,

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great improvements on the sensitivity of the luminol ECL immunosensor were realized, owing to the fact that hemin-reduced graphene oxide catalyze the decomposition of H_2O_2[30]. Meanwhile, Pinar et al.[31] reported that hemin could intercalate into the dsDNA grooves due to its large steric structure and anionic substitution on its porphyrin plane. Inspired by these properties, we proposed an ECL biosensor for miRNA detection based on luminol/H_2O_2 system by intercalating hemin into the sddsDNA grooves. The biocompatible dsDNA could not only improve the amount of immobilized hemin but also efficiently maintain its catalytic activity and improve the stability.

In the present work, we designed a promising and simple biosensor for highly sensitive miRNA-155 detection. With favorable biocompatibility, excellent electrical and electrochemical properties, gold nanoparticles (AuNPs) become an attractive material with application in biosensing and have received considerable propulsion[32-33]. Owing to the above advantages, we firstly deposited AuNPs on the surface of the bare GCE which offered an excellent platform to immobilize abundant helper DNA via Au–thiol chemisorption. Then hexanethiol (HT) was introduced on the electrode to eliminate the possible remaining active groups and block the nonspecific binding sites. Subsequently, H1 was assembled on the resulting electrode and its hairpin structure could be opened in the presence of target miRNA. Furthermore, H1 could hybridize with H2 and simultaneously liberate target for another reaction cycle. The newly emerging part of H2 served as initiator to trigger HCR when H3 and H4 were added, which brought the formation of extended dsDNA polymers. After that, hemin was intercalated into the dsDNA grooves to catalyze the decomposition of H_2O_2. As a result, the catalysis produced increased amounts of ROSs, which considerably enhanced the ECL signal and the sensitivity of miRNA detection. Above all, our study provided a new kind of method for intensity amplification of ECL biosensing and would have a wide application of miRNA in bioanalysis.

Experimental

Reagents and Materials

All the synthetic oligonucleotides used in this study were purchased from TaKaRa (Dalian. China), and were kept at 4 ℃ before used. Helper DNA was modified a free thiol at its 5'terminus to immobilize on the electrode surface. The human serum samples were obtained from the Ninth people's Hospital of Chongqing, China. Oligonucleotides sequences used in this work are listed in Table 1.

Table 1. All the oligonucleotides sequences used in this study.

Helper DNA	5′ -HS-AAC CAA TCA GTC T-3′
MiRNA-155	5′ -UUA AUG CUA AUC GUG AUA GGG GU-3′
H1	5′ -TAA TCG TGA TAG GGG TAT GGA CAT GGA ACC CCT ATC ACG ATT AGC ATT AAA GAC TGA TTG GTT-3′
H2	5′ -ATG GAC ATG GAT AAT CGT GAT AGG GGT TCC ATG TCC ATA CCC CTA TGA AGG AGG GGC GAC T-3′
H3	5′ -GGG GCG ACT TGA AAC AGT CGC CCC TCC TTC-3′
H4	5′ -GTT TCA AGT CGC CCC GAA GGA GGG GCG ACT-3′
MiRNA-101	5′ -UAC AGU ACU GUG AUA ACU GAA-3′
TBA	5′ -GGT TGG TGT GGT TGG-3′
PBA	5′ -CAG GCT ACG GCA CGT AGA GCA TCA CCA TGA TCC TG-3′

Gold chloride tetrahydrate ($HAuCl_4 \cdot 4H_2O$) were purchased from Sinopharm Chemical Reagent Co.Ltd.(China). Ferricyanide solutions ($Fe(CN)_6^{3-/4-}$, 5 mM, pH 7.4) were obtained by dissolving potassium ferricyanide and potassium ferrocyanide with PBS buffer (pH 7.4). Luminol (98%) were bought from Sigma–Aldrich Co. (St. Louis, MO, USA) and the 1.00×10^{-2} M stock solution of luminol was prepared by dissolving it in 0.1 M NaOH, while 1.00×10^{-4} M stock solution of luminol was obtained by diluting the above solution with PBS (pH7.4). Hemin, hexanethiol (96%, HT), were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Hydrogen peroxide (H_2O_2, 30%, *W/V* solution) was purchased from Chemical Reagent Co. (Chongqing, China). Buffer for dispersing the Ferricyanide solutions and luminol was phosphate buffered solution (PBS, pH 7.4), prepared by mixing the solutions of 0.1 M Na_2HPO_4, 0.1 M KH_2PO_4 and 0.1 M KCl. Buffer for preparation of miRNA-155 solutions was 10 mM Tris–HCl containing 0.2 mM NaCl, 10 mM $MgCl_2$ and 1mM EDTA, pH 8.0. Finally, the buffer to dilute the hybridizations H1, H2, H3 and H4 was prepared by the mix of 10 mM Tris-HCl, 1 M NaCl, 1 mM EDTA, pH 7.0. Prior to use, all the hairpin hybridizations (H1, H2, H3 and H4) were heated to 95 ℃ for 2 min and then enabled to cool to room temperature for 1 h. All the chemicals and solvents were of analytical grade or better. Double distilled water was used throughout.

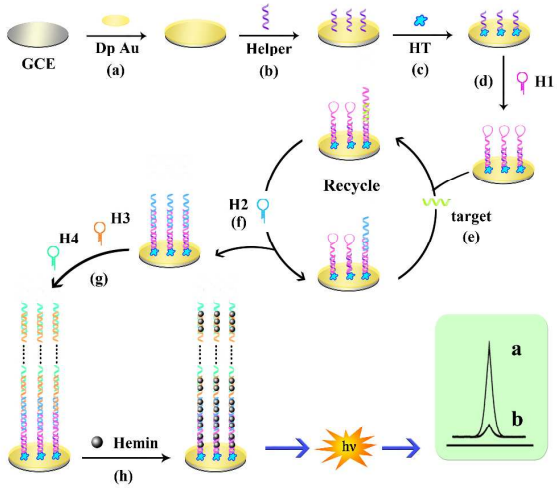
Apparatus and measurements

The corresponding ECL emission measurements were monitored by a model MPI-E electrochemiluminescence analyzer (Xi'an Remax Electronic Science & Technology Co. Ltd., Xi'an, China) with the voltage of the photomultiplier tube (PMT) set at 800 V and the potential scan was from 0.2 to 0.8 V in the process of detection. Cyclic voltammetric (CV) measurements and depositions were accomplished through a CHI 660A electrochemistry workstation (Shanghai CH Instruments, China). All experiments were carried out with a conventional three-electrode system, in which the modified glassy carbon electrode (GCE) was the working electrode, a platinum wire was the

counter electrode and an Ag/AgCl (sat. KCl) was the reference electrode. The pH measurements were performed with a pH meter (MP 230, Mettler-Toledo, Switzerland).

Assembly of the biosensor for detecting miRNA-155

The schematic diagram of the stepwise fabrication procedure for the proposed miRNA biosensor was shown in Scheme 1. Prior to surface modification, the GCE with a diameter of 4 mm was polished repeatedly with 0.3 mm and 0.05 mm alumina powder on fine abrasive paper to obtain a mirror-like surface. After that, the electrode was successively sonicated in anhydrous ethanol and double distilled water each for 5 min and dried in air. Subsequently the cleaned electrode was immersed into $HAuCl_4$ aqueous solution (1%) and electrodeposited at -0.2 V for 30 s to get a gold nanoparticles (AuNPs) layer. Then 10 μL of 2.5 μM helper DNA was dropped onto the surface of AuNPs layer with 16 h incubation at room temperature, followed by immersion in 1 mM HT for 40 min at ambient temperature. The resulting electrode was incubated with 10 μL mixture solution containing H1 (2 μM) and H2 (2 μM) with different concentration of target for 2 h. Afterwards, a droplet of 10 μL mixture solution containing H3 (1 μM) and H4 (1 μM) was casted onto the pretreated electrode for 2 h, too. Finally, for ECL detection, 10 μL of 7 μM Hemin standard solution was deposited onto the modified electrode for approximately 1 h.



Scheme 1. Schematic diagram of the stepwise ECL biosensor fabrication process: (a) electrodepositing Au Nanoparticles onto the bare GCE, (b) modifying helper DNA onto the prepared electrode, (c) HT blocking, (d) introducing of H1, (e) incubating of target, (f) adsorbing of H2, (g) immobilization of H3, H4, (h) intercalating of hemin. The ECL signal (a) and (b) represented the prepared electrode with target and without target respectively.

ECL detection

Under the optimised concentration of hemin, the biosensor was placed in an ECL detector cell containing 2 mL PBS with a final concentration of luminol at 1.00×10^{-4} M and an appropriate concentration of H_2O_2 to record the change of ECL signals at room temperature.

Results and Discussion

The developed method of the miRNA-155 detection

The AuNPs were firstly deposited onto the bare GCE which offered an excellent platform for further immobilizing. Then helper DNA was immobilized on the preparing electrode via strong Au–thiol bonding. Next, HT was introduced on the electrode to block the nonspecific binding sites. Furthermore, H1 was immobilized on the electrode by hybridizing with helper DNA. Subsequently, target RNA was introduced to open the hairpin structure of H1 through hybridizing with H1. Then, H2 was introduced to liberate target by hybridizing with H1. The liberated target was as an initiator to trigger another recycle. Afterwards, H3 was modified on the resulting electrode which was partly complementary with H2, and H4 was further brought on the electrode which was hybridized with H3. The adding of H3 and H4 brought the formation of extended dsDNA polymers. Finally, hemin was intercalated into the dsDNA grooves to enhance the ECL signal.

The characterization of the proposed biosensor for detecting miRNA-155

Electrochemical behaviors of the biosensor

As an outstanding technique, CV was employed to evaluate the changes of electrode behavior of each assemble step by monitoring the electron transfer ability. Fig. 1 exhibited the CV curves characterizing the stepwise modified electrodes in 5.00 mM $Fe(CN)_6^{3-/4-}$ from –0.2 to 0.6 V at a scan rate of 0.1 V s^{-1}. As can be seen, a couple of quasi-reversible redox peaks of the probes were obtained in the bare electrode (curve a). When the bare electrode was treated with the deposition of AuNPs, the current was increased remarkably compared with the bare GCE (curve b) because of AuNPs as electroconductive material for acceleration of electron transfer. After the adsorption of helper probe onto electrode, an obvious decreased peak current was obtained owing to its feature of obstructing the electron transfer (curve c). When HT was introduced to block the non-specific binding sites, there was a decrease which attributed to the ability of HT that can hinder the electron transfer toward the electrode surface (curve d). Then, the CV current was further decreased after the immobilization of miRNA-155 which was mixed with H1 and H2 (curve e), testifying that the interaction of combination among H1, H2 and the target inhibited the transmission of electrons at the electrode interface. At last, the peak current was still decreased when the prepared electrode was assembled with the mixture of H3 and H4 (curve f), owing to the introduction of more negative charges on the electron surface upon formation of the dsDNA polymers.

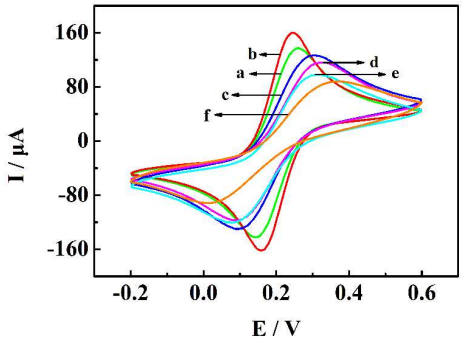


Fig. 1. CVs at the (a) bare GCE, (b) AuNPs/GCE, (c) helper DNA/AuNPs/GCE, (d) HT/helper DNA/AuNPs/GCE, (e) H1, H2 and target/HT/helper DNA/AuNPs/GCE, (f) H3 and H4/H1, H2 and target/HT/helper DNA/AuNPs/GCE in 5.0 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) solution (pH 7.4) containing 0.2 M KCl at a scan rate of 100 mV s^{-1}.

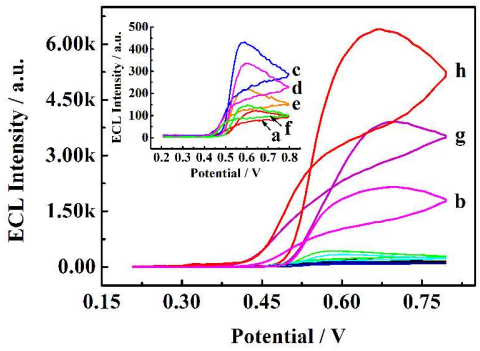


Fig. 2. ECL profiles of (a) bare GCE, (b) AuNPs/GCE, (c) helper DNA/AuNPs/GCE, (d) HT/helper DNA/AuNPs/GCE, (e) H1, H2 and target/HT/helper DNA/AuNPs/GCE, (f) H3 and H4/H1, H2 and target/HT/helper DNA/AuNPs/GCE, (g) H3 and H4/H1, H2 and target/HT/helper DNA/AuNPs/GCE, the working solution was added with H_2O_2, (h) H3 and H4/H1, H2 and target/HT/helper DNA/AuNPs/GCE, the electrode was incubating with hemin and H_2O_2 was added in the working solution.

ECL behaviors of the biosensor

In order to achieve a better comprehension of the proposed biosensor, we also performed the corresponding ECL signals and monitored each step of electrode modification in 2 mL PBS (pH 7.4) with 1.00×10^{-4} M luminol and 3.50×10^{-5} M H_2O_2. Shown in Fig. 2, there was a very low signal of the bare GCE (curve a), after the deposition of the AuNPs, the signal of ECL was almost ten times as higher as the bare one (curve b), which was attributed to the excellent electronic transmission ability of the AuNPs. After the immobilization of helper DNA, a decline of the peak current was observed for the reason that the helper DNA was acting as a nonconductor which could obstruct the electron transfer (curve c). The preparing electrode was then modified with HT to block the non-specific binding sites, leading to a further decrease (curve d). After introduced with the mixture of H1, H2, miRNA-155 (curve e) and incubated with H3 and H4 (curve f), the ECL response declined gradually owing to the formation of dsDNA through hybridization.

After adding H_2O_2 into the detection solution, we got an obvious increase in ECL signal (curve g), attributing to that H_2O_2 was the co-reactant of luminol which could provide ROSs to oxidate luminol. In order to check on whether or not that hemin could catalyze the luminol/H_2O_2 system, we detected the ECL intensity both with the hemin and without hemin in the presence of H_2O_2. As we suspected, the ECL signal which was immobilized both H_2O_2 and hemin was much stronger than that of only H_2O_2 present (curve h), illustrating the fact that hemin could efficiently catalyze thereaction of H_2O_2 in this system.

Optimization of analytical conditions

The relation between ECL intensity and the H_2O_2 concentration was shown in Fig. 4A. The concentration of the H_2O_2 is an important parameter for this reaction on the electrode surface. It could be seen that with the increasing of the concentration of H_2O_2, the ECL signal of the electrode increased and then reached a plateau in 0.7 μM. Besides that, the concentration of hemin was also an important factor that influenced the performance of the biosensor. As is shown in Fig. 4B, the ECL signal was increased with the increasing of the concentration of hemin, finally it reached a constant value at 7 μM. Hence, the concentration of 7 μM was chosen as the optimal concentration of hemin for the miRNA detection.

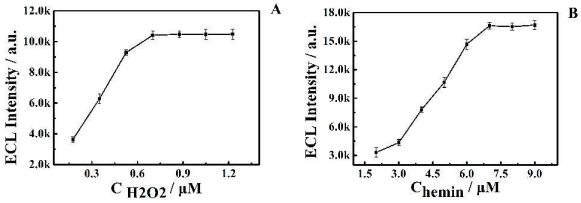


Fig. 4. (A) Effect of the concentration of H_2O_2 on ECL intensity of the biosensor in 2 mL PBS (pH 7.4) with 1.00×10^{-4} M luminol. (B) Effect of the concentration of hemin on ECL intensity of the biosensor in 2 mL PBS (pH 7.4) with 1.00×10^{-4} M luminol.

ECL detection of miRNA-155 with biosensor

On the basis of the optimized experimental conditions, the ECL signal was evaluated with different concentrations of miRNA-155 and we explored the quantitative range of the proposed ECL biosensor. Fig. 5 showed the relationship between ECL intensity and the concentrations of miRNA-155. It was obvious that the anodic ECL intensity of luminol increased with the increasing

concentration of miRNA-155. A linear relationship between ECL signals and the concentrations of miRNA-155 was obtained in the range from 5 fM to 50 pM with the detection limit of 1.67 fM. The linear relationship can be represented as I=11237.2+3996.8c, with the correlation coefficient of R^2=0.9953, where I is the ECL intensity and c is the concentration of the miRNA-155. In a word, the biosensor had a relative low detection limit and can be employed for highly sensitive detection in a wide concentration, which might have a promising future for highly sensitive bioassays applied in clinical detection.

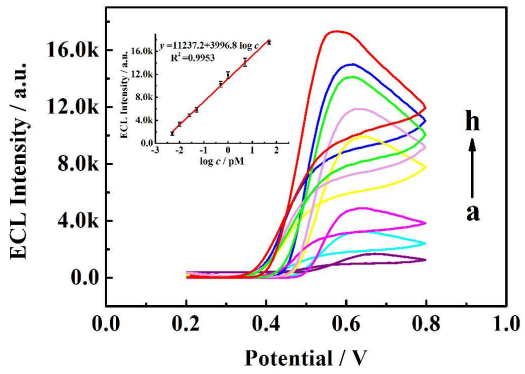


Fig. 5. ECL signals of the developed biosensor to different miRNA-155 concentrations (pM): (a) 0.0005, (b) 0.01, (c) 0.025, (d) 0.05, (e) 0.5, (f) 1, (g) 5, (h) 50. Inset: calibration curve for miRNA-155 detection.

Stability, reproducibility and selectivity of the ECL biosensor

The ECL stability of the proposed biosensor was explored under continuous cyclic potential scans for 20 cycles when the concentration of miRNA-155 was 0.5 pM. As is exhibited in Fig. 6A, the ECL intensity did not show any obvious changes. This results indicated that the newly developed biosensor have considerable stability. The good stability was attributed to the following reasons. Firstly, the AuNPs provided a good matrix for the loading of the hybridizations, which could increase the accessible chance of the hybridizations and provide a good electron transfer tunnel. Secondly, the modified hybridizations loading on the AuNPs kept its good bioactivity and provided strong catalytic effect. What's more, both hemin and H_2O_2 were more stable and highly active to increase the ECL signal.

Simultaneously, the reproducibility of the proposed biosensor was monitored by the relative standard deviation (ECL response) of intra- and inter- assays of miRNA-155 and using five biosensors made at the same GCE with various batches. The relative standard deviations (R.S.D.) of the intra- and inter- assay were not more than 5%. Therefore, the reproducibility of the proposed biosensor was acceptable.

The specificity is another important criterion for the analysis of the biosensor. Under the same experimental conditions, 500 pM of miRNA-101, 500 pM of thrombin aptamer (TBA) and 500 pM of PDGF binding aptamer (PBA) were employed to replace 50 pM of miRNA-155. As is shown in Fig. 6B, it was obvious that the ECL signal observed in 50 pM miRNA-155 led a remarkable increase compared to the other interferences, which indicated that the biosensor displayed good selectivity for the determination of miRNA-155.

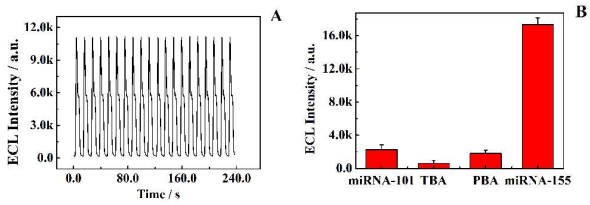


Fig. 6. (A) The stability of the prepared ECL biosensor incubated with 0.5 pM miRNA under consecutive cyclic potential scans for 20 cycles in 2 mL PBS (pH 7.4) with 1.00×10^{-4} M luminol and 0.7 μM H_2O_2. (B) Comparison of ECL responses with 500 pM of miRNA-101, 500 pM of TBA, 500 pM of PBA, 50 pM of miRNA-155.

Application

It is quiet essential to examine the reliability and feasibility potential of using the prepared biosensor for clinical analysis which were performed by standard addition methods in human blood serum. In this study, a series of samples (50, 5, 0.5, 0.05, 0.01pM) were prepared through adding miRNA-155 of different standard concentrations to normal human blood serum samples (standard addition method). As is displayed in Table 2, the recovery (between 95.22 % and 107.2 %) was acceptable, which provided a significant method for determining miRNA-155 in real biological samples.

Table 2. The recovery of the proposed miRNA biosensor in human serum.

Sample number	Add/pM	Found/pM	Recovery/ %
1	0.01	0.01020	102.0
2	0.05	0.04761	95.22
3	0.50	0.4890	97.80
4	5.0	5.230	104.6
5	50	53.60	107.2

Conclusions

In summary, we proposed a novel and ultrasensitive ECL detection method for miRNA-155 based on HCR amplification. The amplification of the luminol ECL intensity could be obtained by H_2O_2 as a coreactant and the catalysis of hemin for the decomposition of H_2O_2 to produce increased various ROSs. In the assay protocol, hemin was intercalated into the dsDNA grooves which efficiently maintain its catalytic activity and improve its stability. The experimental results demonstrated that the as-proposed biosensor had excellent performance for the detection of miRNA-155 with satisfying stability, reproducibility and

selectivity. The characteristics of the assay using ECL detection in this work could satisfy the need for rapid and sensitive methods for miRNA detection which would be valuable and attractive in cancer diagnostics, gene expression and drug discovery. Moreover, the detection of miRNA based on ECL procedure provides a convenient and hopeful way for miRNA detection.

Acknowledgments

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