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Short Communication

Label-Free Electrochemical Detection of MicroRNAs via Intercalation of Hemin into the DNA/RNA Hybridization

Xinyao Yi[1,*], *Zhixuan Lu*[1], *Yu Kong*[1], *Zhuo Chen*[2,*]

[1] School of Materials Science and Engineering, Central South University, Changsha, Hunan, P. R. China 410083
[2] State Key Laboratory of High Performance Complex Manufacturing, Central South University, Changsha, Hunan, P. R. China 410083
[*]E-mail: yixinyao@csu.edu.cn, zhuochen@csu.edu.cn

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Abstract: MicroRNAs (miRNAs) have been emerged as new biomarkers for the detection of a wide variety of cancers. On the basis of the employment of hemin as electron transfer mediator and signal unit, a simple electrochemical assay for sensitive and selective determination of miRNA has been developed. In the presence of miRNA, hemin can intercalate into the hybridization between the pre-immobilized DNA probe and miRNA, thus lead to a reasonable electrochemical signal. In the absence of miRNA, hemin cannot intercalate into the single strand DNA probe which lead to undetectable signal. The current is proportional to the concentration of miRNA in a range between 0.1 μM and 1μM. The proposed assay is label-free, simple and highly selective toward the target miRNA and could be an alternative for detection of miRNA in clinic samples.

Keywords: Electrochemistry; MicroRNAs; Hemin; Label-free

1. INTRODUCTION

MicroRNAs (miRNAs), a class of short, single-strand, endogenously expressed and non protein-coding RNAs with approximately 17−25 nucleotides in length, play a crucial role in physiological processes [1, 2]. Expressions levels of miRNA are closely associated with various diseases, especially the initiation and development of human diseases[3]. Therefore, monitoring miRNA expression levels would provide useful information for clinical diagnosis and drug development [4]. However, the low expression level, small size and high sequence similarity make the detection of miRNAs facing more challenge than genomic DNA and mRNA [5]. Rapid, highly

sensitive and selective miRNA analysis methods which can effectively profile miRNA in minimal amounts of sample and feasible for in situ detection are still challenging.

Northern blotting [6], reverse-transcription polymerase chain reaction (RT-PCR) [7] are the gold standard technical platforms for miRNA determination, but they always suffers from low-throughput and labor intensive. A great effort has been devoted for developing new strategies for sensitive detection of miRNAs[8-14], such as colorimetric measurement [15], fluorescence detection [16, 17], electrochemical detection [18, 19], bioluminescence technology [20] et al. Among these methods, electrochemistry methods provides low cost, portability, rapid and high sensitivity detection which are attractive for point-of-care assays [21, 22]. Electrochemistry methods usually involved direct or indirect labeling integrated with signal amplification strategies to enhance sensitivity.[23-25] Various strategies for amplifying the miRNA detection signals have been employed, such as nanoparticles(NPs) [13], carbon nanotubes [26], grapheme [27] and enzyme [8, 12, 28, 29]. Xia et al reported a label-free and sensitive method for the detection of miRNAs based on the formation of boronate ester covalent bonds and the dual-amplification of gold nanoparticles. [30]Based on the difference in the structures of RNA and DNA, a label-free strategy is demonstrated by Liu et al. [25] In previous, our group have been performed a voltammetric detection of miRNA via hybridization with DNA probes followed by the attachment of Fc-capped gold nanoparticle/streptavidin conjugates [31].

Although the detection limit of the above methods are low enough for miRNA detection but signal amplifier were introduced which means the procedure are more complicated and time-consuming. Porphyrins widely exist in organism and possess physiological activity. Hemin as porphyrin derivatives has the potential of intercalation into the double strand DNAs. In the current work, by introducing hemin as the signal producer, the current produced by hemin is proportional to the target miRNA concentration in a wide range. Although the method is less sensitive than the previous reported electrochemical method[8, 12, 24], it was label-free and simple may presents a new strategy for the rapid detection of miRNAs.

2. EXPERIMENTAL

2.1 Chemicals and reagents

$KClO_4$, KH_2PO_4, K_2HPO_4, $MgCl_2$, NaCl, $HAuCl_4$, H_2O_2, $NaBH_4$, poly (vinyl alcohol), absolute ethyl alcohol, 6-mercapto-1-hexanol (MCH), bovine serum albumin (BSA), and 6-ferrocenyl-1-hexanethiol were acquired from Sigma-Aldrich (St. Louis, MO). The hairpin probe with a sequence of 5’-biotin-GGG GTT GGA GTG TGA GTT CTA CCA TTG CCA AAC CAA CCC CTT TTT-$(CH_2)_6$-SH-3’ was obtained from Sangon Biotech (Shanghai, China). All miRNAs were purchased from GenePharma Co., LTD. (Shanghai, China). Target miRNAs used to hybridize with the ODN probe have the following sequences: 5’-UUU GGC AAU GGU AGA ACU CAC ACU-3’ (fully complementary), 5’-UUU GGC AAU GGU CGA ACU CAC ACU-3’ (one base mismatch), 5’-GUU GGC AAU AGU AGA AAU CAC AAU-3’(four-base mismatch), and 5’-GCG UAU GGC AAC CUC CAG AGA GAC-3’ (non-complementary). All solutions were prepared with diethypyrocarbonate-treated deionized water under an RNase-free environment. Hairpin DNA probe and target miRNAs

were diluted with 10 mM phosphate buffer (pH 7.4) containing 100 mM NaCl and 5 mM $MgCl_2$. Unless otherwise stated, all hybridization reactions were carried out at room temperature.

2.2 Immobilization of hairpin DNA probe onto Au-electrode

Immobilization of the hairpin DNA probe onto Au-electrode was carried out by casting DNA probe onto the electrode for 1h. This was followed by washing the electrode thoroughly with phosphate buffer (0.1 M NaCl, 10 mM sodium phosphate, pH 7.4, PBS). The electrode was then treated with 0.1 mM MCH for 30 min to block the unreacted sites.

2.3 MiRNA hybridization and intercalation of hemin

100 μL miRNA-182 with varied concentrations was added onto the above electrode for 1 h, followed by washing the electrode thoroughly with phosphate buffer (0.1 M NaCl, 10 mM sodium phosphate, pH 7.4, PBS) to remove the unreacted targets, the electrode was then exposed to hemin for 1 h. Finally, the electrode was extensively washed with PBS and water.

2.4 Electrochemical detection

Differential pulse voltammetry (DPV) and electrochemical impedance spectroscopy (EIS) were performed on a CHI 620E electrochemical workstation (CH Instruments, Shanghai, China) and a Gamry Reference 600 potentiostat (Gamry Instruments, USA), respectively. A gold electrode with a diameter of 2 mm (CH Instruments, Austin, TX) was used as working electrode. A platinum wire and a Ag/AgCl electrode were used as the auxiliary and reference electrodes, respectively.

3. RESULTS AND DISCUSSION

3.1 Principle for miRNAs detection by intercalation of hemin

The principle behind the simple voltammetric detection of miRNA is illustrated in Figure 1. The signal amplified by labeling miRNAs with nanoparticles offers higher selectivity and sensitivity, but limited by its high operational complexity and cost. Label-free biosensors are simple, rapid and cost effective thus widely used in miRNA detection. The novel miRNA sensor was constructed by immobilizing a hairpin DNA probe on the Au electrode through Au-S bond. The hairpin DNA probe consists of an 8-bp-long stem and a loop with sequences that are complementary to target miRNA. The empty sites on the Au electrode were blocked with MCH to prevent the nonspecific adsorption. The hybridization of miRNA to a conformation change of the DNA probe thus opens up the loop region to form the double strand DNA/RNA. The electrode was them immersed into hemin solution, hemin has the ability to intercalate into the double strand DNAs,[12] the himin intercalated into the double strand of DNA/RNAs produced reasonable signals. For samples without miRNA, hemin was prevented to intercalate into the single strand DNA. As a result, no detectable voltammetric signal was obtained. By

intercalating hemin into the double strand of DN/RNA, a label-free, simple assay of miRNA was realized. Comparing with other miRNA detection strategy, our method takes the advantage of single step detection by hemin intercalation thus avoid the complicated amplification strategy.

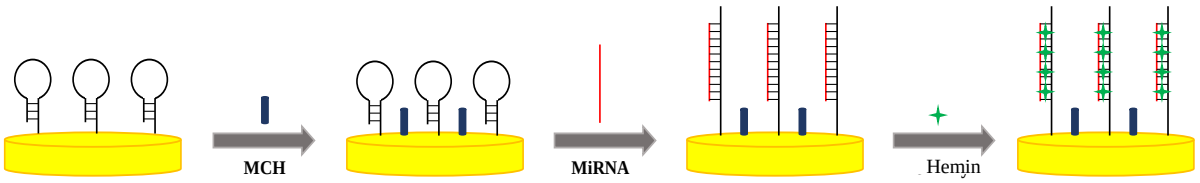

Figure 1. The principle behind the electrochemical detection of miRNA by porphyrin.

3.2 Characterization of the hybridization on electrode

The success immobilization of DNA probe and the hybridization of miRNA was the crucial step for the electrochemical sensor. To ensure the electrode was well modified and the hybridization of miRNA to DNA probe, electrochemical impedance spectroscopy (EIS) has been employed to characterize the modified electrode (Figure 2). R_{et} was estimated from the diameter of the semicircle in the Nyquist plot. A small semicircle domain was obtained at the bare magnetic gold electrode (Ret = 50.5 Ω, curve a). The modification of Au electrode with negatively charged hairpin DNA probes, followed by MCH, increase the R_{et} to 2.92 kΩ. After hybridization of hairpin DNA probe-modified Au electrode with miRNAs, R_{et} was further increased to 3.34 kΩ (curve d). Intercalate hemin into the hybridization further increased R_{et} to 4.13 kΩ (curve e). Evidenced by the EIS data, with the increasing of the R_{et} value, the step-wise construction of the hybridization of DNA/RNA was viably achieved.

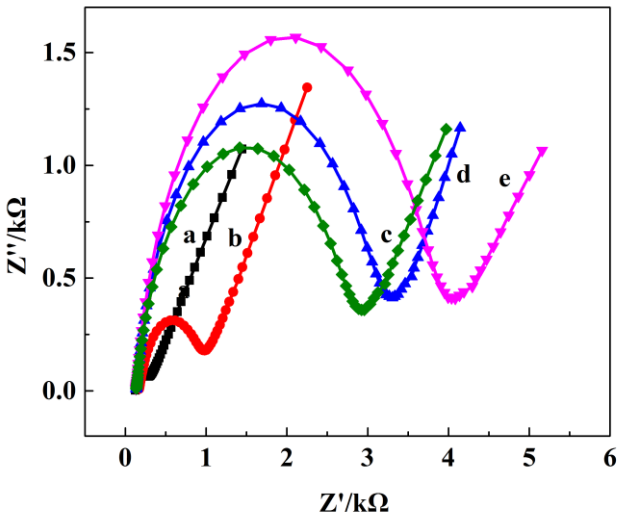

Figure 2. Electrochemical impedance spectra of step-wise assembly of DNA, miRNA, hemin on Au electrode in aqueous solution containing 5 mM $[Fe(CN)_6]^{3-/4-}$ (1:1) and 0.1 M KCl. (a) bare Au electrode, (b) hairpin DNA probe/Au electrode, (c) MCH /DNA/Au electrode, (d) miRNA/MCH/DNA/Au electrode, (e) hemin/miRNA/MCH /DNA/Au electrode. The impedance spectra were obtained at the frequency range from 100 mHz to 100 kHz and R_{et} values were interpreted by Randles equivalent circuit model.

3.3 Feasibility for miRNAs detection

MiRNA detection has been attract a lot of attentions in recent years [32,33] due to its up or down regulation under many pathological conditions, including cancer and cardi- ovascular diseases. Most of the electrochemical sensors are based on modification of the electrode or using signal amplification strategies to achieve a higher sensitivity, however, the signal amplification increase the detection cost and the complexity.[9,12] Figure 3 shows the voltammetric responses acquired at Au electrode modified with the hairpin DNA in the absence (a) or in the presence of 1μM (b) miRNA. The peak was observed at 0.21 V (curve b). The feasibility of the method for sensitive miRNA assay results from the intercalation of hemin into the double strand DNA/RNA, the intercalation produces the detectable signals. For samples without miRNA, no hybridization happened, hemin cannot intercalate into the single strand of DNA caused undetectable signal (curve a). In the presence of miRNA, hemin intercalate into the double strand DNA/RNA which lead to reasonable responses (curve b). The signal caused by interaction of hemin into the double strand DNA/RNA was much higher than the single strand DNA probe prove the feasibility of the method. Moreover, the background signal was decreased by inducing MCH to take the unreacted sites and the optimized concentration of hemin. Noticed the sensor surface was easy to establish and the time for detection was significantly shortened due to the one step of hemin intercalate. Thus, the current miRNA assay was highly simplified the signal amplification assay and shortened the assay time.

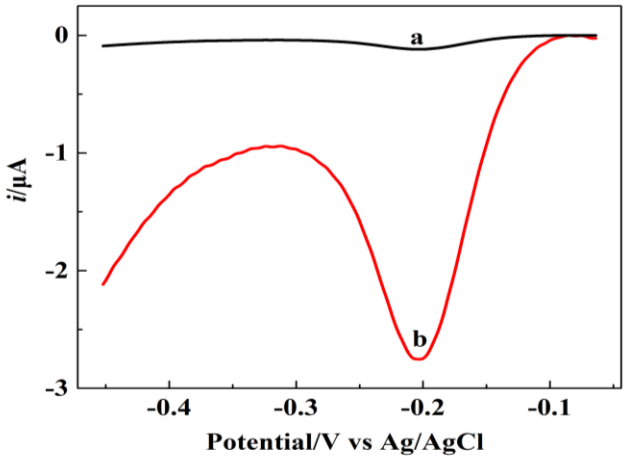

Figure 3. Cyclic voltammograms (CVs) acquired at hairpin probe immobilized Au electrode(a) after hybridization miRNA (b) at room temperature. Followed by intercalation of hemin. The scan rate was 0.1 V/s, and the arrow indicates the scan direction.

3.4 Selectivity of the assay

The short sequences, the sequence similarity and low abundance of miRNAs made it hard to detect and discriminate from the similar miRNA family (similar sequences or mature miRNAs).[5] A false positive signal was easily produced in the hybridization-based assay due to the sequence mismatch. Strategies are explored for improving the specificity of miRNA. Due to the sequence

similarity of miRNAs, the selectivity of the method for miRNA assay was examined. The single mismatch, four base mismatch and non-compliment miRNAs were chosen for testing the specificity of the assay. As depicted in Figure 4, in comparison with that for a complement, the peak current for a single mismatch and a four-base mismatch decreased by 47% and 75%, respectively. For a non-complement miRNA, the miRNA cannot hybridize with the DNA pre-immobilized on Au electrode, which hinders the intercalation of hemin. Thus, tiny voltammetric signal was attained. The simple miRNA assay is highly selective and serves as a viable means for identification of single nucleotide polymorphism.

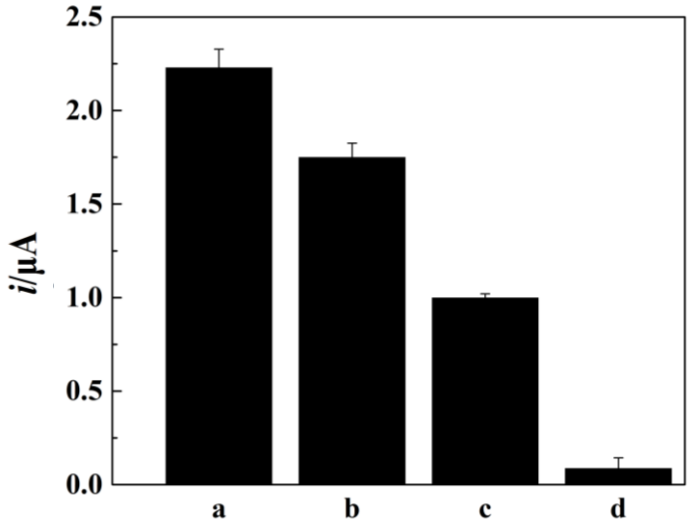

Figure 4. Specificity of miRNA assay. The peak currents were measured at electrodes modified with probe DNA hybridize with 0.1 nM complement (a), single mismatch (b), four-base mismatch (c), and non complement(d) miRNAs with hemin insertion. The absolute errors deduced from three replicate measurements were shown as the error bars. Other experimental conditions are the same as those in Figure 3.

3.5 Calibration curve of miRNA assay

The concentration of miRNAs in clinical samples or cell lysate are as low as a few molecules per cell. In addition, the expression level of miRNAs varies by four orders of magnitude, thus, a dynamic range of detection is necessary for miRNA detection. As demonstrated in Figure 5, the increasing concentrations of miRNA leads to the opening up of more hairpin ODN probes, and the intercalation of more hemin were realized. The dependence of the peak currents of hemin on the concentrations of miRNA was shown in Figure 5. The linear portions in range of 0.1–1 μM for miRNA and the equations are presented as I (μA) = 2.15 [miRNA182] (μM) + 0.18 (R^2 = 0.99). Although the method is less sensitive than the previous electrochemical methods[8, 12, 24, 29] and not suitable for detection of clinical samples,[32] it presents a new strategy for the simple, label-free and rapid detection of miRNAs, may serve as a proof-of-concept for helping improve the simple detection method for miRNA. In comparison with previous reported method using signal amplification strategy,

such as nanoparticles, enzymes et al [8, 9, 33, 34] , the time of detection is highly shortened to 60 min due to the single-step detection avoidance of the complicated label procedure. The analytical performances of different assays are compared in Table 1. As can be seen clearly, the amplification-free method shows highly shortened detection time which makes the single step detection strategies more suitable for point-of-care detection. Although the single step detection of miRNA without signal amplification compromises the sensitivity, the shortened detection time, simple detection procedure may provide new alternative strategy for miRNA detection. We are hoping the new simple, rapid assay can be extend to other method or combined with other strategies to reach a higher sensitivity thus serve in point-of-care detection.

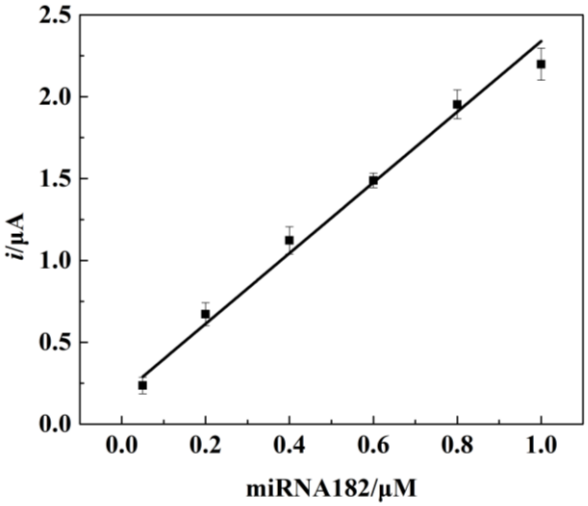

Figure 5. Dependence of the peak currents on the concentrations of target miRNA. The absolute errors were deduced from three replicate measurements and shown as the error bars.

Table 1. Comparison of the current assay to other sensors for miRNA detection.

Method	Amplification strategy	Detection time	Linear range	Refs
Fluorescence	Exponential isothermal amplification	100 min	5-100 fM	[9]
Surface plasmon resonance	Charged silver nanoparticles	120 min	0.6 fM	[33]
Electrochemistry	Multifunctionalgold nanoparticles, enzymesandredox-cyclingreaction	90 min	0 fM- 5 pM	[34]
Electrochemistry	Amplification-free	30 min	5 nM- 5 μM	[30]
Electrochemistry	Amplification-free	60 min	0.1–1 μM	This work

4. CONCLUSION

In summary, intercalation of hemin into the DNA/RNA hybridization for electrochemical detection of miRNA targets was demonstrated and the feasibility was characterized. In the presence of

miRNA, hemin can intercalate into the DNA/RNA hybridization to produce detectable voltammetric signals. MiRNA concentration as low as 0.1 μM were determined with excellent reproducibility (RSD<5%). With the assay being straightforward, label-free, rapid and sensitive, the proposed assay may extended to wide applications in the clinical detection of miRNAs.

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