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Short communication

Molecular beacon structure mediated rolling circle amplification for ultrasensitive electrochemical detection of microRNA based on quantum dots tagging

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ABSTRACT

MicroRNA-16 (miR-16) frequently deleted or downregulated in patients with B cell chronic lymphocytic leukemia and thus can be used as promising biomarkers in B-CLL diagnosis and prognosis. Herein, an ultrasensitive miR-16 detection was carried out by combining rolling circle amplification (RCA), quantum dots tagging and anodic stripping voltammetric detection (ASV). The method could specifically quantify miR-16 over a 6-decade dynamic range and reach an ultra-low detection limit of 0.32 aM.

1. Introduction

B-cell chronic lymphocytic leukemia (B-CLL) is a common adult leukemia associated with the deregulation of miR-15 and miR-16 [1]. The miR-15 and miR-16 can be used as promising biomarkers in B-CLL diagnosis and prognosis. Therefore, hsa-miR-16 (miR-16) was chosen as the target miRNA in the detection.

During last decades, various approaches have been proposed for miRNA detection, such as northern blotting [2], microarray [3], and RT-PCR [4]. Among them, RT-PCR was a commonly applied amplification method, however, thermal cycling and other harsh conditions restrict its extensive application. More recently, an isothermal amplification method defined as rolling circle amplification (RCA) was proposed. In typical RCA, a long linear concatenated DNA product containing thousands of tandem repeats complementary to the circular template can be produced within 1–2 h under constant temperature. The technique is much simpler than that of PCR and has been used as an amplification strategy by combining with fluorescence [5], colorimetry [6], UV–vis spectroscopy [7], SERS [8], electrochemiluminescence [9] and electrochemical methods [10,11] for sensitive detection of protein [12], DNA [13], and RNA [14]. Compared with other amplification methods, it has many significant advantages such as simplicity, speediness and linear kinetic model [15].

In this work, a new strategy is proposed for the ultrasensitive detection of miRNA due to the integration of molecular beacon (MB) structure mediated RCA and quantum dot (QD) tagging. The hairpin structure of MB is beneficial for binding target efficiently and discriminating from mismatched sequence, since the presence of the stem makes the structure thermodynamically unfavorable for binding a mismatched sequence to the loop. Moreover, QD has been widely used as electroactive label for the detection of DNA and proteins due to its unique amplification feature, which makes it as an ideal labeling candidate in the sensitive detection of miRNAs. The design of Locked nucleic acid (LNA)-MB probe and the RCA reaction conveniently transferred the miRNA to QDs which can be detected rapidly through electrochemical methods.

2. Experimental

2.1. Reagents

LNA-MB probe, 5′-SH-CGTACCACAAGCAAACGCCAATATTTACGTG CTGCTAAAACAGCATGGTACG-3′, linear template, 5′-p-ATGCTGACT AACGGTGGCCGGTTGAAATTCAGTCGGCTTCGAATGCGTACC-3′, primer, 5′-AAACAGCATGGTACG-3′ and detection probe, 5′-NH_2-CGGTTG AAATTCAGT-3′ were synthesized by Takara Biotechnology Co., Ltd. (Dalian, China). The miR-16, 5′-UAGCAGCACGUAAAUAUU GGCG-3′ and the central mismatched miRNA, 5′-UAGCAGCACGAAAAUAUU GGCG-3′ were received from Gene Pharma Co., Ltd. (Shanghai, China). Phi29 DNA polymerase, T4 DNA ligase, and dNTPs were obtained from Fermentas (Lithuania). N-hydroxy-succinimide (NHS), 6-mercapto-1-hexanol (MCH), and Tween-20 were obtained from Sigma (St. Louis, MO, USA). 1-ethy-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was purchased from Pierce (Rockford, IL). Ligation buffer

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was composed of 40 mM Tris–HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM DTT and 0.5 mM ATP. Washing buffer was 30 mM, Tris–HCl buffer (pH 7.5) containing 0.05% Tween 20. 0.1 μM RCA template, 40.0 mM Tris–HCl buffer (pH 7.5), 50.0 mM KCl, 10.0 mM $MgCl_2$, 5.0 mM $(NH_4)_2SO_4$, 4.0 mM DTT, and 2 mM dNTPs formed RCA reaction buffer.

2.2. Apparatus

The UV–vis absorption and PL spectra were recorded with an UV-3600 spectrophotometer (Shimadzu, Japan) and a F900 fluorescence spectrometer (Edinburgh Instruments Ltd., UK), respectively. All electrochemical measurements were performed on a CHI 660 electrochemical analyzer (Co. CHI, TX). The fluorescence image was taken by a TE2000-U inverted fluorescence microscope (Nikon, Japan). The fluorescence gel imaging was carried out using Bio-Rad Imaging System (Serial No. 76S/06725).

2.3. Preparation of QD-modified detection probe

The water-soluble CdSeTe/CdS QDs was synthesized according to our previous work via a microwave method [16]. QDs was activated in 150 μL solution containing 20 mg EDC and 10 mg NHS for 30 min and then linked to detection probe (50 μL, 1 μM) under 37 °C for 1 h. The obtained conjugates were washed thrice by ultrafiltration.

2.4. RCA reaction and QDs tagging

10 μL of 1 μM padlock probe and 10 μL of 1 μM primer were mixed in 98 μL of ligation buffer and incubated at 37 °C for 30 min. Then, 2 μL of T4 DNA ligase (5 U/μL) was added and incubated at 22 °C for 1 h. After ligation, T4 DNA ligase was inactivated by heating.

20 μL of a thiolated-MB solution (1 μM) was dropped onto the pretreated Au electrode [17] and incubated at 37 °C for 16 h. After rinsing twice, the electrode was incubated in 500 μL of 1 mM MCH at 37 °C for 1 h. Then the MB probe-modified electrode was exposed in a series of 100 μL solution containing 10 μL miR-16 sample of different concentrations. After incubation at 37 °C for 2 h, the electrode was washed twice. Then the modified electrode was immersed into 100 μL RCA reaction buffer including 2 units of Phi29 DNA polymerization. The RCA reaction continued 1 h at 37 °C. After washing thrice, the electrode was incubated in 100 μL solution containing 10 μM QD-modified detection probe at 37 °C for 30 min.

2.5. Electrochemical detection

After the modification of QDs, the electrode was rinsed and then treated by 200 μL of 0.1 M HNO_3 solution for 2 h. 200 μL of the obtained solutions were mixed with 1.8 mL of 0.2 M, HAc–NaAc buffer (pH 5.2) to perform ASV. The mercury film modified glassy carbon electrode was prepared by 4 cycles of alternate deposition at −1.0 V for 40 s and scanned from −0.9 V to −0.2 V at 0.1 V/s in 0.2 M, HAc–NaAc buffer (pH 5.2) containing 40 μg/mL Hg^{2+} under N_2 atmosphere. The ASV detection involved pretreatment at 0.6 V under N_2 for 2 min, electrodeposition of cadmium at −1.1 V for 9 min, and stripping from −0.9 V to −0.2 V under N_2 atmosphere using a square wave voltammetric waveform, with 4 mV potential steps, 25 Hz frequency and 25 mV amplitude.

3. Results and discussion

3.1. Principle of miR-16 detection based on rolling circle amplification

The schematic principle for miR-16 detection is illustrated in Fig. 1. The LNA-MB probe was immobilized on the surface of Au electrode, followed by reacting with the target miRNA to open up MB so that the loop of the LNA-MB probe could hybridize with miRNA to release the RCA primer. After binding RCA template, dNTPs and Phi29 DNA polymerase were introduced to initiate the RCA reaction. The obtained RCA product was a long single-strand DNA containing thousands of repeated sequences for linear periodic hybridization with the QDs-modified detection probes. The Cd^{2+} released by dissolving QDs attaching to the resulting electrode was quantified by anodic stripping voltammetry (ASV).

The QD-tagged RCA products spontaneously collapsed into a random coil of DNA in aqueous solution and showed micrometer-sized fluorescent objects as shown in Fig. 2A. The fluorescence intensity of the QD-tagged RCA products was much stronger than the surrounding single QD-DNA probe in solution, which proved the efficient amplification of RCA. According to the characterization of polyacrylamide gel electrophoresis (PAGE) applying the 100–10,000 bp DNA marker (Fig. 2B), Compared with lane(1)–(3), RCA was performed in lane (4)–(7) and high molecular weight products were observed with red oval emphasized. In lane (5) and (7), the RCA primer was obtained from the reaction between LNA-MB probe and miR-16, thus, the lower band in accordance with the band in lane (2) was observed. The result of PAGE further confirmed the high molecular weight RCA product.

3.2. Anodic stripping voltammetric detection of Cd^{2+}

The correlation between the stripping peak current and the concentration of target miRNA is shown in Fig. 2C. For the whole range from 10 aM to 1 μM, the stripping peak current of Cd^{2+} and the logarithm value of miRNA concentration fitted a curve equation analyzed by sigmoidal fit method. Fig. 2C (inserted) shows the ASV signal of the Cd^{2+} obtained from QD-tagged RCA products. An obvious electrochemical response of Cd^{2+} responding to 1 fM miRNA target could be observed at −0.71 V in 0.2 M, pH 5.2 HAc–NaAc buffer after performing rolling circle amplification. Compared to RCA results, the current signal without RCA reaction showed a much lower response. The contrast experiment confirmed the significant effectiveness of RCA to improve sensitivity and indicated very little nonspecific adsorption of QD-DNA probe on the Au electrode. More comparative experiments were performed to investigate the RCA efficiency for sub-picomole miRNA as shown in Fig. 4D. Obviously, the signal was significantly enhanced after performing RCA reaction. In addition, blank signal by performing RCA and without performing RCA was indistinguishable, which indicated that the RCA process did not increase the background signal. Moreover, the signal for 0.1 fM obtained from RCA amplification procedure was even higher than that for 100 fM without performing RCA, further exhibiting that the desired route can increase detection sensitivity and reach low detection limit. Thus, the remarkable signal enhancement provided a possibility for detecting ultra-low concentration of target miRNA.

3.3. Anodic stripping voltammetric analysis of miR-16

Fig. 3B shows the ASV profile of the sensor after incubation with different amounts of miR-16. The increase of stripping peak current of Cd^{2+} is proportional to the logarithm value of miR-16 concentrations over a 6-decade range from 10 aM to 10 pM with a linear correlation coefficient $R = 0.997$. The broad linear dynamic range was attributed to the linear kinetic model of RCA. The detection limit was observed to be 0.32 aM. The specificity of the proposed sensor was also investigated. The signal of complementary target was nearly 6-fold than that of central mismatched oligonucleotide, exhibiting good performance to distinguish target oligonucleotide. In this regard, the MB-RCA-QD method has remarkable advantage compared with other reported miRNA analysis methods [18,19].

4. Conclusions

A novel ultrasensitive proposal has been developed for the detection of miR-16. The method showed good specificity to miR-16 and

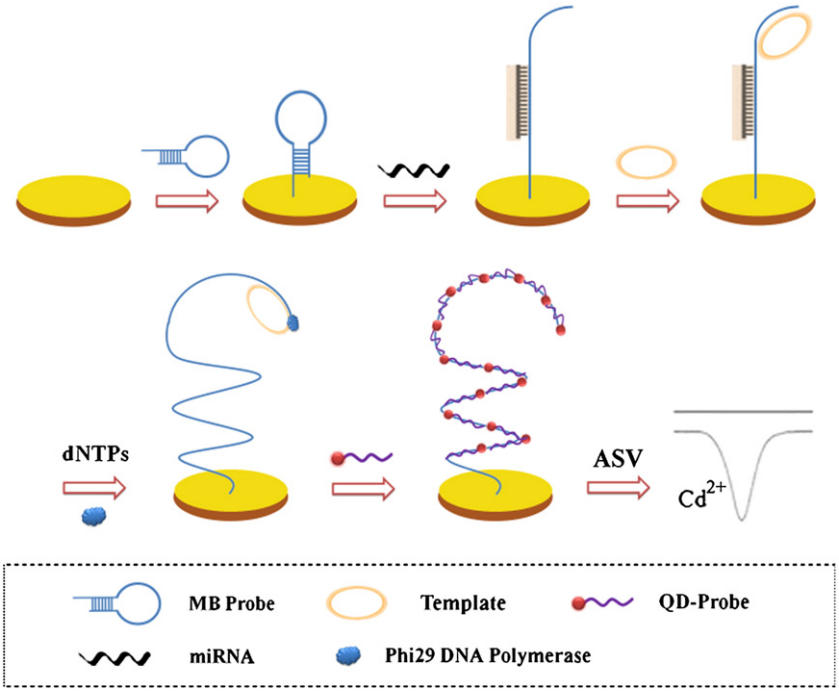

Fig. 1. Schematic representation of the designed strategy for miRNA detection.

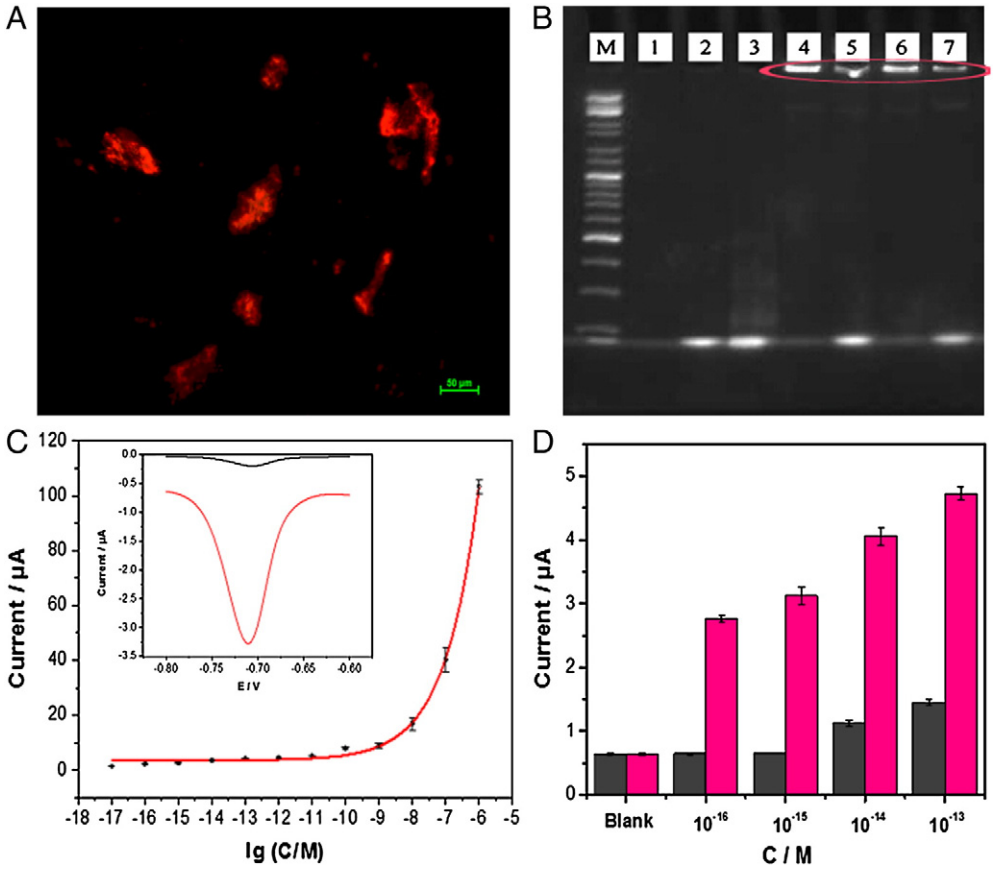

Fig. 2. (A) Fluorescence microscopic images of QD-tagged RCA product. (The scale length is 50 μm.) (B) Characterization of the assembly procedure by polyacrylamide gel electrophoresis: markers (M) followed by (1) LNA-MB; (2) product of the reaction between LNA-MB and miR-16; (3) circular template; (4) product of RCA procedure using original prime; (5) product of RCA procedure using the primer released by reaction between LNA-MB and miR-16; (6) hybridization between (4) and an excess of detection DNA probe; and (7) hybridization between (5) and an excess of detection DNA probe. (C) The concentration profile for the detection of miRNA in the range of 10 aM to 1 μM. (Inserted graph) ASV signal comparison responding to 1 fM of miRNA between with (red line) and without (black line) RCA. (D) ASV signal of Cd^{2+} responding to different concentrations of miR-16 with (red column) and without (black column) RCA.

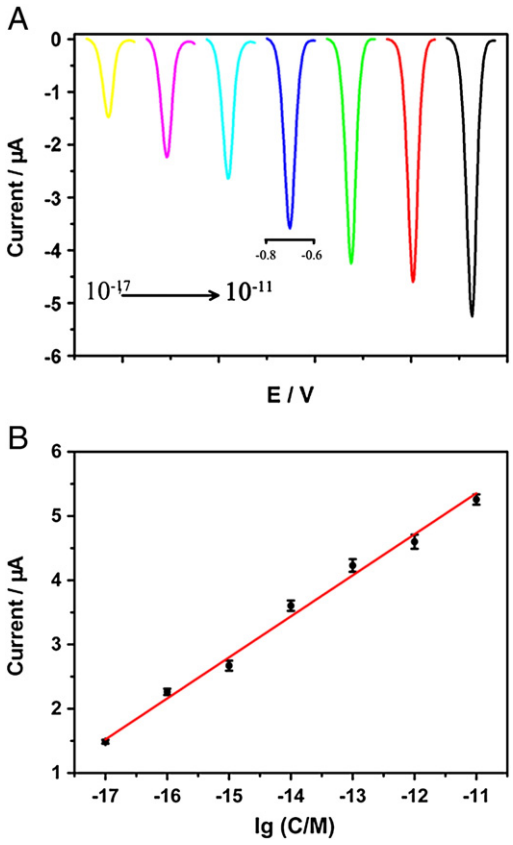

Fig. 3. (A) ASV signal of Cd^{2+} responding to 10^{-17}, 10^{-16}, 10^{-15}, 10^{-14}, 10^{-13}, 10^{-12}, and 10^{-11} M miR-16 (left to right). (B) The quantitative linear dynamic range of the designed method.

could quantify miR-16 over a 6-decade dynamic range and reach an ultra-low detection limit of 0.32 aM. Additionally, the assay is easy to manipulate and can be used for detecting other cancer related miRNAs by changing bases of the capture MB probe conveniently, thus providing a promising diagnostic approach for early cancer prevention and detection.

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