Digital Quantitative Detection of Serum Circulating miRNAs Using a Dual-enhanced Magnetobiosensors Based on Cascaded Nucleic Acid Circuits

Chunxue Liu, a Yayun An, a Yuanfu Zhang, Xia Li, Qingwang Xue, a Huaisheng Wang, a*

a Department of Chemistry, Liaocheng University, Liaocheng, 252059, Shandong, China.

Corresponding author:
Tel: +86-635-8239001; fax: +86-635-8239001
Email: xueqingwang1983@163.com; hswang@lcu.edu.cn
Experimental section

Chemicals and materials

HPLC purified miRNAs and RT-PCR kit were obtained from Takara Biotechnology (Dalian, China). All oligonucleotide sequences were synthesized and purified by HPLC at Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China). Streptavidin magnetic beads (MBs) were purchased from Bangs Laboratories Inc. (Fishers, IN). Sulfosuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC), Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) and N,N,N′,N′-tetraaceticacid (EGTA) were obtained from Sangon Biotechnology Co., Ltd. (Shanghai, China). Invertase from baker’s yeast was ordered from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were of analytical reagent grade. Deionized water obtained from a Millipore filtration system was used all throughout the experiment.

Table 1 Sequence of oligonucleotides
Gel electrophoresis analysis

SDS-PAGE (5% polyacrylamide stacking gel and 8% polyacrylamide resolving gel) was used to analyze the products of H3-invertase and H4-invertase. SDS-PAGE was performed in tris-glycine electrophoresis buffer. Polyacrylamide stacking gel was firstly performed for 30 min at a current of 80 V, polyacrylamide resolving gel was then performed for 1.5 h at a current of 120 V. Finally, the gels were stained, decolorized, and photographed by Gel DocXR+ imaging system.

In the gel electrophoresis, a sample containing 10 μL each reaction sample, 2 μL 6× loading buffer was subjected to 15% polyacrylamide gel electrophoresis (PAGE), running about 1 h at a current of 120 V in 1× TBE buffer. Subsequently, the gels were stained with ethidium bromide, and finally photographed with Gel DocXR+ imaging system.
Synthesis of DNA-invertase conjugate

To 20 μL of 0.1 mM thiol-DNA (H3 and H4) in Millipore water were added and mixed 30 μL of 1 M sodium phosphate buffer (1 M Na₂HPO₄•12H₂O, 1M NaH₂PO₄•2H₂O, pH=5.5) and 2 μL of 0.3 mM TCEP in Millipore water. This mixture was kept at room temperature for 1 hour and then purified by Amicon-10K using PBS buffer by 5 times. For invertase conjugation, 200 μL of 5 mg/mL invertase in PBS buffer was mixed with 1 mg of sulfo-SMCC. After vortexing for 5 minutes, the solution was placed on a shaker for 1 hour at room temperature. The mixture was then centrifuged, and then purified by Amicon-100K using PBS buffer by 5 times. The purified solution of sulfo-SMCC-activated invertase was mixed with the above solution of thiol-DNA (H3 and H4). The resulting solution was kept at room temperature for 48 h. To remove unreacted thiol-DNA (H3 and H4), the solution was purified by Amicon-100K for 5 times using PBS buffer.

Magnetobiosensors fabrication for miRNA-21 detection

2 μL of streptavidin-functionalized magnetic beads (MBs) (10 mg/mL) was first rinsed three times with PBS in a magnetic environment. The MBs were then resuspended in 100 μL of PBS. Subsequently, 8 μL biotinylated DNA probe (H1, 1.5 μM) was added into the MBs solution, followed by shaking for 40 min at room temperature. The conjugates then were rinsed three times with PBS in a magnetic environment. Next, miRNA-21 was added to the solution containing MB-H1, and the mixture was vortexed at room temperature for another 0.5 h, followed by washing three times with PBS by magnetic separation. Next, H2 (2.0 μM) were added and reacted at 37 °C for CHA reaction 60 min, followed by washing three times with PBS by magnetic separation to remove free H2. Then, DNA(H3/H4)-invertase conjugates (1.0 mg mL⁻¹) were added and reacted at room temperature for HCR reaction 60 min, followed by washing three times with PBS by magnetic separation to remove free DNA(H3/H4)-invertase. Finally, 50 μL of sucrose (1.0 M) were added to the above attained MB and incubated for 30 min at 37 °C. a portion of 10 μL of the final solution was measured using a commercially available PGM.
Sample analysis.

Human plasma samples were provided by Liaocheng People’s Hospital. Circulating miRNA-21 in serum were extracted using the miRNeasy RNA isolation kit from Qiagen, with the miRNeasy spike-in control (C. elegans miR-39 miRNA mimic) as an internal control for miRNA recovery and reverse transcription efficiency\(^1\). Reference values of miRNA were obtained by qPCR and normalized according to the parallel recovery of Cel_miR-39. For direct profiling of circulating miR-21 in serum, each 10 μL of serum sample, obtained from patients and healthy persons, respectively, was diluted with PBS to a final volume of 50 μL (1:5 dilution), heated at 95 °C for 5 min, and then cooled rapidly on ice for 5 min. Then, the heat-denatured serum lysates were centrifuged at 15000 g for 20 min at 4 °C. Finally, 20 μL of the supernatant (equal to 4 μL of serum) was added to each cascaded nucleic acid circuits reaction.

**RT-PCR experiments.**

According to the manufacturer’s instructions, the total RNA content of serum was extracted from human blood using the Trizol Reagent (Invitrogen, Carlsbad, CA, USA). To quantify the expression of mature miRNA-21, the following procedure was carried out. 1.5 μg of total RNA was reverse-transcribed to cDNA using AMV reverse transcriptase and looped antisense primers. The resulted cDNA was then quantified by RT-PCR. The reaction conditions are described as follows: 95 °C for 5 min, followed by 40 cycles with a 15 s interval at 95 °C and a 1 min interval at 60 °C. All reactions were performed in triplicate, and U6 was used as the internal control. Notably, all primers used for these assays are listed as follows\(^2-3\): miR-21 forward primer: 5’-ACA CTCCAG CTG GGT AGC TTA TCA GAC TGA-3’; miR-21 reverse primer: 5’-CTCAAC TGG TGT CGT GGA GTC GGC AAT TCA GTT GAG TCA ACA TC-3’; U6 forward primer: 5’-CTC GCT TCG GCA GCA CA-3’; miR-21 reverse primer: 5’-AAC GCT TCA CGA ATT TGC GT-3’.

Results and Discussion

Characterization of invertase-DNA conjugate
Fig. S1 SDS-PAGE images of invertase/DNA-invertase: Lane 1: invertase, Lane 2: H3, Lane 3: H3 DNA-invertase conjugate, Lane 4: H4, Lane 5: H4 DNA-invertase conjugate.

**Optimization of the reaction conditions**

To obtain the best performance, some experimental conditions were optimized in the proposed magnetobiosensors assay. Since the H1 recognition and CHA reaction was a prerequisite for signal transduction, the concentration of H1 probe, H2 probe and CHA reaction time were firstly investigated, respectively. As shown in **Fig. S2A**, when other conditions were kept unchanged, the signal-to-background ratio (S/N) of PGM signal increased dramatically as the concentration of H1 probe increased from 0.1 to 1.5 μM. Further increasing the concentration of H1 probe led to little change in the S/N of PGM glucose signal. Thus, 1.5 μM was chosen as the optimal H1 probe concentration to modify the MMB as recognition element. Subsequently, the concentration of H2 probe was investigated. As shown in **Fig. S2B**, the S/N of PGM glucose signal increased with the increasing of the H2 concentration and reached the maximum at 2.0 μM, suggesting that 2.0 μM H2 was enough for the HCA reaction. So, 2.0 μM H2 was chosen as the best condition in the following study. In addition, when the CHA reaction time was carried out from 20 to 90 min, the S/N of PGM signals were analyzed. The results are shown in **Fig. S2C**. Obviously, the S/N of PGM signals increase significantly with the extension of the elongation reaction time up to
60 min. Afterward, there was a slight increase in the S/N of PGM signal with the reaction time further extended from 60 to 90 min. Hence, 60 min was chosen as the optimal reaction time for CHA reaction.

HCR was a critical factor for signal response. Thus, the concentration of invertase-H3 probe or invertase-H4 probe, HCR reaction time and temperature were also investigated. The concentration of invertase-H3 probe affects the signal. It was found from Fig. S3A that the changes in S/N of PGM signal rise gradually as the concentration of invertase-H3 probe increased. A plateau was observed when the concentration of invertase-H3 probe was more than 1.5 mg mL$^{-1}$. Therefore, 1.5 mg mL$^{-1}$ invertase-H3 probe was chosen in the HCR reaction. In addition, the amplification efficiency of HCA depended on the reaction time. It was found from Fig. S3B that the changes in S/N of PGM signal rise gradually as the reaction time increased. A plateau was observed when the reaction time was more than 1 h. Therefore, 1 h was designated as the HCR reaction time. Moreover, temperature affects the amplification efficiency of HCA and the stability of the hairpin DNA probes. By monitoring the change in S/N of PGM signal, it was discovered that the optimal temperature was 37 °C (Fig. S3C).
Fig. S2 Optimization of the detection conditions: (A) effect of the concentration of H1 probe on the S/N of PGM signal. (B) effect of the concentration of H2-invertase probe on the S/N of PGM signal. (C) effect of the CHA reaction time on the S/N of PGM signal. The error bars represent the standard deviation of three repeated measurements.
Fig. S3 Optimization of the detection conditions: (A) effect of the concentration of H3-invertase probe on the S/N of PGM signal. (B) effect of HCR reaction time on the S/N of PGM signal. (C) effect of the temperature on the S/N of PGM signal. The error bars represent the standard deviation of three repeated measurements.
The PGM signals corresponding to the analysis of different concentration of miRNA by (A) the CHA-based magnetosensors, (B) the HCR-based magnetosensors. The error bars represent the standard deviation of three repeated measurements.

**Determination of miR-21 in human serum samples**

The standard adding method was used to determine miR-21 in the breast cancer patient’s sera. We choose a cancer patient serum sample as an example. A series of synthetic miR-21 at concentrations of 1, 5, 10, 15, 25 fM were spiked into serum sample, respectively, with equal volume to establish a calibration curve (in this case, the concentration of serum sample and synthetic miR-21 were both equivalent to be diluted 2-fold). The other steps were followed by the same experimental procedures described in experimental section.

As shown in **Fig. S5**, the PGM signal intensities of the system increased with the increasing of the spiked concentration of the synthetic miR-21 and exhibited a fairly
good linear relationship in the range from 1 to 25 fM (Shown as inset of Fig. S5). According to the standard addition method, the concentration of miR-21 in the diluted serum sample was estimated to be 3.2 fM. Thus, the content of miR-21 in the original serum sample was calculated to be 32 fM because 20 μL of the supernatant (equal to 4 μL of serum) was added to each cascaded nucleic acid circuits reaction.

Fig. S5 (A) linear graph for fluorescence intensity with the concentrations of miRNA-21. The error bars represent the standard deviation of three repeated measurements.

Fig. S6 (A) Quantitative real-time fluorescence monitoring of the PCR amplification reaction triggered by different starting quantity of miRNA-21. (B) Variance of the $C_T$ value as a function of the logarithmic starting quantity of miRNA-21.
