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Colorimetric detection of microRNA based hybridization chain reaction for signal amplification and enzyme for visualization

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ABSTRACT

MicroRNAs (miRNAs) have key roles in gene expression and can be employed as biomarkers for early diagnosis of various diseases, especially cancers. Detection of miRNAs remains challenging and often requires detection platforms. Here, a horseradish peroxidase (HRP)-assisted hybridization chain reaction (HCR) for colorimetric detection of miR-155 was described. In the presence of target miRNA, the capture probe immobilized on the microplate sandwiched the target miR-155 with the 3′ end of the reporter probe. Another exposed part of the RP at the 5'end triggered HCR producing double-stranded DNA polymers with multiple fluorescein isothiocyanates (FITC) for signal amplification. Finally, multiple HRP molecules were immobilized onto the long-range DNA nanostructures through FITC/anti-FITC monoclonal antibody interactions on the microplate for visualization by tetramethylbenzidine/H_2O_2 system and the colorless substrate turned into the blue product. To obtain accurate data, the absorbance at 450 nm was calculated by microplate reader. The detection limit was 31.8 fM (3.18 amol). Furthermore, this biosensor showed high specificity and was able to discriminate sharply between target miRNA and mismatched sequences. And this approach could be easily applied to the detection of miR-155 in serum sample, thereby ascribing it for a wide application.

Introduction

MicroRNA is a single chain, evolutionarily highly conserved, endogenous, non-protein-coding small RNA molecule (19–23 nucleotide), which regulates the gene expression [1]. Presently, more than 2000 miRNA targets have been discovered that regulate more than 30% of the human genome [2,3]. Many studies found that human disease occurrence, early development, and immune system dysfunction are associated with miRNA expression [4,5]. MiRNAs have become a new generation of diagnostic markers. Overexpression of miRNA-155 (miR-155) is observed in multiple types of cancers, such as chronic lymphocytic leukemia and hematopoietic malignancies (malignancies of the hematopoietic system), colon, cervical, lung, breast, pancreas, thyroid cancer, and other solid tumors [6,7]. Therefore, miR-155 can be used as a biomarker to predict tumor and disease progression.

Owing to their small size, low abundance, and high homology between the phylogeny of human miRNAs, detection of miRNAs is challenging [8]. Northern blotting, microarray, and quantitative real-time PCR (RT-qPCR) are the conventional strategies providing ideal platforms for miRNA expression profiling. However, northern blotting has a low sensitivity and specificity for the detection of miRNA. It is laborious and time-consuming, and radioactive isotope labeling is not environmentally friendly. RT-qPCR and microarray have adequate detection limits but require expensive equipment and materials, which limits their use [9]. In recent years, fluorescence [10] and surface plasmon resonance (SPR) [11] have been used for miRNA detection because of their precise quantitative assessment. But they also require specialized laboratory conditions, expensive equipment.

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Enzyme-linked assays have been routinely used for miRNA detection as they can cost-effectively amplify the detection signal with relative ease as a consequence of the rapid conversion of substrates into products that can be readily visualized by the naked eye without requiring additional instruments [7]. However, they often suffer from low sensitivity, especially during the assessment of real samples. To enhance the detection sensitivity, several groups have employed nucleases to amplify the microRNAs detection signals [12]. Moreover, the nuclease-assisted target recycling renders the experimental process complicated and the detection process cost-ineffective.

HCR is a new type of non-enzymatic nucleic acid isothermal amplification technology. At room temperature (RT) and in the presence of the initiator sequence, two stable hairpin probes self-assemble to extend the double-stranded DNA polymers, which avoids using enzymes under stringent conditions [13–15]. Extended double-stranded polymers, and the signal molecules of fluorescence [16–18] electrochemical techniques [19–21] etc can be detected.

Here, we present a sensitive and visual detection platform for miRNA detection based on HCR in conjunction with horseradish peroxidase (HCR-HRP). MiR-155 is a model target in this study. HCR can produce modified long dsDNAs with multiple FITCs, through which anti-FITC monoclonal antibody-HRP (anti-FITC-HRP) conjugation can be attached for the visual and measurable signal. This method exhibited high sensitivity to target miRNA with the limit of detection (LOD) of 31.8 fM. Furthermore, the assay was also capable of discriminating the target miRNA from even a single base mismatched nucleic acid, which is promising in medical diagnosis and biological therapy in the future.

Materials and methods

Reagents and chemicals

All DNA sequences (Table 1) were synthesized and purified by Shanghai Sangon Biological Engineering Technology & Services (Shanghai, China). All oligonucleotides were dissolved in sodium phosphate sodium chloride buffer solution (SPSC; 150 mM Na_2HPO_4, 0.75 M NaCl, pH 7.4) to a storage concentration of 10 μM. Streptavidin (SA)-coated 96-well plates (catalog no.15120), 4,4′-diamino-3,3′,5,5′-tetramethylbenzidine (TMB) substrate (product code: N301), ethidium bromide, bovine serum albumin, acrylamide, N,N-methylene bisacrylamide, ammonium persulfate, and tetramethylethylenediamine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (HyClone) were purchased from Thermo Fisher Scientific (Waltham, MA). Anti- FITC-HRP mouse monoclonal antibody (catalog no. MAB045P) was purchased from Millipore (Billerica, MA, USA). All the other reagents used in the experiments were analytical grade. Nuclease-free water was used for all aqueous solution preparations. The sequences adopted from the literature are listed in Table 1.

Polyacrylamide gel electrophoresis

H1-FITC (1 μM) and H2-FITC (1 μM) were heated to 95 °C for 5min separately and then cooled to RT for 1 h before use. Ten microliters of different concentrations of RP were incubated with the same volume of H1-FITC and H2-FITC at RT for 2 h. A gel stock solution (30%, m/v) was prepared by dissolved 29 g acrylamide and 1 g N, N-methylene bisacrylamide in 100 mL purified water. The separating gel was prepared by mixing 3 mL gel stock solution, 6.890 mL of 5 × TBE buffer (0.45 M Tris, 0.45 M Boric acid, 10 mM Na_2EDTA, pH = 8.3), 100 μL ammonium persulfate and 10 μL of tetramethylethylenediamine. The native gel was run at 140 V for 1 h with 10 μL sample/well, stained for 15 min with 0.5 μg/mL ethidium bromide, and visualized under UV light.

Best ratio of hairpin-FITC/hairpin probes

Four groups of probes (H1-FITC, H2-FITC, H1, and H2) were diluted to 1 μM, respectively, heated at 95 °C, and cooled to RT to form the hairpin structure. Ten microliters of the reaction systems of FITC-labeled and unlabeled hairpin probes with different volume ratios (10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9 and 0:10) were separately established. 10 μL of the RP (0.2 μM) was added to a final volume of 30 μL. The reaction mixtures were incubated at room temperature for 2 h. The bands of dsDNA polymers formed via HCR were analyzed on native polyacrylamide gel (9%, m/v) electrophoresis.

MiR-155 detection by HCR-HRP on the plate

SA-coated 96-well plate was used. Each well of the plate was incubated with 100 μL biotin-labeled capture probe (CP, 0.5 μM) in SPSC buffer at RT for 1 h. Subsequently, the microwells were washed thrice with washing buffer (25 mM Tris, 150 mM NaCl, 0.1% (m/v) bovine serum albumin, 0.05% (v/v) Tween 20) to remove the unreacted CP. Then, 100 μL miR-155 of different concentrations from 100 fM to 100 nM were added to the microplate and incubated at RT for 30 min. Thus, the target miR-155 was captured by hybridization with CP to form a duplex. After washing away the unreacted target miR-155, 100 μL HCR products from the mixture of RP (0.2 μM), H1 and H2 (The volume ratio of Hairpin-FITC/Hairpin was 9:1 and the concentration of each hairpin probe was 1 μM separately) were added to each well and maintained at RT for 30 min to form a sandwich of "CP/miRNA/RP-HCR". Next, 100 μL of HRP-Ab solution (1:2000 in SPSC buffer) was added to each well, and the plate was agitated on a shaker for 30 min. The plate was washed thrice to remove the unreacted HRP-Ab, following which, 100 μL of TMB substrate solution was added to each well and incubated in the dark at RT for 10 min. The colorless substrate solution turned into a visible blue product. The image of the reaction product captured using a digital camera was used without further modification. Finally, 20 μL H_2SO_4 (2 M) was added to each well to

Table 1
The sequence used in this assay. (The sequences used are as follows (5′-3′)).

Name	Abbreviation	Sequence (5′-3′)
Capture probe	CP	Biotin-TTTTTT**ACCCCTATCAC**
miRNA-155	miR-155	UUAAUGCUAAUCGUGAUAGGGGU
Reporter probe	RP	**GATTAGCATTAA**AGTCTAGGATTCGGCGTGGGTTAA
Hairpin1-FITC	H1-FITC	FITC—TTTTTTTTAACCCACGCCGAATCCTAGACT***CAAAGT***AGTCTAGGATTCGGCGTG
Hairpin2-FITC	H2-FITC	AGTCTAGGATTCGGCGTG***GGTTAA***CACGCCGAATCCTAGACTACTTTGTTTTTT—FITC
Hairpin 1	H1	TTAACCCACGCCGAATCCTAGACT***CAAAGT***AGTCTAGGATTCGGCGTG
Hairpin 2	H2	AGTCTAGGATTCGGCGTG***GGTTAA***CACGCCGAATCCTAGACTACTTTG

*In the hairpin sequences, loops are italicized, and sticky ends are underlined; in the CP and RP, the parts that are complementary to the miR-155 are bold.