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A colorimetric biosensor for detection of attomolar microRNA with a functional nucleic acid-based amplification machine

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A functional nucleic acid-based amplification machine was designed for simple and label-free ultrasensitive colorimetric biosensing of microRNA (miRNA). The amplification machine was composed of a complex of trigger template and C-rich DNA modified molecular beacon (MB) and G-rich DNA (GDNA) as the probe, polymerase and nicking enzyme, and a dumbbell-shaped amplification template. The presence of target miRNA triggered MB mediated strand displacement to cyclically release nicking triggers, which led to a toehold initiated rolling circle amplification to produce large amounts of GDNAs. The formed GDNAs could stack with hemin to form G-quadruplex/hemin DNAzyme, a well-known horseradish peroxidase (HRP) mimic, for catalyzing a colorimetric reaction. The modified MB improved the stringent target recognition and reduced background signal. The proposed sensing strategy showed very high sensitivity and selectivity with a wide dynamic range from 10 aM to 1.0 nM, and enabled successful visual analysis of trace amount of miRNA in real sample by the naked eye. This rapid and highly efficient signal amplification strategy provided a simple and sensitive platform for miRNA detection. It would be a versatile and powerful tool for clinical molecular diagnostics.

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1. Introduction

MicroRNAs (miRNAs) are short (18–23 nucleotides), endogenous, single-stranded RNA molecules that regulate the expression of mRNA in many cellular processes [1–3]. Since the discovery of miRNAs in circulation, large amounts of miRNA-related biological studies have confirmed that the dysregulated expression of miRNAs contributes to the pathogenesis of most human malignancies [4–8]. Therefore, miRNAs have been emerged as novel biomarkers in the diagnosis and treatment of cancers [9–11], which calls for rapid, simple, reliable, sensitive and selective assays for miRNAs detection.

Generally, northern blot and real-time PCR have been widely used for analysis of miRNA expression [12–14]. Though these methods have showed some merits, they still suffer from some drawbacks in practical applications, i.e., semi-quantitative, laborious, and requiring expensive equipment [15–16]. Hence, biosensing strategies, including electrochemical biosensors, luminescence sensors and colorimetric sensors, have become a class of powerful tools for accurate and quantitative miRNA expression [17–19]. Among them, colorimetric sensors have recently gained special attention due to the extreme simplicity and the visual detection of miRNAs in homogeneous solution without any separation and washing steps.

To improve the analytical performance of biosensing strategies, various amplification strategies, including exonuclease (Exo)-assisted signal amplification [20–21], strand displacement amplification (SDA) [22] and rolling circle amplification (RCA) [23–28] have been developed. Among these methods, RCA has received particular interest and has been widely used for the development of ultrasensitive biosensors. Though RCA has excellent property of signal amplification, it is apt to generate nonspecific amplification due to the impurity of circular template. Also, RCA products are large fragment of ssDNAs, which decreases the solubility and greatly increases the steric hindrance to hinder their hybridization with probe. All of these features may counteract the specificity of amplification methods based on RCA and compromise the analytical performance.

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Aiming at improving the analytical performance of RCA-based amplification strategies for miRNAs detection, this work designed a complex of trigger template and C-rich DNA co-modified molecular beacon (MB) and G-rich DNA (GDA) as a probe, and integrated the MB mediated SDA with toehold initiated rolling circle amplification (TIRCA) to design a novel functional nucleic acid-based amplification machine for specific and ultrasensitive detection of miRNAs. Here, the amplification machine contained the MB/GDNA probe, polymerase and nicking enzyme, and a dumbbell-shaped amplification template as seal probe. The recognition of MB/GDNA probe with target miRNA triggered the MB mediated SDA to cyclically release nicking triggers, which then hybridized with the seal probe to trigger multiple TIRCA and produce large amounts of GDNAs. Specifically, the 3′-OH of MB and GDNA were inactivated by C6 spacer, which could decrease the nonspecific polymerization along with 5′ protruding termini of MB, thus improving the stringent target recognition and reducing background signal. In addition, the same specific nicking site recognized by Nb. BbvCI was sequestered in the modified MB and seal probe, which enabled only one nicking endonuclease being used to simplify the whole miRNA detection process. Also, it made sure that TIRCA products could be nicked to large amounts small fragments, which reduced the steric hindrance and facilitated solution of TIRCA products, resulting in greatly increased sensitivity.

The functional nucleic acid-based amplification machine produced large amounts of GDNA to form DNAzyme, a horseradish peroxidase (HRP) mimic [29–31]. The formed DNAzyme could catalyze the conversion of a colorless 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{2-}$) to a green $ABTS^{\cdot -}$, and thus led to an ultrasensitive visual method for detection of miRNAs. Due to the presence of nicking enzyme and the nicking site in the seal probe, the amplification machine allowed the input of target miRNA to be converted to multiple outputs of DNAzyme, thus produced a mass of green $ABTS^{\cdot -}$ for ultrasensitive colorimetric and visual detection of miRNAs. This signal amplification and biosensing method provided a simple, rapid and sensitive platform for miRNA detection and could become a versatile tool for clinical molecular diagnostics.

2. Experimental

2.1. Materials and reagents

Phi29 DNA polymerase, Nb.BbvCI, T4 DNA ligase, dNTP, Exo-I, Exo-III and RNase inhibitor were purchased from New England Biolabs (USA). Hemin and $ABTS^{2-}$ were obtained from Sigma-Aldrich (St Louis, MO, USA). MiRNAs were obtained from TaKaRa Biotech. Inc. (Dalian, China). HPLC-purified DNA oligonucleotides were synthesized by Sangon Biotechnology Co. Ltd. (Shanghai, China). The sequences of nucleic acids employed in this study were shown in Table 1. All oligonucleotides were dissolved in tris-ethylenediaminetetraacetic acid (TE) buffer (pH 8.0, 10 mM Tris–HCl, 1 mM EDTA) and stored at −20 °C, which were diluted in appropriate buffer prior to use.

The stock solution of 1.0 μg/μL total RNA extracted from breast adenocarcinoma (MCF-7) cells was purchased from Ambion (California, USA). All solutions and deionized water used were treated with diethyprocarbonated (DEPC) and autoclaved to protect from RNase degradation. All aqueous solutions were prepared using Millipore-Q water (≥18 MΩ, Milli-Q, Millipore). The stock solution of hemin (5 mM) was diluted to the required concentrations with 25 mM 4-(2-hydroxyethyl)-1-piperazineëthanesulfonic acid (HEPES) (pH 8.0) containing 20 mM KCl, 200 mM NaCl, 0.05% Triton X-100 and 1% dimethyl sulfoxide (DMSO).

2.2. Apparatus

A UV-visible spectrophotometer (UV-2550, Shimadzu, Kyoto, Japan) was used to collect the signal. The gel electrophoresis was performed on the DYY-6C electrophoresis analyzer (Liuyi Instrument Company, China) and imaged on a Bio-rad ChemDoc XRS (Bio-Rad, USA). The concentrations of DNA suspensions were measured by ultraviolet spectrophotometry using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

2.3. Preparation of MB/GDNA and seal probes

The MB/GDNA probe was prepared by simply mixing MB and GDNA at a ratio of 1.2:1 to incubate at room temperature (RT) for 1 h. The resulting hybridization complex was stored at −20 °C before use. Seal probe was prepared by self-templated ligation of 5′-phosphorylated dumbbell-shaped DNA sequence using T4 DNA ligase. The ligation reaction was conducted in a 20-μL reaction mixture containing 1 × T4 DNA ligase reaction buffer (50 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM ATP, 10 mM dithiothreitol, pH 7.5), 1 μL DNA seal probes (100 μM), 17 μL DEPC-treated H_2O and 100 U of T4 DNA ligase. Ligation process was performed at 16 °C overnight and followed by inactivation at 65 °C for 10 min to terminate the ligation reaction. After ligation, Exo-III (100 U/μL) and Exo-I (20 U/μL) were added to digest the leftover dsDNA and ssDNA to yield closed DNA. Following digestion, the Exos were denatured by heating at 80 °C for 20 min. These prepared probe was then stored at −20 °C until use.

2.4. Signal measurement

0.5 μL of target miRNA or sample was added in 100 μL mixture of 1 μM MB/GDNA probe, 0.2 U/μL Phi29 DNA polymerase, 0.15 U/

Table 1
Nucleic acids employed in the present work.

Nucleic acid[a]	Sequence (5′-3′)[b]
miRNA21	UAGCUUAUCAGACUGAUGUUGA
miRNA222	AGCUACAUCUGGCUACUGGGUCUC
SM	UAGCUUAUCAGACUGAUGUUUA
DM	UAGCUUAUCAGACUGAUUUUUA
NC	AUUGAAUAUUCUUAUUAUAAUU
MB	GTCAGATGAATTCGTGTGAGAGCACCTCAGCAACCCGCCCAACGCGCGTCAACATCAGTCTGATAAGCTAGACGCGCG – C6 SPACER
GDNA	GGGTTGGGCGGGTTGGG – C6 SPACER
Seal probe	GTGAGAGCCCAACCCGCCCTACCCTAGACCTCAGCTCTCACACGAATTCCCGTCAGATGAATTCGT

[a] SM, single-base mismatched strand; DM, double-base mismatched strand; NC, non-complementary mismatched strand; MB, trigger template and C-rich DNA modified molecular beacon with C6 SPACER protected 3′-end.
[b] The colors correspond to the sequences shown in Fig. 1.

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