Electronic Supplementary Material (ESI) for Chemical Communications

Electronic Supplementary Information

Rolling circle amplification-based DNA machine for miRNA coupling catalytic hairhin assembly with DNAzyme formation

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EXPERIMENTAL SECTION

Reagents and Chemicals. Target miRNA was obtained from TaKaRa Biotech. Inc. (Dalian, China). Oligonucleotides, 10× TE buffer, 10 mM dNTP, T4 RNA ligase 2, and Phi29 DNA polymerase were purchased from Dingguo Biotechnol. Co., Ltd. (Beijing, China). 3,3′,5,5′-Tetramethylbenzidine sulfate (TMB) was achieved from Sigma-Aldrich (Shanghai, China). Hemin was gotten from Tokyo Chem. Re. Co., Ltd. (Japan). All the oligonucleotides used in this work were purified by HPLC and confirmed by mass spectrometry. Total RNAs extracted from normal cells were purchased from Ambion with a stock solution of 1.0 μg/μL. All other chemicals were of analytical grade, and used as received without further purification. Ultrapure water obtained from a Millipore water purification system (≥ 18 MΩ, Milli-Q, Millipore) was used in all runs. The stock solution of hemin (5 mM) was diluted to the required concentration with 25 mM Tris-HCl (pH 8.0) containing 0.05% Triton X-100 and 1% DMSO. TMB substrate solution was freshly prepared with TMB (1.0 mg/mL, dissolved in ethanol), 0.1 M citrate acid and 0.2 M Na_2HPO_4 (pH 5.2), and 30% H_2O_2 at the ratio of 100 : 900 : 1. The sequences of oligonucleotiedes employed in this work are listed as follows:

Target miRNA: 5'-UGAGGUAGUAGGUUGUAUAGUU-3'

Padlock probe: 5'-phosphate-CTACTACCTCATACACCTATAACGAC**GAGAAAGGGCTGCCA**GATACTCT TCGCAATTTTAACTATACAAC-3'

Hairpin H1: 5'-GGGTAGGGC**GGGTTGGGATG**AGAAAG*GGCTGCCA****CATCCCAACCC****ATA*-3'

Hairpin H2: 5'-*TATGG****GTTGGGATG****TGGCAGC*C**CATCCCAAC**-3'

Single-base mismatched miRNA: 5’-UGAGGUAGUAGGUUGUCUAGUU-3’

Two-base mismatched miRNA: 5’-UGAGGUAGUAGGUUGUCUAUUU-3’

Non-complementary miRNA: 5'-UAUGAUACUUGCUAGUGUAUCG-3'

In the probe H1, the hemin-binding aptamer was underlined. The italic letters of H1 probe (22 nt at the 3' end) were complementary to those of H2 probe (22 nt at the 5' end). The bold letters of hairpin DNA were complementary to the bold letters of the corresponding hairpin DNA. The bold letters for the padlock probe were the catalytic sequence.

Assay Protocol for Target miRNA. The ligation reaction was carried out with a 10 μL reaction mixture containing 1 × ligation buffer [40 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 0.5 mM ATP (pH 7.8)], 2 U of T4 RNA ligase 2, 2 μL the padlock probe (10 nM) and 2 μL target miRNA with various concentrations. Before adding T4 RNA ligase 2 and ligation buffer, the oligonucleotide mixture was denatured at 65 °C for 3 min, and cooled slowly to room temperature over a 10-min period. After annealing, T4 RNA ligase 2 and ligation buffer were added to the mixture and incubated at 37 °C for 2 h. During this process, the padlock probe was ligated in the presence of target miRNA and ligase (Scheme 1A). Afterward, the added T4 RNA ligase 2 was denatured by heating at 90 °C for 10 min.

Next, the resulting product was used for the rolling circle amplification reaction. Initially, the above-prepared mixture was diluted to 20 μL with Tris-Ac buffer (33 mM, pH 7.9) containing 10 mM $MgAc_2$, 66 mM KAc, 0.1% Tween 20, 1 mM DTT, 1 mM each dNTP and 2 U Phi29 DNA polymerase. Then the RCA reaction was conducted at 30 ºC for 3 h, and terminated by heating at 90 °C for 10 min. During this process, the RCA product autonomously replicated a multiple machinery cutter cycle and generated accumulated amount of products (Scheme 1B). After that, 50 nM NaCl, 400 nM H1 and 400 nM H2 were simultaneously injected into the mixture, and incubated for 60 min at 45 ºC. Finally, 0.3 μM of freshly prepared hemin solution was added in the product, and the mixture was incubated at room temperature (RT) for 30 min to form the DNAzyme through the interaction between hemin and G-quadruplex (Scheme 1C).

Measurement Procedure. Initially, 30 μL of DNAzyme substrate solution including TMB and H_2O_2 was added to the above-prepared mixture, and then the mixture was incubated for 30 min at RT. The enzymatic reaction was stopped by adding 40 μL H_2SO_4 (2.0 M) to the mixture. Following that, the resulting solution was removed into a low-binding polystyrene microtiter plate (Greiner, Frickenhausen, Germany) or a quartz cuvette. Meanwhile, the absorbance was read at 450 nm with a plate reader (DNM-9602, Beijing Perlong Medical Instrument Ltd, China) or measured by UV-vis absorption spectrophotometer (Techcomp 1102, China). All measurements were conducted at RT (22 ± 1.0 ºC). Analyses were made in triplicate.

Gel Electrophoresis. Gel electrophoresis was performed on a 2.0 wt % agarose gel with 0.5 × TBE (pH 8.0) as running buffer. 6.0 μL of each DNA sample was loaded into the lanes and performed at a constant potential of 100 V for 45 min. After Stains-All staining by EB solution for 30 min, gels were photographed by gel image system.

Assay Principle and Validation for Target miRNA with RCA-based DNA Machine. Scheme 1 represents the assay procedure based on RCA-based DNA machine for *in situ* amplified monitoring of miRNA accompanying catalytic hairpin assembly with DNAzyme formation. The process mainly consists of three steps and one catalytic recycling for hairpin assembly. At the first step, the RCA module is formed on the padlock probe through the ligase in the presence of target miRNA. The padlock probe consists of a hybridization sequence with target miRNA at the 5′ and 3′ ends (green) and a catalytic sequence for the hairpin assembly at the ZipCode region (red) (designated as "catalyst"). Upon target miRNA introduction, miRNA hybridizes with the terminal sequences of the padlock probe. The bases both ends are specifically ligated and circularized with target miRNA as a template in the presence of T4 RNA ligate 2. In this case, the DNA machine is initiated by replication of the circularized probe through rolling circle amplification in the presence of Phi29 DNA polymerase, thus resulting in the formation of a repeatedly concatenated sequence of the padlock probe. The carried catalysts within the long single-strand DNA further catalyze the automatic assembly of hairpin H1 and hairpin H2 to produce numerous H1/H2 duplexes. Meanwhile, the formation of H1/H2 duplex can cause the release of the catalytic sequence, thereby promoting the hairpin recycling assembly. Because of the hemin-binding aptamer at the 5' end of hairpin H1, DNAzyme can be formed upon addition of hemin. The formed DNAzyme by the RCA product can act as the peroxidase mimics to catalyze the TMB-H_2O_2 system, thus resulting in the amplification of detectable signal.

The concrete principle for catalytic hairpin assembly and the formation of DNAzyme is schematically illustrated in the inset of Scheme 1. Hairpins H1 and H2 sever as the substrates for the catalytic hairpin assembly with the help of catalytic sequence in the padlock probe. Hairpin H1 contains three regions, which are defined as regions '*a*', '*b*' and '*c*' according to different functions, respectively. The region '*a*' represents the hemin- binding aptamer, which can also partially hybridize with region '*c*'. Similarly, hairpin H2 is equipped with two regions (named as regions '*d*' and '*e*'). H1 and H2 can potentially hybridize to form a H1/H2 duplex and activate the initially blocked DNAzyme sequence (*Note*: The spontaneous hybridization of two hairpins is kinetically hindered by occluding complementary regions within intramolecular hairpin secondary structures). Upon hairpins H1/H2 introduction, the catalytic sequence hybridizes with region '*b*' to open hairpin H1 and form the Cat/H1 intermediate (step 1). The exposed region '*c*' acts as a "toehold" to hybridize with region '*d* 'of hairpin H2 and initiates a branch migration reaction to form a Cat/H1/H2 complex (step 2). The formed Cat/H1/H2 complex is inherently unstable similar to a "transient state". Meanwhile, the catalytic strand rapidly dissociates from Cat/H1/H2 complex and hybridizes with another hairpin H1 to initiate the next cycle, thus resulting in the produce of numerous H1/H2 complexes (step 3). Upon addition of hemin, the region '*a*' in the H1/H2 complex can cause the formation of DNAzyme (step 4). By this means, target miRNA at a very low concentration can trigger the operation of DNA machine, and form a large number of DNAzyme molecules for the signal amplification.

Optimization of Experimental Conditions. To achieve an optimal analytical performance for the RCA-based DNA machine, some experimental conditions, *e.g.* the reaction time for the RCA, hybridization temperature and hybridization time for the CHA, and the concentration of the added hemin for DNAzyme formation, should be investigated in detail by using 1.0 pM miRNA as an example. Because of the difference of the operating conditions between experiments, the judgment was mainly based on the change in the signal-to-background (S/B) ratio (*i.e.* relative to the background signal). As shown in Fig. S1-a, the S/B ratio increased with the increasing reaction time for the RCA, and tended to level off after 3 h. Longer reaction time did not almost cause the significant change in the S/B ratio. Thus, 3 h of reaction time was chosen as the RCA progression.

Another important parameter for catalytic hairpin assembly is the incubation temperature, since it directly affects the formation of DNAzyme. As seen from Fig. S1-b, the S/B ratio increased with the increment of hybridization temperature, and decreased after 45 ºC. The reason might be attributed to the fact that the catalytic strand could not efficiently open the H1 at below 45 °C, while high temperatures would result in the nonspecific opening of H1 and H2 and cause a high background signal. At this condition, we also investigated the effect of hybridization time for hairpin assembly on the S/B ratio of the RCA-based DNA machine. An optimal S/B ratio was obtained at ~75 min (Fig. S1-c). Therefore, 45 ºC and 75 min were utilized as the hybridization temperature and hybridization time for the catalytic hairpin assembly, respectively.

In this work, the detectable signal mainly derives from the DNAzyme, which indirectly relies on the concentration of H1 and H2. To acquire a high-amount H1/H2 complex, the ratio between H1 and H2 was studied. As indicated from Table S1, the maximum S/B ratio was obtained at 400 nM H1 and 400 nM H2. In this case, we monitored the effect of different hemin concentrations on the S/B ratio of the RCA-based DNA machine. The optimal signal was achieved at 0.3 μM hemin (Fig. S1-d). Although the high-concentration hemin is conducive to form the DNAzyme, it often causes high background signal. So, 400 nM H1 + 400 nM H2 and 0.3 μM hemin were selected for the CHA and DNAzyme formation, respectively.

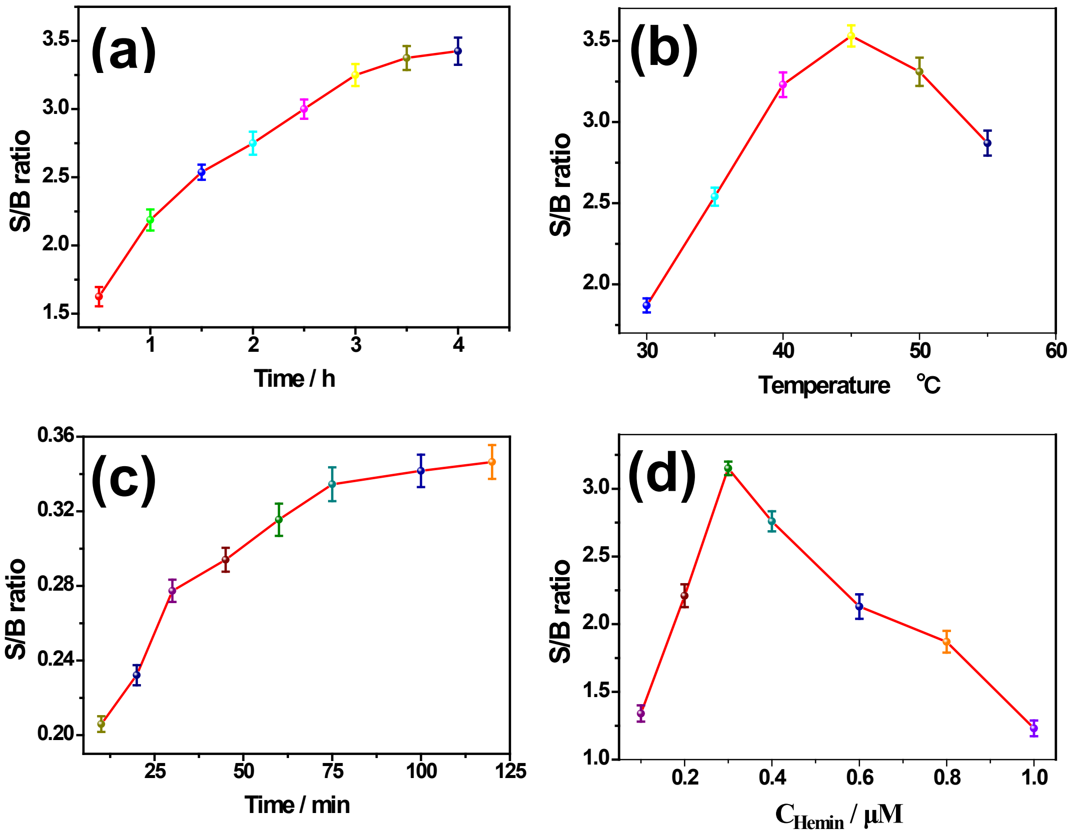

Fig. S1 The effects of (a) reaction time for the RCA, (b) reaction temperature for the CHA, (c) hybridization time for the CHA, and (d) hemin concentration on the signal-to-background (S/B) ratio of the RCA-based DNA machine. Padlock probe: 10 nM, miRNA: 1.0 pM, Ligation reaction: 37 °C for 2 h, RCA reaction: 30 °C for 3 h. The error bar represents the standard deviation of three measurements.

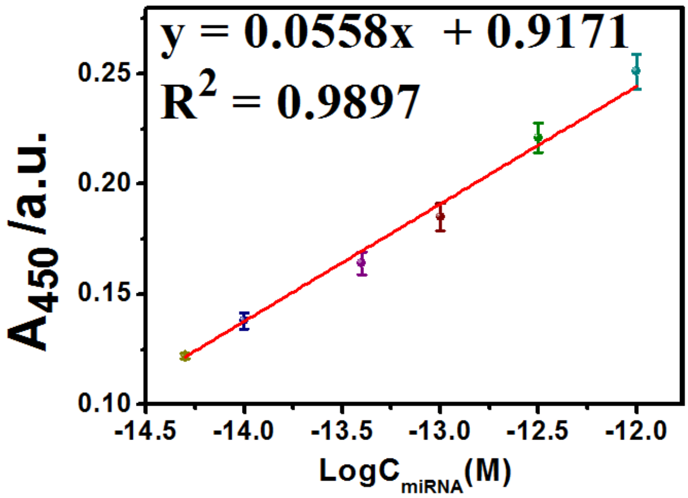

Fig. S2 Calibration curves of the RCA-based DNA machine towards standard target miRNA spiked in total extracted RNAs.

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Table S1 Effect of different ratios between H1 and H2 on the signal-to-background (S/B) ratio of the RCA-based DNA machine.

	$C_{[H2]}$, nM; signal-to-background (S/B) ratio (mean ± SD, RSD, n = 3)				
$C_{[H1]}$, nM	150	200	400	600	800
150	1.12 ± 0.03 (2.7%)	1.34 ± 0.04 (3.0%)	1.56 ± 0.06 (3.8%)	1.43 ± 0.06 (4.2%)	1.22 ± 0.03 (2.5%)
200	1.95 ± 0.04 (2.1%)	2.35 ± 0.07 (3.0%)	2.85 ± 0.05 (1.8%)	3.13 ± 0.06 (1.9%)	2.66 ± 0.05 (1.8%)
400	2.34 ± 0.06 (2.6%)	2.75 ± 0.06 (2.2%)	3.54 ± 0.09 (2.5%)	3.32 ± 0.07 (2.1%)	3.01 ± 0.07 (2.3%)
600	2.03 ± 0.06 (3.0%)	2.87 ± 0.05 (1.7%)	3.34 ± 0.08 (2.4%)	2.98 ± 0.06 (2.0%)	3.13 ± 0.07 (2.2%)
800	1.77 ± 0.02 (1.2%)	2.67 ± 0.05 (1.9%)	3.23 ± 0.07 (2.3%)	2.75 ± 0.07 (2.5%)	3.04 ± 0.06 (2.0%)

Table S2 Comparison of analytical properties of RCA-based DNA machine with other miRNA detection methods.

Detection method	LOD	Readout	Ref.
branched RCA	18 fM	fluorescence	S1
graphene oxide/ ISDPR	2.1 fM	fluorescence	S2
loop-mediatedisothermal amplification (LAMP)	1 pM	fluorescence	S3
exponential amplification reaction (EXPAR)	0.1 aM	fluorescence	S4
competitive hybridization with Fc-AuPNs label	10 fM	electrochemistry	S5
duplex-specific nuclease signal amplification	100 fM	fluorescence	S6
RCA-based DNA machine	0.68 fM	colorimetric assay	this work

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