Supplementary Materials

Dual Amplification Ratiometric Biosensor Based on DNA Tetrahedron Nanostructure and Hybridization Chain Reaction for Ultrasensitive Detection of MicroRNA-133a

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1. Experimental Section

Reagents. 2-(Dibutylamino)ethanol (DBAE) and Tris(2-carboxyethyl)-phosphine hydrochloride (TCEP) were purchased from Sigma-Aldrich (St. Louis, MO). The TM buffer (pH 7.4) was prepared by 10 mM Tris-HCl and 50 mM $MgCl_2$. The PBS solution (0.1 M, pH 7.4) containing 0.1 M KCl was prepared by the stock solution of 0.1 M Na_2HPO_4 and 0.1 M NaH_2PO_4. Ultra-pure water (18.25 MΩ·cm) was used throughout the experiment. All DNAs and miRNAs were synthesized and purified by Sangon Biotech. Co., Ltd. (Shanghai, China) and the sequences are listed in Table S1.

The binding regions between miRNA-helper and S1, H1 or miRNA-133a are marked in the same colors. The sticky ends and loops of hairpin DNA strands are shown underlined.

Apparatus. Square wave voltammetry (SWV) and electrochemical impedance spectroscopy (EIS) were measured using a CHI 760e electrochemistry workstation (Shanghai Chenhua Apparatus Inc., China). The EIS measurements were conducted in 5 mM $[Fe(CN)_6]^{3-/4-}$ solution containing 0.1 M KCl with the frequency range of 0.1-10 kHz and amplitude of 5 mV. ECL measurements were performed on MPI-E ECL instrument (Xi'an Remex Analysis Instruments Co., Ltd., China). Atomic force microscope (AFM) image was obtained by a LEXT OLS4500 microscopy (Olympus, Japan).

Preparation of DTN. DTN was synthesized from a 90-nucleotides of DNA strand (S1) and three thiolated DNA strands with 55 nucleotides (S2, S3 and S4). Briefly, S2, S3 and S4 were incubated with TCEP (100 mM) in TM buffer for 30 min at room temperature, respectively. Afterwards, equal volume of S1, S2, S3 and S4 with the same concentration of 4 μM were mixed together and gently stirred for 1 min. The resulting mixture was heated to 95 °C for 5 min, and rapidly cooled to 4 °C to form stable DTN. The finally obtained DTN solution (1 μM) was stored at 4 °C for further use.

Native Polyacrylamide Gel Electrophoresis (PAGE). 15% native polyacrylamide gel electrophoresis was performed in TBE buffer (89 mM Tris-Boric Acid, 2 mM EDTA). The resulting electrophoresis strips were stained by silver ions

and scanned by a HP Scanjet G4050. Prior to the HCR, the miRNA-helper, H1 and H2 probes were heated to 95 °C for 2 min, respectively, and then cooled to room temperature at a cooling rate of 6 ℃/min. After the electrophoretic run was terminated, the sample holes on the gel slice were cut off to facilitate the following staining and imaging.

Fabrication of the ECL-EC ratiometric biosensor. Firstly, the gold electrode (GE) (Φ = 3 mm) was pretreated with a piranha solution (98%H_2SO_4:30% H_2O_2 = 3:1) for 20 min. Then, the GE was polished sequentially with alumina powder (0.3 and 0.05 μm) and ultrasonically cleaned with ethanol and ultrapure water. Afterwards, the cleaned GE was electrochemically activated in 0.5 M H_2SO_4 solution by circulating the potential from -0.2 to 1.6 V with a scan rate of 100 mV/s until the stable characteristic peaks were obtained. Finally, the electrode was rinsed with ultrapure water and dried with N_2.

Secondly, 8 μL of DTN solution (1 μM) was dropped on the surface of the cleaned GE and incubated at 4 °C for 10 h. Next, the DTN/GE was incubated with 8 μL of miRNA-helper (1.5 μM) at 37 °C for 2 h. Subsequently, 8 μL of miRNA-133a solution with various concentrations was casted on the surface of the modified electrode at 37 °C for 75 min. Finally, 8 μL of H1+H2 mixture (1 μM) was spread on electrode surface to react at 37 °C for 1.5 h. After each step of the modification, the electrode was rinsed with PBS buffer for three times.

Measurement of ECL and EC signals. The ECL and SWV signals were measured in PBS solution (0.1 M, pH = 7.4) containing 20 mM DBAE and 0.1 M KCl

at room temperature by a three-electrode system (the reference electrode was a saturated calomel electrode and the auxiliary electrode was a platinum wire). The scanning potential for ECL was in the range of 0 to 1.4 V with a scan rate of 100 mV/s, and the voltage of photomultiplier tube (PMT) was set at 1000 V. SWV measurements were performed from 0 to -0.5 V, the modulation amplitude was 25 mV and the step potential was 4 mV. We firstly measured the ECL signal and then immediately performed negative potential scanning to obtain SWV signal.

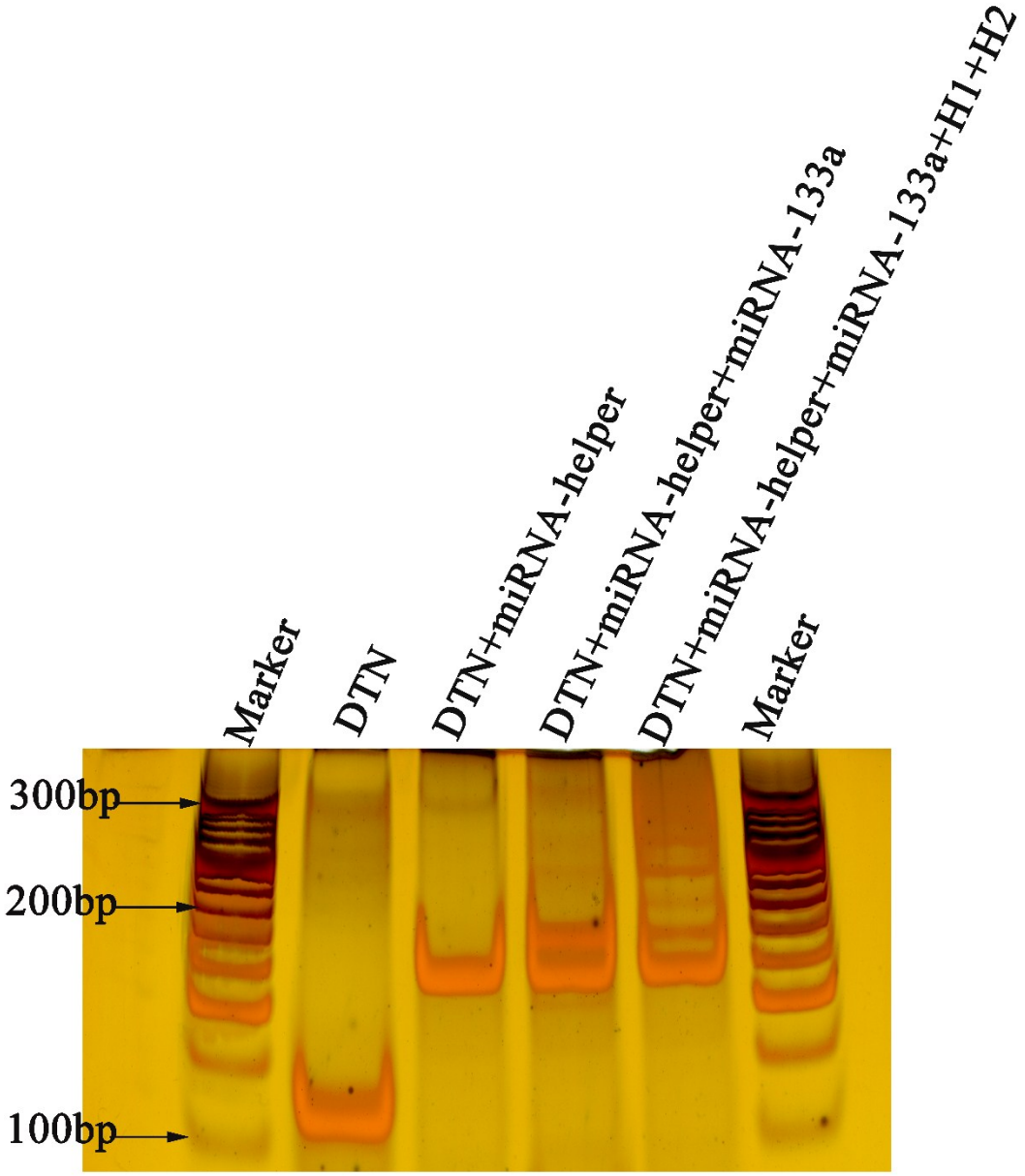

Fig. S1 PAGE image of tetrahedron after HCR to show indeed the double stranded DNA has expanded upon miRNA binding.

2. Optimization of detection conditions

In order to obtain excellent detection performance, the concentration of miRNA-helper, the incubation time between miRNA-helper and miRNA-133a, as well as the HCR time were optimized. As shown in Fig. S2A, the current signal enhanced with the increase of miRNA-helper concentration and reached a relatively stable value at the concentration of 1.5 μM, indicating that the reaction of miRNA-helper and DTN has reached equilibrium at this concentration. Therefore, 1.5 μM was used as the optimal concentration of miRNA-helper. The effect of incubating time between miRNA-helper and miRNA-133a on the ratio of $ECL_{Ru}/Current_{MB}$ was investigated (Fig. S2B). As the incubating time increased from 15 to 75 min, more and more miRNA-133a reacted with miRNA-helper, thus exposing more initiator chains and triggering more hybridization reaction events. Consequently, gradually enhanced ECL signals and the $ECL_{Ru}/Current_{MB}$ ratios were obtained. However, when the reaction time increased further, the ratio levelled off, indicating that the appropriate incubation time for miRNA-helper and miRNA-133a was 75 min. In addition, the $ECL_{Ru}/Current_{MB}$ ratio improved with the increase of HCR time and then reached a plateau at 90 min (Fig. S2C). Thus, 90 min was selected as the optimal hybridization time.

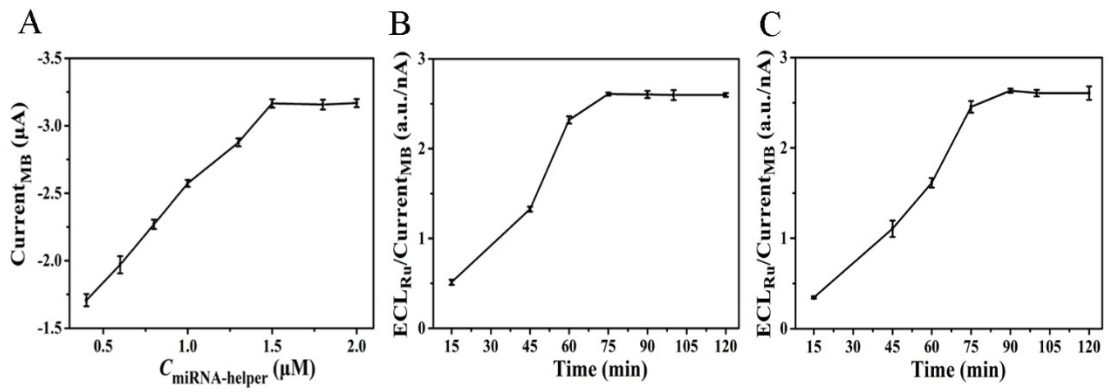

Fig. S2 (A) Effect of the miRNA-helper concentration on the current intensity. Effects of (B) the incubation time between miRNA-helper and miRNA-133a and (C) HCR time on the ratio of signals. The concentration of miRNA-133a was 50 fM.

Table S1 Sequences of DNAs and RNAs used in this study.

name	sequence (5′ to 3′)
S1	GTATCCAGTGGCTCAGACTTTTGACTTTTTTTTTTACAT TCCTAAGTCTGAAACATTACAGCTTGCTACACGAGAAG AGCCGCCATAGTA
S1*	GTATCCAGTGGCTCAGACTTTTGACTTTTTTTTTTACAT TCCTAAGTCTGAAACATTACAGCTTGCTACACGAGAAG AGCCGCCATAGTA-$(CH_2)_6$-SH
S2	SH-$(CH_2)_6$-TATCACCAGGCAGTTGACAGTGTAGCAAG CTGTAATAGATGCGAGGGTCCAATAC
S3	SH-$(CH_2)_6$-TCAACTGCCTGGTGATAAAACGACACTAC GTGGGAATCTACTATGGCGGCTCTTC
S4	SH-$(CH_2)_6$-TTCAGACTTAGGAATGTGCTTCCCACGTAG TGTCGTTTGTATTGGACCCTCGCAT
miRNA-helper	MB-$(CH_2)_6$-GTCAAAAGTCTGAGCCACTGGATACATTTG GTTCCATTTTACCAGCTGCAATTAGCTGGTAAAATGGA A
H1	AGCTGGTAAAATGGAAGTTTGATTCCATTTTACCAGCT A ATTGC-$(CH_2)_6$-$Ru(bpy)_3^{2+}$
H2	$Ru(bpy)_3^{2+}$-$(CH_2)_6$- TCAAACTTCCATTTTACCAGCTGCAATT AGCTGGTAAAATGGAA

miRNA-133a	AGC UGG UAA AAU GGA ACC AAA U
miRNA-499a	UUA AGA CUU GCA GUG AUG UUU
miRNA-208b-5p	AAG CUU UUU GCU CGA AUU AUG U
miRNA-328	CUG GCC CUC UCU GCC CUU CCG U

Table S2 Comparison of different biosensors for miRNAs detection.

Method	Target	linear range (fM)	LOD (fM)	References
Electrochemical	miRNA-21	10-10^7	1.95	[1]
Electrochemical	miRNA-21	500-10^7	176	[2]
Fluorescence	miRNA-155	100-10^6	33.4	[3]
SERS	miRNA-133a	1-10^7	0.306	[4]
CL	miRNA-133a	10^4-10^8	300	[5]
ECL	miRNA-141	0.1-10^6	0.0295	[6]
ECL	miRNA-155	0.1-10^5	0.036	[7]
ECL	miRNA-21	0.1-10^5	0.0186	[8]
ECL	miRNA-141	1-10^4	0.3	[9]
ECL	miRNA-133a	0.05-10^4	0.01217	This work

Table S3 Recovery experiments for miRNA-133a in real human serum.

Samples	Spiked (fM)	Found (fM)	RSD (%, n=3)	Recovery (%)
Human serum (15%)	0.1	0.0991	1.08	99.1
	10	10.15	2.09	101.5
	100	100.23	2.91	100.23
Human serum (10%)	0.1	0.1012	0.91	101.2
	10	10.27	1.89	102.7
	100	99.07	2.34	99.07
Human serum (2.5%)	0.1	0.1035	0.78	103.5
	10	10.41	2.07	104.1
	100	99.43	2.89	99.43

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