Supporting information

Sensitive SERS detection of miRNA via enzyme-free DNA machine signal amplification

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EXPERIMENTAL SECTION:

Materials and Reagents. All oligonucletides designed in this study were synthesized by Sangon Biotech Co., Ltd. (Shanghai China),and their sequences are listed in Table S1. Magnetic microbeads (MBs) modified with carboxyl group (1.0-2.0 μm, 10 mg/mL) were purchased from Baseline ChromTech Research Center (Tianjin, China). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and Chloroauric acid ($HAuCl_4 \cdot 3H_2O$) were obtained from Sigma-Aldrich (St. Louis, MO). The washing buffer was phosphate-buffered saline (PBS, 0.01 M NaH_2PO_4/ Na_2HPO_4, pH 7.4) . The MBs washing buffer were imidazol-HCl solution (0.1 M HCl, 0.1 M imidazol, pH 6.8). Buffer A contains 10 mM HEPES (pH 7.9), 1.5 mM $Mg(NO_3)_2$, 10 mM KNO_3 and 0.5 mM DTT. Buffer B contains 20 mM HEPES (pH 7.9), 25% (v/v) glycerol, 0.42 M $NaNO_3$, 1.5 mM $Mg(NO_3)_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 0.5 mM DTT. Buffer C contains 20 mM HEPES (pH 7.9), 20% (v/v) glycerol, 0.1 M KNO_3, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT. All chemicals employed were of analytical grade and used without further purification. The cells were obtained from tumor maker research center Cancer Hospital Chinese Academy of Medical Sciences (Beijing China). Doubly distilled deionized water was used throughout the experiments.

Apparatus. Transmission electron microscopy (TEM) images were recorded on a JEM-2100 transmission electron microscope (JEOL, Japan). UV/Vis absorption spectra were obtained with a Cary 50 UV/Vis-NIR spectrophotometer (Varian, USA). SERS was performed on an inVia Raman microscope (Renishaw, England).

Synthesis of gold nanoparticles (AuNPs). AuNPs were synthesized by citrate reduction method. The distillation flask and stir bar were soaked by nitric acid solution and rinsed thoroughly with H_2O before use. 100 mL of 0.01% (w/w) $HAuCl_4$ solution was constantly stirred and heated to its boiling point.

Then, 1.5 mL of 1% (w/w) trisodium citrate solution was added to the solution quickly. After several minutes, the solution changed from a pale yellow to a wine-red color, then refluxed for 30 min to ensure complete reduction. The colloidal solution was then slowly cooled to room temperature with continuous stirring. The synthesis of AuNPs was characterized by TEM. The prepared gold colloidal solutions were stored in brown glass at 4 °C until use.

Synthesis and characterization of the oligonucleotide functionalized AuNP. The preparation of oligonucleotide functionalized AuNP was made as follows according to a literature. Briefly, 10 μL of 1×10^{-7} M 3'-thiol modified capture DNA, 50 μL of 1×10^{-6} M bio-barcode (5'-thiol and 3'-Rox) and freshly prepared AuNPs (1 mL) into the glass vials, and shaken gently overnight (approximately 20 h) at 37 °C. Subsequently, the DNA-AuNPs conjugates were aged in 0.05 M salts (NaCl, 200 μL) for 6 h and in 0.1 M salts (NaCl, 200 μL) for 6 h, respectively. Excess reagents were removed by centrifugation at 10,000 rpm for 30 min. Then, the red precipitate was washed and centrifuged three times. The resulting oligonucleotide functionalized AuNP was finally dispersed into 30 μL of 0.01 M pH 7.4 phosphate buffer (PBS, 0.01 M, pH 7.4) and stored at 4 °C.

Cell Culture and Preparation of Cellular Extracts. Cells were grown in RPMI 1640 (Hyclone, penicillin 100 U/mL, streptomycin 100 μg/mL) with 10% fetal bovine serum. The cells were maintained at 37 °C in a humidified atmosphere (95% air and 5% CO_2). The process of cellular extracts were prepared according to this method: Briefly, 10^7 cells were washed once with phosphate-buffered saline and twice with Buffer A, orderly. The cell pellet was cultured in Buffer B with 0.1% Nonidet P-40 (20 mL per 10^7cells).and incubated for 20 min on ice, the lyzed cellular suspension was briefly mixed on a vortex and centrifugated for 10 min at 4 ºC. Then supernatant was diluted with 60 mL per 10^7 cells of Buffer C and stored at -80 ºC.

Preparation of DNA-MBs. The process of the immobilization of DNA (H2) onto MB was enforced as follows: Amino group modified H2 (40 μL, 10^{-7} M) was annealed in the water bath at 90 °C for 5 min and cooled naturally to room temperature. Then a 10 μL suspension of carboxylated MB was placed in a 0.5 mL Eppendorf tube (EP tube) and separated from the solution on a magnetic rack, after washing three times with the imidazole-HCl buffer. Then the MBs were dispersed in EDC (100 μL, 0.1 M) solution and shaken gently for 30 min at 37 °C to activate the carboxyl groups on the MBs, followed by washing three times with 200 μL of 0.01M PBS buffer. Finally, 40 μL of 10^{-7} M H2 was added into the activated MBs and shaken gently overnight at 37 °C. Then the DNA modified MBs were washed three times with PBS buffer, resuspended in 100 μL of 0.01 M PBS buffer, and stored at 4 °C for further use.

Enzyme-free DNA Machine signal amplification assay. Firstly, the H1, H3 and H4 were heated at the water bath 90 °C for 5 min in PBS buffer and allowed to cool to room temperature to form the hairpin structure. Then, the DNA of H1, H3 and H4 (40 μL, 10^{-7} M) were injected into the DNA-modified MBs and the miR-141 samples at different concentration (10 μL) were put into the reaction solution, and incubated for 2 h at 37 °C. Finally, the DNA-MB complex was magnetic separated and removed the supernatant, the MBs was washed three times with PBS buffer, and resuspended in 100 μL of 0.01 M PBS buffer,. After that, the solution of AuNPs modified bio-barcode (10 μL) was added into DNA-MBs solution for 2 h at 37 °C. Then the MBs incorporated SERS bio-barcodes were performed through magnetically controlled separation to remove the excess bio-barcode, washed with PBS for three times and redispersed in 10 μL of 0.01 M PBS (pH 7.4).

SERS Detection. After casting 1 μL of DNA-MBs solution onto the Au slides, air-dried at room temperature ahead of SERS analysis. SERS spectra were obtained by using a Renisaw in Via Raman Spectrometer at an excitation laser of 633 nm. The laser power was 5 mW and the resolution of the

spectra was over cm^{-1}. The collection time for each spectrum was 10 s. Ten spectra were obtained from different cites of each sample and averaged to represent the SERS results, and ten repeated experiments were carried out. Error bars showed the standard deviation of the ten experiments.

qRT-PCR procedure for miRNA analysis. Total RNAs (miRNA and mRNA) were extracted using Trizol (Invitrogen) in accordance with the manufacturer's procedure. RNA concentration was measured with NanoDrop (Thermo Scientific). CDNA was obtained by reverse transcription of total RNA using one step SYBR Green Mix including HiScript II Reverse Transcriptase, RNase Inhibitor and Champagne TaqTM DNA Polymerase (Vazyme)), respectively. The expression level of mature miR-141 was measured by real time PCR with HiScript II One Step QRT-PCR SYBR Green Kit (Vazyme). The threshold cycle (CT) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold (Figure S7). Experiments for each data point were run in triplicate, and data analysis was performed by use of 7500 Software v2.3.

Results and Discussions

Characterization of AuNPs. The AuNPs synthesized were characterized by TEM (Figure S1)

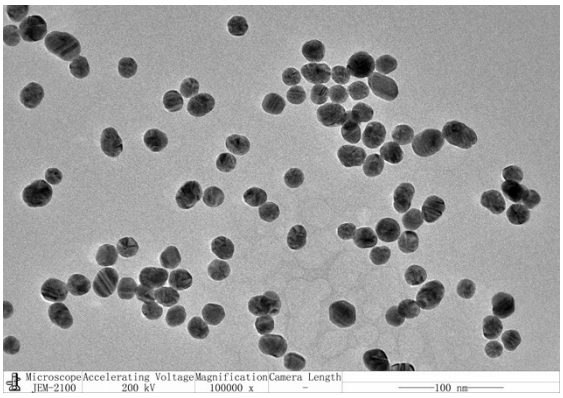

Figure S1. TEM image of AuNPs synthesized (about 20 nm)

UV-visible spectra of the ROX-DNA conjugates. The UV-visible spectra of signal probe (Rox-DNA), capture DNA probe, AuNPs and bio-barcode were carried out on a Cary 50UV/Vis spectrophotometer.

As revealed in Figure S2, curve a showed the AuNPs characteristic absorbance (~520 nm), curve b exhibited the characteristic absorbance of the capture DNA (~260 nm) and curve c showed the characteristic absorbance of the signal probe (~260 nm and 500~600 nm, respectively, DNA characteristic absorbance and Rox characteristic absorbance). Curve d revealed both the characteristic absorbance of AuNPs and Rox-DNA which meant the successful conjugation of Rox-DNA with AuNPs (bio-barcode).

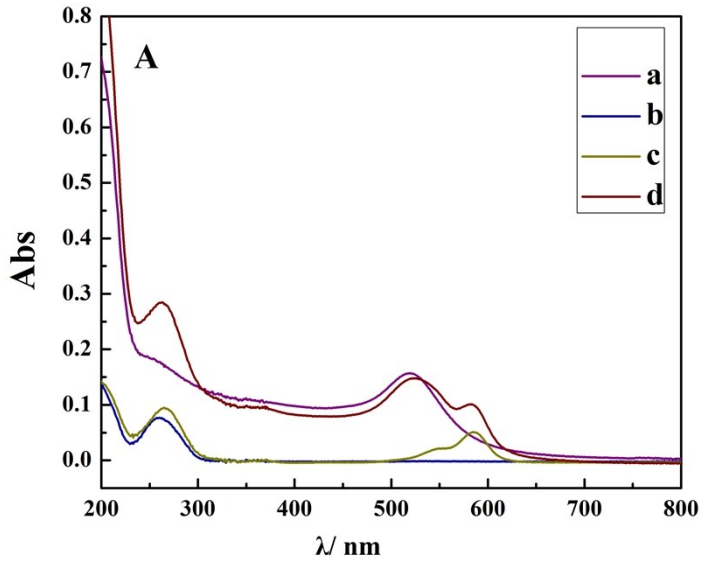

Figure S2. UV spectra of a) AuNPs , b) Capture DNA, c) Rox-DNA, d) bio-barcode

Optimization of the number of complementary bp for the H2 probe.

As a multifunctional probe, H2 probe play an important role in the TSD-HCR. It is the compete strand in the TSD recycle reaction, the trigger strand in the HCR, and the capture strand of HCR products. Thus, to achieve the best assay performance, the sequences of the probes need to be optimized. The number of complementary base pairs (bp) in the range from 8-15. (the length of the stem) was tested, as shown in Figure S3, the highest discrimination factor was achieved by using a seal probe with 13-bp.

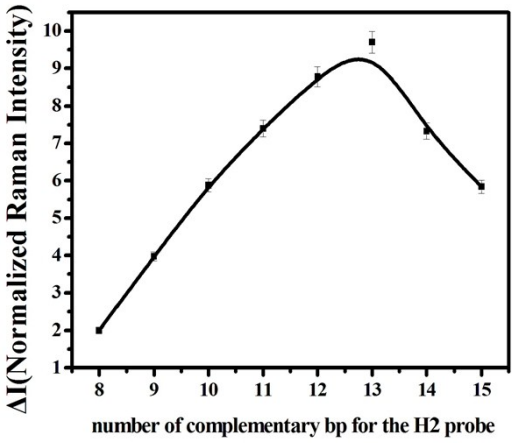

Figure S3. Effect of the number of complementary bp for the H2 probe on the Raman signal, the concentration of miR-141, 10^{-12} M.

Optimization of the concentration of the H2 probes immobilized on the MB.

Figure S4 shows the variance of SERS intensity along with the concentration change of H2 probes. It can be seen that the SERS intensity increases with the increase of the H2 probes concentration, and reaches a maximum at 1.0×10^{-7} M, upon analyzing target RNA at a concentration corresponding to 1.0×10^{-12} M. These results demonstrated that low concentration hairpin probes might not hybridize with sufficient target RNA, while high-concentration H2 probes might increase the steric hindrance of the microenvironment, adversely preventing their hybridization with target miRNA molecules. Therefore, 1.0×10^{-7} M was employed as the optimum concentration of H2 probes.

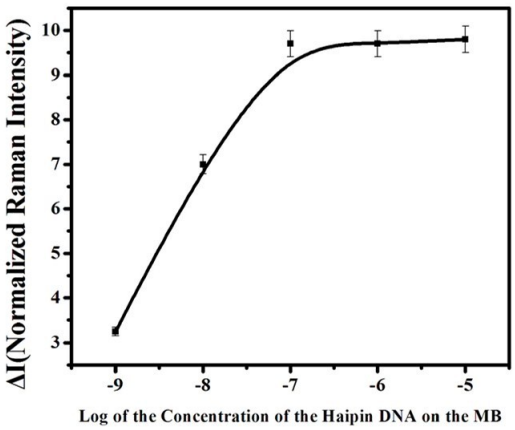

Figure S4. Effect of the concentration of the H2 probe immobilized on the MB on the Raman signal, the concentration of miR-141, 10^{-12} M.

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Optimization of the reaction temperature.

Temperature is an important parameter of reaction kinetics and determines the probability of collisions between the molecules. In this method, incubating temperature was the key factor that influences the stability and interaction of the hairpins. As shown in Figure S5, the Raman intensity (ΔI) changed as the temperature increasing from 20 °C to 45 °C, and reached a maximum at 37 °C, and decreased gradually as the temperature was increased further. Thus, 37 °C was chosen as the optimal temperature.

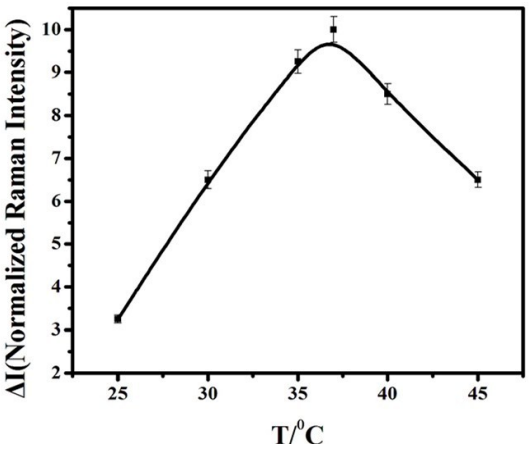

Figure S5. Influence of temperature of the reaction on the Raman signal, the concentration of miR-141, 10^{-12} M.

Optimization of the reaction time.

Figure S6 shows the changes of Raman signals generated by performing the experiments at different time intervals. The results revealed that the Raman intensity increased rapidly as the incubation time prolonged and reached a plateau after 120 min. Therefore, 120 min was selected as the best incubation time for the assay.

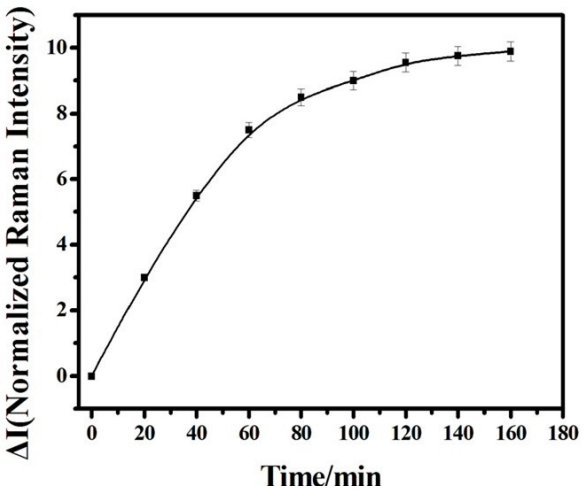

Figure S6. Influence of incubation time on the Raman signal, the concentration of miR-141, 10^{-12} M.

The Quantitative real-time fluorescence monitoring of the qRT-PCR reactions:

To assess the accuracy of this method, we also investigated the sensitivity of the conventional method by testing the same miRNA. Figure S7A, B indicates that the detection range of the conventional method is from 10 fM to 100 pM, and the correlation equation is $\Delta C_T = 55.5+2.3\lg C_{miRNA}$, with an R of 0.9972, and this sensing performance is much poorer than that of the developed assay.

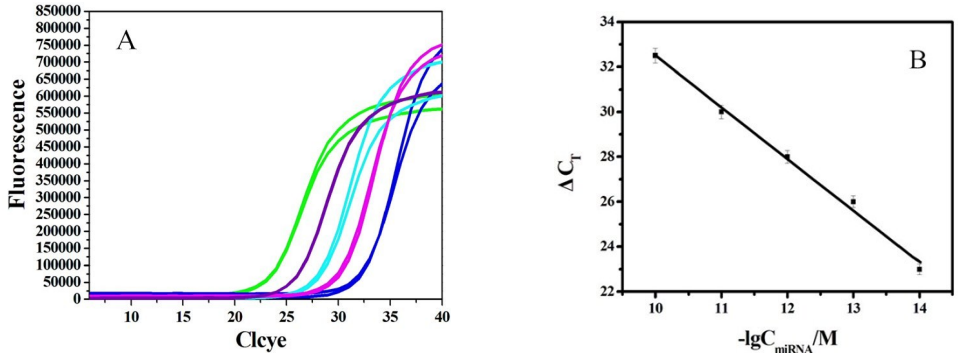

Figure S7. (A) Quantitative real-time fluorescence monitoring of the qRT-PCR reactions triggered by miR-141 at different quantity. (B) The relationship between ΔCt value and the logarithm of the concentration of miR-141 .

Table S1. Oligonucleotide sequences used in our experiments.

Oligonucleotides name	Sequences(5'-3')
H1	CCATCTT TACCAGAC AGTGTTA GGTGTGTGTGG TAACACT GTCTGGTA CTTTCCCTCTC
H2	AGTGTTA CCACACACACC TAACACT GTCTGGTA GGTGTGTGTGG TTT--NH_2
H3	CGACATGTTCA CTTTCCCTCTC TTCGGCTT GAGAGGGAAAG TACCAGAC
H4	AAGCCGAA GAGAGGGAAAG GTCTGGTA CTTTCCCTCTC CGACATGTTCA
Capture strand	TGAACATGTCG-SH
miR-141	UAA CAC UGU CUG GUA AAG AUG G
miR-200	CUG UGC GUG UGA CAG CGG CUG A
miR-200b	UAA UAC UGC CUG GUA AUG AUG A
miR-429	UAA UAC UGU CUG GUA AAA CCG U
Signal DNA	Rox-TTTTTTCCTAGCGAC-SH
H2-1	AGTGTTA CCACACACACC TAACACT GTCTGGTA GGTGTGTGTGG TTT--NH_2
H2-2	AGTGTTA CCCACACACACC TAACACT GTCTGGTA GGTGTGTGTGGG TTT--NH_2
H2-3	AGTGTTA CCCACACACC TAACACT GTCTGGTA GGTGTGTGGG TTT--NH_2
H2-4	AGTGTTA CCCACACAC TAACACT GTCTGGTA GTGTGTGGG TTT--NH_2
H2-5	AGTGTTA CCACACAC TAACACT GTCTGGTA GTGTGTGG TTT--NH_2
H2-6	AGTGTTA CCCACAC TAACACT GTCTGGTA GTGTGGG TTT--NH_2
H2-7	AGTGTTA CCACAC TAACACT GTCTGGTA GTGTGG TTT--NH_2

Table S2. The comparison of different methods for miRNA detection

Method	Transducer	Detection limit
bio-barcoded nanoparticles and HRP catalyzed H_2O_2	chronoamperometry	0.06 Pm[1]
catalyzed hairpin assembly reaction (CHA) and hybridization chain reaction (HCR)	celectrochemistry	10 fM[2]
used as templates for copper nanocluster (CuNC)	electrocatalysis	8.2 fM[3]
isothermal amplification reaction	DPV	5.36 fM[4]
CdTe QDs-based ERET	ECL	21.7 fM[5]
Using quadratic isothermal amplification	fluorescence	10 fM[6]
DSN-assisted target recycling	SWV	4.2 fM [7]
The electrocatalytic property of heteroduplex-templated copper nanoclusters	electrochemical	8.2 fM[8]
DNAzyme-Assisted Target Recycling and Rolling Circle Amplifications	ECL	0.3 fM[9]
DNAase-Triggered Signal Amplification	DSN-SPR	3 fM[10]
DNA detection based on hybridization chain reaction	fluorescence	67.5 fM[11]
This work	SERS	0.17 fM

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