Supporting Information for

A New Enzyme-Free Quadratic SERS Signal Amplification Approach for Circulating MicroRNA Detection in Human Serum

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EXPERIMENTAL SECTION

Materials and instruments. All oligonucleotides sequences were dissolved in highly pure water (sterile Millipore water, 18.3 MΩ) as stock solutions. The concentrations of the solution were estimated by UV absorption using published sequence-dependent absorption coefficients. Streptavidin-coated silica microbeads (SiMBs, non-porous, 2 μm) were purchased from Bangs Laboratories, Inc. and dispersed at 0.1 mg/mL in 100 mM phosphate-buffered saline (PBS, pH 7.4). 4-aminobenzenethiol (4-ABT) was purchased from Alfa Aesar. All other chemicals were of analytical reagent grade and were used as received, unless otherwise stated. Human serum were provided by the Hunan Provincial Tumor Hospital, Central South University (China). The size and shape of SiMBs under different conditions were determined by SEM images obtained on JSM-6700F microscopes (JEOL, Ltd., Japan), respectively. Atomic Force Microscopy (AFM) images were performed on a SPI3800N-SPA400 (Seiko Instruments, Inc.). SERS measurements were performed using a confocal microprobe Raman instrument (Ram Lab-010, Horiba Jobin Yvon, France), and spectra were acquired at 25 ^{o}C using an 632.8 nm He-Ne laser and a 50×long working objective lens (8 mm). For each Raman spectrum, the laser output power was 3 mW, the diameter of laser focus point was 2 μm, the collecting time was 20 s, and the width of the slit and the size of the pinhole were set as 100 and 100 μm, respectively.

Quantitative analysis of P_1/P_0 on the surface of SiMBs. The biotin-labeled P_1 was first incubated with streptavidin-coated SiMBs. The mixtures were vortexed at room temperature for 1 h, followed by washing three times with PBS using centrifugation at

1 000 rpm to remove any P_1 that did not conjugate to the SiMBs. The absorption maximums (measured at 260 nm) of the supernatant, containing free P_1 removed from the SiMBs, were converted to molar concentrations of DNA by UV-Vis absorption using published sequence-dependent absorption coefficients. Finally, the average number of successfully conjugated oligonucleotides on the surface of SiMBs was obtained. The concentration of hybridized P_0 was also determined using the same method.

Preparation of AgNPs. All glassware was thoroughly cleaned overnight with freshly prepared 3:1 HCl/HNO_3 (aqua regia) and rinsed thoroughly with Mill-Q water prior to use. AgNPs were prepared following the reported protocols published by Lee and Meisel. Briefly, 100 μL of 100 mM $AgNO_3$ were added into 10 mL of water and cooled to ice cold temperature. Then, 30 mL of 2×10^{-3} M $NaBH_4$ solution was prepared. Under vigorous stirring and ice cold conditions, 10 mL of $AgNO_3$ solution were added dropwise to the above $NaBH_4$ solution. The stirring was stopped, and the clear yellow sol showed the characteristic surface plasmon absorption band of nanosized Ag particles at *ca.* 396 nm. The colloid was stored at 4 °C until use.

Preparation of AuNPs@4-ABT. The vigorously stirred 1.0×10^{-4} g/ml $HAuCl_4$ solution was heated to boiling followed by rapid addition of sodium citrate (1.0×10^{-4} g/ml), following by a color change from pale yellow to red. The solution was kept boiling for 15 min and then stirred at room temperature for another 15 min. The resulting AuNPs were used as seed particles for the synthesis of larger particles. A typical synthesis for 60 nm AuNPs was carried out as follows: 4 mL preformed seed AuNPs was added to 80 mL water, followed by the quick addition of 7.6 ml 1.0×10^{-3} g/ml $HAuCl_4$. The mixture was vigorously stirred at room temperature, then hydroxylamine hydrochloride (250 μM) was added to the mixture and the reaction was completed within 10 minute. The resulting gold colloids were stored at 4 °C in the dark. The resulting gold colloids were diluted with ultrapure water by 1:1 volume ratio (3.3×10^{-8} M) following by the incubation with 4-ABT solution (1.0×10^{-5} M) with alcohol-water mixed solvent for 5 minutes. Determined by UV-vis absorption spectra, the surface coverage of 4-ABT molecules on AuNPs is evaluated to be 2.85 pmol/cm^2.

Fabrication of long DNA polymer-conjugated AgNPs on the surface of SiMBs. The biotin-labeled P_1 was first incubated with streptavidin-coated SiMBs. The

mixtures were vortexed at room temperature for 1 h, followed by washing three times with PBS using centrifugation at 1 000 rpm to remove any DNA that did not conjugate to the SiMBs. The conjugates were dispersed in PBS and stored at 4 ^{o}C at a concentration of 0.1 mg/mL. Then, P_0 was added to the solution containing P_1-conjugated SiMB and the mixture was vortexed at room temperature for another 1 h, followed by washing three times with PBS using centrifugation at 1 000 rpm to remove free P_0. Next, miR-21 was added to the solution containing SiMB-P_1/P_0, and the mixture was vortexed at room temperature for another 0.5 h, followed by washing three times with PBS using centrifugation at 1 000 rpm to remove P_0/miR-21 complex. Next, SH-labeled M1/M2 were added and reacted at room temperature for HCR 12 h, followed by washing three times with PBS using centrifugation at 1 000 rpm to remove free SH-labeled M1/M2. Finally, AgNPs were added to the above attained SiMBs and incubated for 2 h, followed by washing three times with PBS using centrifugation at 1 000 rpm to remove free AgNPs and the conjugation of AgNPs and M1/M2.

SERS detection. Afterwards, 30% H_2O_2 solution was used to dissolve AgNPs retained on SiMBs to yield free Ag^+. For each assay, 20 μL sample solution containing Ag^+ was added into 500 μL AuNP@ 4-ABT colloid with phosphate buffer at pH 7.0, and the SERS spectrum was collected after incubation in sealed tube in darkness for 10 min. Finally, the supernatant was collected and mixed with buffer as sample solution, and then incubated with AuNPs@4-ABT to further SERS detection.

Sample analysis. Circulating miR-21 in serum were extracted using the miRNeasy RNA isolation kit from Qiagen. Reference values of miRNA were entgeltlich provides by AxyBio Company (Changsha, China). For direct profiling of circulating miR-21 in serum, each 100 μL of serum sample, obtained from CCL patients and two healthy persons, respectively, was diluted with PBS to a final volume of 500 μL (1:5 dilution), heated at 95 °C for 5 min, and then cooled rapidly on ice for 5 min. Then, the heat-denatured serum lysates were centrifuged at 15000 g for 20 min at 4 °C. Finally, 50 μL of the supernatant (equal to 10 μL of serum) was added to each HCR reaction.

RT-PCR experiments. The total RNA content of serum was extracted from human blood using the Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. To quantify the expression of mature miR-21, the

following procedure was carried out. 1.5 μg of total RNA was reverse-transcribed to cDNA using AMV reverse transcriptase (TaKaRa, Dalian, China) and looped antisense primers. The resulted cDNA was then quantified by RT-PCR by the Step-One Plus Detection system (Applied Biosystems, Foster City, CA, USA). The reaction conditions are described as follows: 95 °C for 5 min, followed by 40 cycles with a 15 s interval at 95 °C and a 1 min interval at 60 °C. All reactions were performed in triplicate, and U6 was used as the internal control. Notably, all primers used for these assays are listed as follows[1]: miR-21 forward primer: 5'-ACA CTC CAG CTG GGT AGC TTA TCA GAC TGA-3'; miR-21 reverse primer: 5'-CTC AAC TGG TGT CGT GGA GTC GGC AAT TCA GTT GAG TCA ACA TC-3'; U6 forward primer: 5'-CTC GCT TCG GCA GCA CA-3'; miR-21 reverse primer: 5'-AAC GCT TCA CGA ATT TGC GT-3'.

Live subject statement. All experiments were performed in compliance with the relevant laws and institutional guidelines, and Hunan Provincial Tumor Hospital, Central South University (Changsha, China) have approved the experiments. The informed consent was obtained for any experimentation with human subjects.

Table S1. Oligonucleotides Used in This Work*

Entry	Sequence (5′-3′)
P_0	TCAACATCAGTCTGATAAGCTA
P_1	ACCTGGGGGAGTATTGCGGAGGAAGGT
M1	GACTGATCAGCTTAGCGCCGGCAGCGCTAAGCTGATCAGTCTGATATTTTTTTTTT-SH
M2	SH-TTTTTTTTTTTGCCGGCGCTAAGCTGATCAGTCTATCA GACTGATCAGCTTAGCGC
miR-21	UAGCUUAUCAGACUGAUGUUGA
SM-21	UAGCUUAUCUGACUGAUGUUGA
miR-141	UAACACUGUCUGGUAAAGAUGG

Table S2. Performance of Various miRNA Sensor

Signal output	Enzyme-assisted	LOD	Ref
Fluorescence	Yes	10 fM	2
Fluorescence	Yes	2.1 fM	3
Fluorescence	Yes	1 fM	4
Electrochemiluminescence	No	0.68 fM	5
Electrochemical	No	67 fM	6

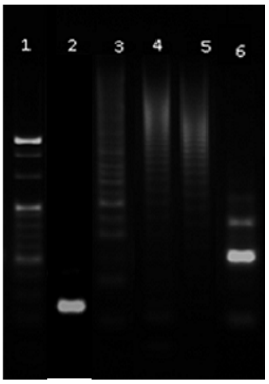

Figure S1. EB-stained agarose gel electrophoresis demonstrates P_1-initiated HCR. Lane 1, 25 bp marker; lane 2, mixture of 1.0 μM M1 and M2; lanes 3-6, the presence of P_1 (100 nM, 200 nM, 500 nM and 1 μM) with a mixture of M1 and M2. HCR time: 12 h.

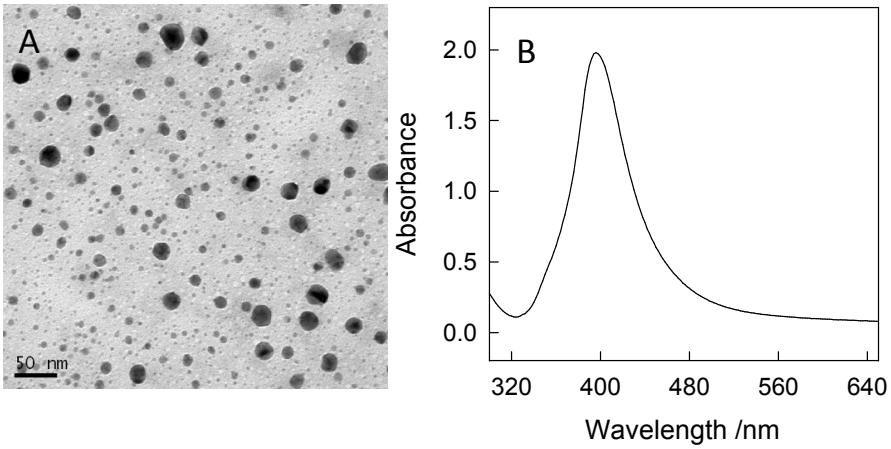

Figure S2. TEM images (A) and UV-visible spectra (B) of the 12 nm AgNPs.

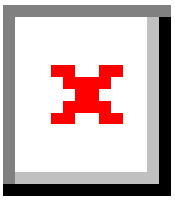

Figure S3. Representative TEM images of AgNPs loaded on the formed long nicked double-helix by HCR.

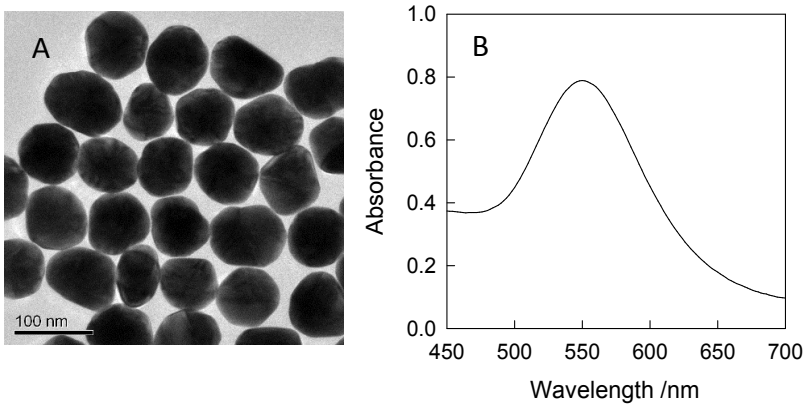

Figure S4. (A) TEM image and (B) of UV-visible spectra AuNPs.

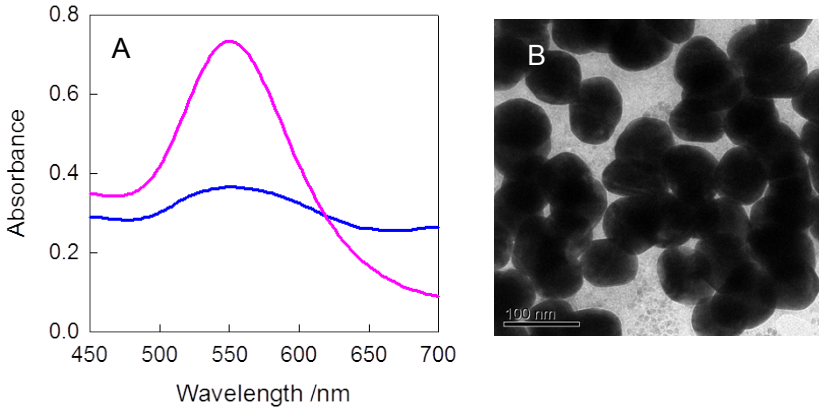

Figure S5. (A) UV-visible absorption of AuNPs@4-ABT before (blue trace) and upon (pink trace) the addition of 1 μM Ag^{+}. (B) TEM images of AuNPs@4-ABT upon the addition of 1 μM Ag^{+}.

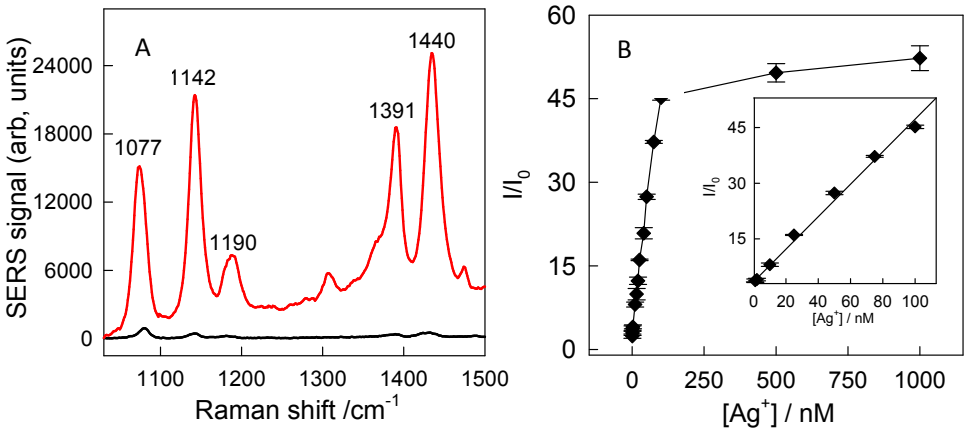

Figure S6. (A) SERS spectra of AuNPs@4-ABT before (black trace) and after (red trace) the addition of 1 μM Ag^+ along with the peak assignment. (B) SERS intensity ratio, I/I_0, of the 1439 cm^{-1} band, is plotted against the concentrations of Ag^+. The inset shows a linear relationship between I/I_0 and Ag^+ concentration at 1.0×10^{-9} to 1.0×10^{-7} M. Error bars show the standard deviation of three experiments. The collection time for each spectrum was 15 s and the accumulation was set as 3.

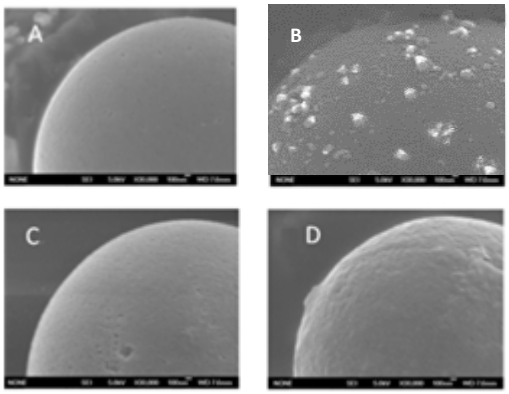

Figure S7. Representative SEM images of P_1/P_0-SiMBs (A) before and (B) after miR-21 addition and subsequent following by HCR. For comparison, (C) shows the surface of SiMB after the same treatment upon miR-141 addition. (D) is the surface of (B) after dissolution with H_2O_2.

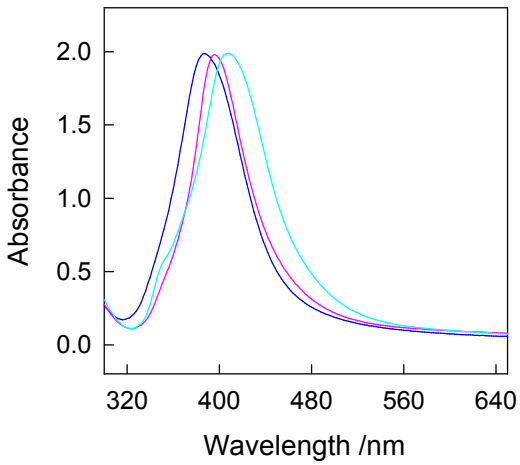

Figure S8. UV-visible spectra of 4 nm (blue curve), 12 nm (pink curve) and 30 nm (pale blue curve) AgNPs.

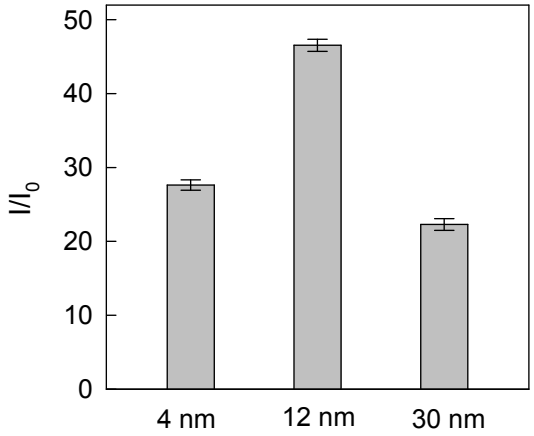

Figure S9. SERS signal enhancements of the 1440 cm^{-1}-band, I/I_0, of the DNA assemble-aided Ag^+-mediated cascade amplification with different sizes of AgNPs, in the presence of 100 pM miR-21. The collection time for each spectrum was 15 s and the accumulation was set as 3. The measuring conditions as shown in Figure 2.

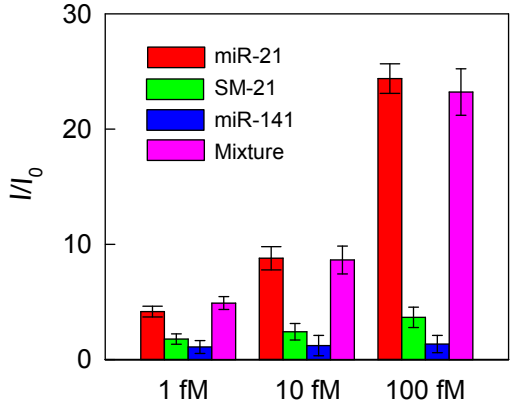

Figure S10. Specificity of miRNA assay. Bars representing the SERS enhancement I/I_0 from different inputs of miR-21, SM-21, miR-141 and the mixture with the same concentrations of 1 fM,10 fM, and 100 fM, respectively. Error bars represent standard deviations for measurements taken from at least five independent experiments. The measuring conditions as shown in Figure 2.

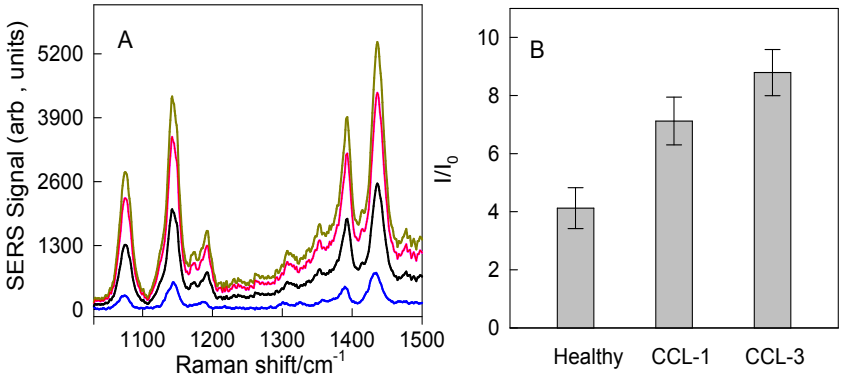

Figure S11. (A) Representative SERS spectrum and (B) SERS signal enhancements of our constructed DNA assemble-aided Ag^+-mediated cascade amplification SERS detector upon miR-21 extracted from healthy donor serum (black curve), CCL-1 patient (red curve), CCL-3 patient (grass green curve) addition. The spectrum of the DNA assemble-aided Ag^+-mediated cascade amplification SERS detector without any miR-21 addition is shown as blue curve. The measuring conditions as shown in Figure 2.

Reference:

[1] H. F. Dong, J. P. Lei, H. X. Ju, F. Zhi and F. Yan, *Angew. Chem., Int. Ed.,* 2012, **51**, 4067.
[2] Y. Li, L. Liang and C. Y. Zhang, *Anal. Chem.,* 2013, **85**,11174.
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