Electronic Supplementary Information (ESI)

for

Detecting miRNA by producing RNA: A Sensitive Assay that Combines Rolling-Circle DNA Polymerization and Rolling Circle Transcription

*Xuemei Li[a], Fuwei Zheng[b] and Rui Ren[a,] **

a Shandong Province Key Laboratory of Detection Technology for Tumor Makers, College of Chemistry and Chemical Engineering, Linyi University, Linyi 276005, P. R. China,

b Key Laboratory of Sensor Analysis of Tumor Marker, Ministry of Education, College of Chemistry and Molecular Engineering, Qingdao University of Science and Technology, Qingdao 266042, P. R. China

Table of Contents

S1. Materials and Instruments

DNA sequences. Four DNA oligonucleotide strands were used in the reaction system for the reported assay: two strands were for constructing the circular probe; one "primer strand" was used for starting the RCP, and one "attaching strand" was used to label the gold nanoparticle, so that the latter can bind to the transcripts from RCT and enhance the Raman scattering of MG.

These DNA oligomers were synthesized by Sangon Biotech Co. Ltd. (Shanghai, China). Their detailed sequences are listed in Table S1.

Table S1. DNA Oligomers Sequences

Strand Name	**Sequence**
Circular Probe strand 1	5'- P - CCG CCC GC|C GCC CC|C CCT GAG GTA CTA TAC AAC CTA CTA CCT CAG GGT CTC CCT ATA GTG AGT CGT ATT AGC▲ TGA GGA AAA AAA AAG GGG CG-3'
Circular Probe Strand 2	5'- P - GCG GGC GGG *GGA TCC* **CGA CTG GCG AGA GCC AGG TAA CGA ATG** *GAT CC*C- 3'
RCP Primer	5'- CTC AGG G|GG GGC G|GC GGG CGG| G -3'
Attaching Strand	5'- SH - CCC TAT AGT GAG TCG TAT TAG C -3'
Complementary Strand of Circular Probe strand 1	5'- CGC CCC TTT TTT TTT CCT CAG CTA ATA CGA CTC ACT ATA GGG AGA CCC TGA GGT AGT AGG TTG TAT AGT ACC TCA GGG GGG GCG GCG GGC GG -3'
Complementary Strand of Circular Probe strand 2	5'- GGG ATC CAT TCG TTA CCT GGC TCT CGC CAG TCG GGA TCC CCC GCC CGC -3'

*** Notes:**

1) The small triangle ▲ indicates the potential nicking point of Nb.BbvCI. A functional Nb.BbvCI nicking point would form on the complementary strand upon hybridization.
2) Color codes of the sequences are in accordance with those of the segments in Scheme S1 as well as in Scheme 1.
3) 5'-P- stands for the phosphate group.

RNA sequences were synthesized by Genebase Co. Ltd. (Shanghai, China). These include the target in this study, three mismatched miRNAs and one negative control for the selectivity tests. The simulated transcript had the sequence of one repetitve unit of the expected RCT product, i.e. it had the RNA sequence complementary to that of the circular probe. This strand was used in the measurement of the SERS responses with no amplification applied, to provide a basepoint for the comparison of amplification.

Table S2. RNA Oligomers Sequences

Strand Name	**Sequence**
Target miRNA (hsa-let-7a)	5'- UGA GGU AGU AGG UUG UAU AGU U -3'
Precursor of the Target (pre-hsa-let-7a)	CUU CAC UGU GGG A UGA GGU AGU AGG UUG UAU AGU U UUA GGG UCA CAC CCA CCA CUG GAG AUA ACU AUA CAA UCU ACU GUC UUU CCU AAC GUG AUA
Mismatched miRNA #1 (hsa-let-7b)	5'- UGA GGU AGU AGG UUG UGU GGU U - 3',
Mismatched miRNA #2 (hsa-let-7c)	5'- UGA GGU AGU AGG UUG UAU GGU U - 3'
Mismatched miRNA #3 (hsa-let-7d)	5'- AGA GGU AGU AGG UUG CAU AGU U -3'
Negtive Control	5'- UUG UAC UAC ACA AAA GUA CUG G-3'
Simulated transcript	5'- GCU AAU ACG ACU CAC UAU AGG GAG ACC CUG AGG UAG UAG GUU GUA UAG UAC CUC AGG GGG GGC GGC GGG CGG GGG AUC CAU UCG UUA CCU GGC UCU CGC CAG UCG GGA UCC CCC GCC CGC CGC CCC UUU UUU UUU CCU CA -3'

Note: the red nucleotides are the mismatched ones; the blue segment in the precursor shows the location of the mature miRNA sequence.

Buffer solutions:

Imidazole-HCl solution was prepared by mixing 0.1 mol HCl with 0.1 M imidazole solution at appropriate ratio to reach a pH of 6.8.

TAE (Tris-acetate EDTA) buffer contains 40 mM tris(hydroxymethyl)aminomethane (tris), 2 mM EDTA and 20 mM acetic acid, and the pH was adjusted to 7.9.

PBS (phosphate buffered saline) buffer contains 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.76 mM KH_2PO_4. The newly prepared PBS has a pH of 7.4; and the pH can be adjusted with HCl or NaOH if needed.

HEPES buffer contains 10 mM HEPES [4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid], 100 mM KCl and 5 mM $MgCl_2$.

Enzymes and relating buffers:

T4 DNA Ligase (EP0011) and the relating **buffer** were provided by Fermentas Inc. The buffer contains 400 mM Tris-HCl (pH 8.0 at 25°C), 100 mM $MgCl_2$, 100 mM DTT, and 5 mM ATP, and was diluted 10-fold when use.

***E. coli* DNA Polymerase Klenow Fragment (EP0051)** and the relating **Klenow buffer (10×)** were provided by Fermentas Inc. The buffer contains 500 mM Tris-HCl (pH 8.0 at 25°C), 500 mM NaCl, 100 mM $MgCl_2$ and 10 mM DTT, and was diluted 10-fold when use.

Nb.BbvCI (R0631) and the relating **NEBuffer 2 (10×)** were provided by New England Labs. The buffer contains 100 mM Tris-HCl (pH 8.0 at 25°C), 50 mM $MgCl_2$ and 10 mM DTT, and was diluted 10-fold when use.

T7 RNA RNA Polymerase (2540A) and its relating **Buffer** were provided by TaKaRa Bio Co. The buffer contains 40 mM Tris-HCl (pH 8.0 at 25°C), 8 mM $MgCl_2$ and 2 mM spermidine. DTT (dithiothreitol) were added separately.

Gold nanoparticles (AuNPs) for the enhancement of the Raman scattering signals were prepared through the chemical reduction of chloroauric acid. 2.0% sodium citrate solution and chloroauric acid solution (0.01%) were filtered through filter membrane with average aperture of 0.2 μm, respectively. 1.0 mL sodium citrate solution was added dropwise into 100 mL boiling chloroauric acid solution, and stirred for 10 min in persistent boiling. The prepared gold nanoparticles were stored under 4 °C before use. TEM examination indicated the prepared AuNPs were 15~20 nm in average diameter (Figure S1).

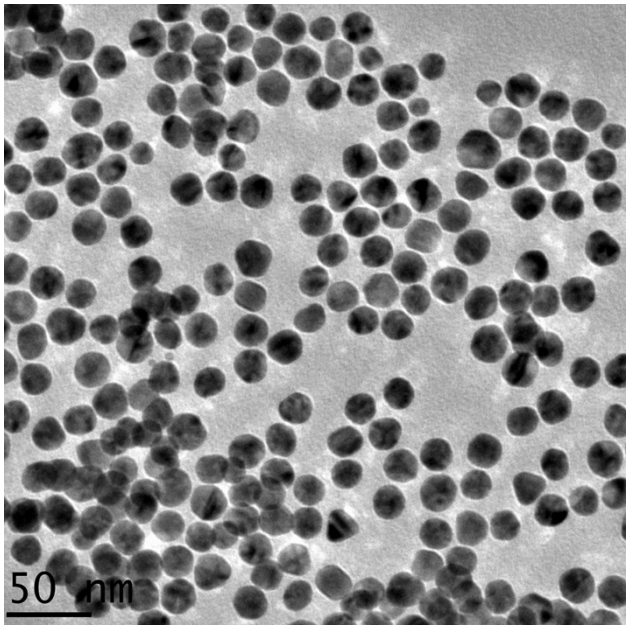


Figure S1. TEM image of the prepared AuNPs

The attaching strands were labeled on the AuNPs with the following procedures. AuNP was dispersed in the presence of TCEP (10 mM) in 1.5 mL Tris-acetate buffer (pH 5.2). After being stirred in the dark for 16 h, the DNA-AuNP conjugate was aged in 0.1 M NaCl solution containing 20 mM Tris-acetate (pH 7.0) for 24 h, and was separated through centrifugation at 15,000 rpm for 30 min. The red oily residue was washed with 100 μL solution containing 300 mM NaCl and 25 mM Tris-acetate (pH 8.2) and then re-centrifuged. The washing and centrifuging were repeated twice, and the residue was re-dispersed in 1 mL solution containing 100 mM NaCl and 25 mM tris-sodium acetate, and kept under 4 °C before use. UV-Vis spectra verified the attaching strand have been bound with AuNPs (Figure S2).

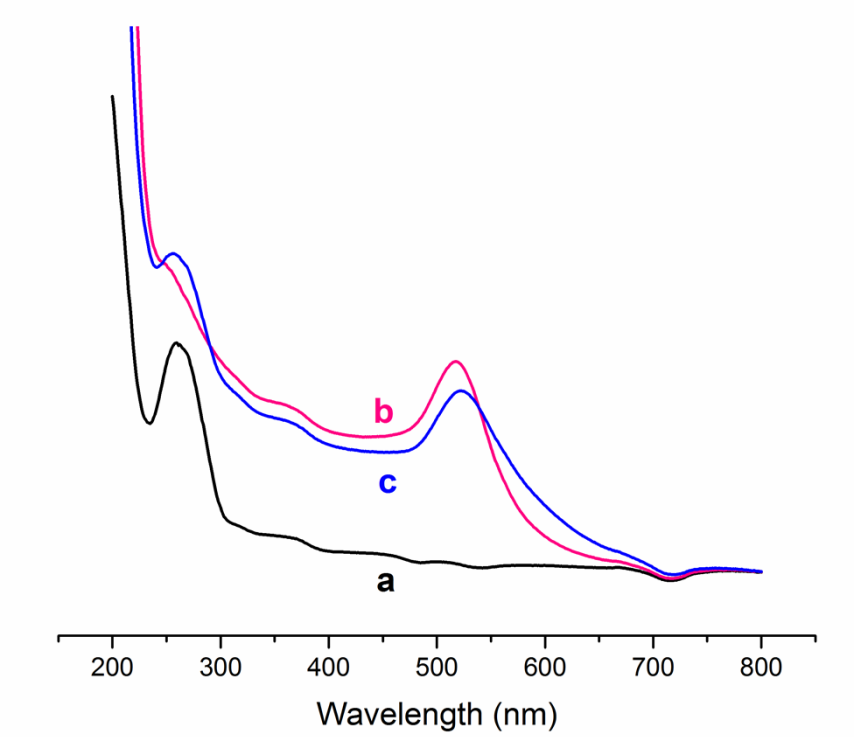


Figure S2. UV-Vis characterization of the binding between AuNP and attaching strand.

a) AuNP; b) attaching strand; c) attaching product

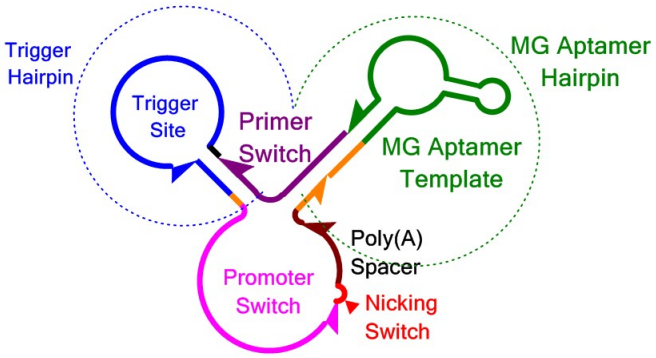
All the SERS measurements were carried out on the Renishaw inVia Raman Microscope. The excitation wavelength was set to 785 nm, and the Raman scattering spectra were recorded in the wavenumber range from 500 cm^{-1} to 1900 cm^{-1}.

Non-denaturing polyacrylamide gel electrophoresis (PAGE) was carried out on a DYCZ-24DN cell with Liuyi DYY-6C power supply (Beijing Liuyi Instruments, Beijing, China), and the PAGE patterns were imaged on a WD-9413B gel imaging system (same supplier as above). Typically, the PAGE was run at 120 V for 2 h. The TIRF image was recorded on a Leica AM TIRF MC apparatus using 635 nm as the excitation wavelength.

S2. Structure and Construction of the circular probe.

The reaction system in this assay was based on the circular probe, whose structure is illustrated in Scheme S1. It is a single-stranded closed DNA circle. Functionally, it served as both the template for RCP and the anti-sense part of the template for RCT (which means the RCT transcripts would contain the sequences complementary to that of the probe); it also contains

several sub-structures and several functional segments on which the triggering and interlinking of the reactions were based. These included:



Scheme S1. The detailed structure of the circular probe. The color codes are in accordance with those in Table S1; the arrows indicate the direction of the strand (5' > 3')

Sub-structures. The circular probe was 140 deoxynucleotides in total length. Two hairpin structures were designed on the circular probes: the "**trigger hairpin**" (33-nt in total, including a 15-nt loop and a 9-nt stem) and "**MG aptamer hairpin**" (68-nt in total, including a 26-nt loop and a 21-nt stem). The rest part of the circular probe was 39-nt long.

Functional units

The "**trigger site**" (color-coded blue in Table S1 and Scheme S1) is the site where the target recognition occurred, i.e. where the target miRNA specifically hybridized. Residing in the trigger hairpin, it included the whole loop (15-nt) and part (6-nt out of the 9-nt total) of the 3'-half of the stem, and was complementary to the 21 of the 22-nt target miRNA (hsa-let-7a), while the nt at the 3'-end of the target was excluded from hybridization. Such a design enabled the circular probe to recognize the target miRNA by sufficient hybridization, while excluded the possibility that the bound target would trigger any unexpected polymerization.

The MG aptamer template (color-coded green) is the anti-sense template for the transcription of the RNA aptamer of MG. It resided in the MG aptamer hairpin, and was complementary to the 38-nt sequence of MG aptamer. This segment took the shape of a hairpin because the reported MG aptamer also assume this shape. [S2, S3]

The "**primer switch**" (color-coded violet) was designed to be the switchable site for the binding of the RCP primer: in the initial status, the binding was blocked, but was enabled once the switch was "activated". It was complementary to the 22-nt primer, and comprised two connected

fractions, part of the trigger hairpin and part of the MG aptamer hairpin. On the trigger hairpin side, it occupied a 7-nt fraction in the 5'-half of the stem (opposite to the 3'-half, where the 6-nt fraction of the "trigger site" resided). 4-nt in this 7-nt fraction was complementary to the "trigger site". On the MG hairpin side, it occupied 15-nt fraction on the 3'-half of the stem.

The binding of the 22-nt primer to the primer switch was sufficiently ***blocked*** in the initial status of the probe, since the total length of the two double-stranded stems in the two hairpins was 41-nt (15-nt on the Trigger hairpin side and 26-nt on the MG aptamer hairpin side), much longer than that of the primer (22-nt). The blocking was supported by the stimulation with NuPack (developed by CalTech, http://www.nupack.org).

However, once the "primer switch" was release to be single-stranded in a considerable length, the binding would be "***enabled***". According to the design of this probe, such a release could be achieved once the target miRNA bound with the "trigger site", which would lead to the strand separation in the stem of the "trigger hairpin", meaning the release of the 5'-half of the stem.

In this sense, the "trigger site" and the "primer switch" were coupled in their actions: recognition of the target on the former would cause the activation of the latter. This coupling was essential in the design of the reaction system.

.

There were also several functional units in the rest of the circular probe:

A 9-nt poly(A) segment (color-coded brown) that was added to facilitate the formation of the RCT template[s1].

The nicking switch (color-coded red) was a 7-nt segment that was the anti-sense half of the double-stranded functional recognizing site of the nicking endonuclease Nb.BbvCI. Such site could be formed once this switch hybridizes with its complementary strand; and after this, the nicking would occur at where the red triangle in Scheme S1 marked, on its complementary strand.

The promoter switch (color-coded magenta) was the anti-sense half of the double-stranded functional T7 promoter. The promoter could be formed once this switch hybridizes with its complementary strand

The complementary strands to the circular probe were also prepared for the amplification comparison experiments.

In practice, the sequence of the circular probe initially existed in two strands, Circular Probe strand 1 and 2. Circular Probe Strand 1 contained the "trigger site", "primer switch", "nicking switch" and "promoter switch"; and Circular Probe Strand 2 mainly consisted of the "MG aptamer template". Each strand bore a double-stranded segment with a sticky end so that they can hybridize with each other. The circular probe was prepared by ligating two hybridized strands. Such an arrangement rendered the reported assay generalizable: by altering the Circular Probe Strand 2, one can change the signal production approach, so that different signal production approaches can be coupled with the recognition and amplification protocols.

The RCP primer strand was for primering rolling circle amplification. It was complementary to the "primer switch" in the circular probe.

The Attaching strand was used to label the gold nanoparticles, so that the latter could be attached on the RCT transcripts. This strand was complementary to part of the transcripts, and same as part of the circular probe, at the "promoter switch" plus 2-nt from the "nicking switch".

Preparation of the circular probe. To prepare the circular probe, circular probe strand 1 and circular probe strand 2 were mixed in equal concentration (typically, 2×10^{-8} M). And then, T4 DNA ligase and its buffer were added, and the system was incubated at 25 °C for 2 h. The formation of the circular probe can be testified by non-denature PAGE (see section 4 in this ESI).

S3. Detailed diagrams and descriptions of five stages

The reaction system recognizes the target and provides dual signal amplifications in four stages. Their diagrams and detailed descriptions are listed below.

Triggering and RCP cycle (Scheme S2)

Function: recognizing target miRNA; providing primary signal amplification.

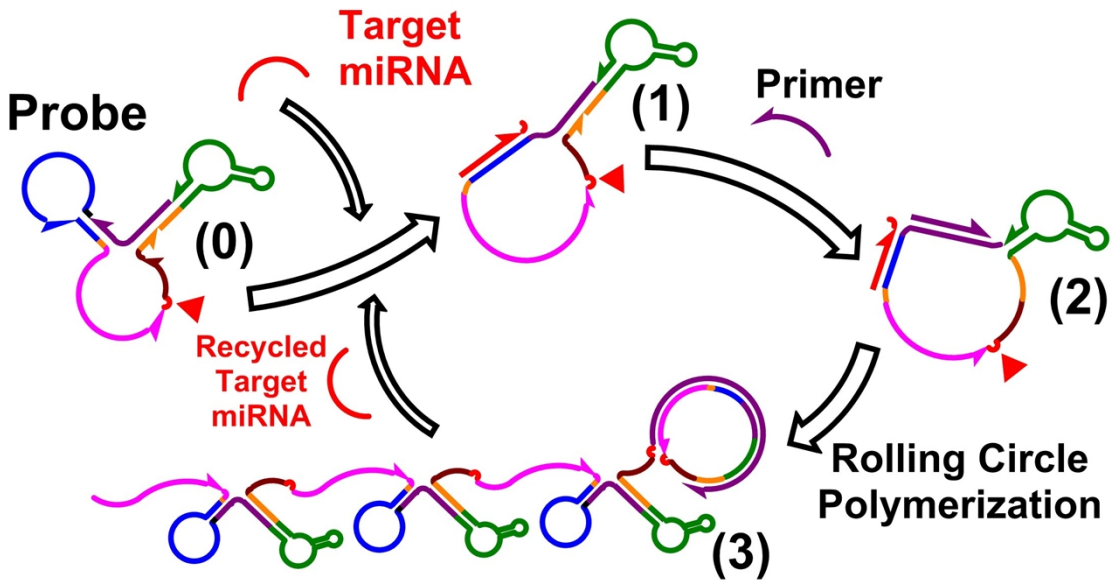
Triggering: hybridization recognition of miRNA at the triggering site in the circular probe.

Output: RCP product, the long DNA strand with repetitive units, with the sequence complementary to that of the circular probe.

Cycling: Target miRNA is displaced and released in RCP.

The first two stages, "triggering" and "RCP", formed a cycle. They recognized and circulated target miRNA, and generated TCP products. As the triggering, the target hybridized to the "trigger site", causing the strand separation of the stem of the trigger hairpin, in which the 5'-half of the stem was released to be single-stranded, thus open to the binding of the RCP primer, which then attached to form the **initialization complex** in Scheme S2.

In the presence of Klenow polymerase, its buffer and dNTPs, RCP occurred using **the circular probe** as the template, producing the RCP product. On the other hand, in RCP, the extended new strand displaces the target miRNA, which was then released into the solution, and was then available for another triggering on one of the excess probe. Thus the triggering and the RCP were carried out in a cycle.



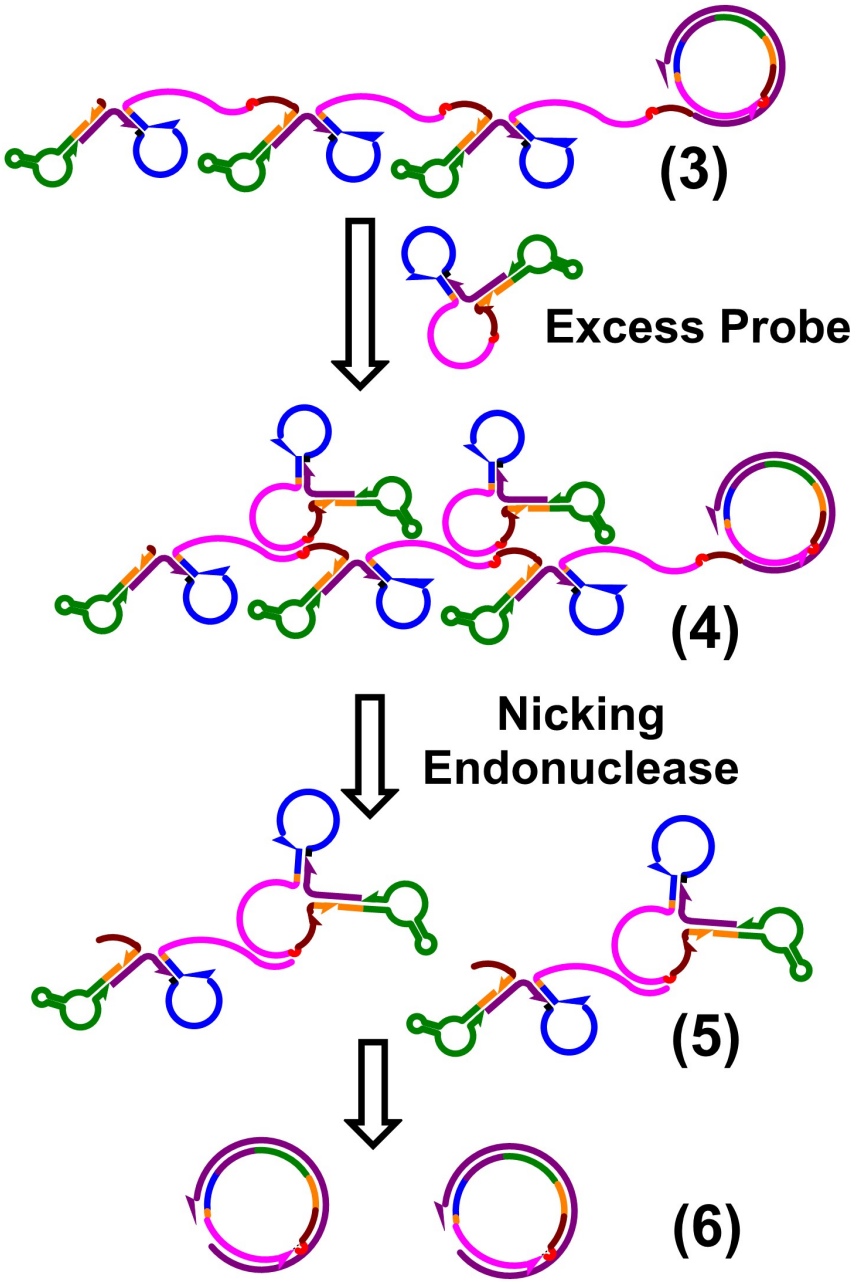
Scheme S2. Diagram of the cycle of triggering stage and the RCP.

Interlinking Stage (Scheme S3)

Triggering: hybridization of the RCP product with the excess circular probe

Output: double-stranded RCT template

The interlinking stage links the RCP and RCT, and conveys the amplification from the former to the latter. This stage occurred based on the fact that the circular probe was excess in quantity to the probe. Since the repetitive units in the RCP product were complementary to that of the circular probe, the former and the later would hybridize, preferably at the nicking switch (for this site was spaced from both two hairpins and would pose the least hindrance to the hybridization). The RCP product would be nicked at this site, forming fragments containing one or multiple units of the RCP products. These fragments could hybridize with excess probes to form double-stranded DNA circle, in which the promoter switch also hybridized with its complementary strand, forming the functional T7 promoter; thus this ds-circle could serve as RCT template.

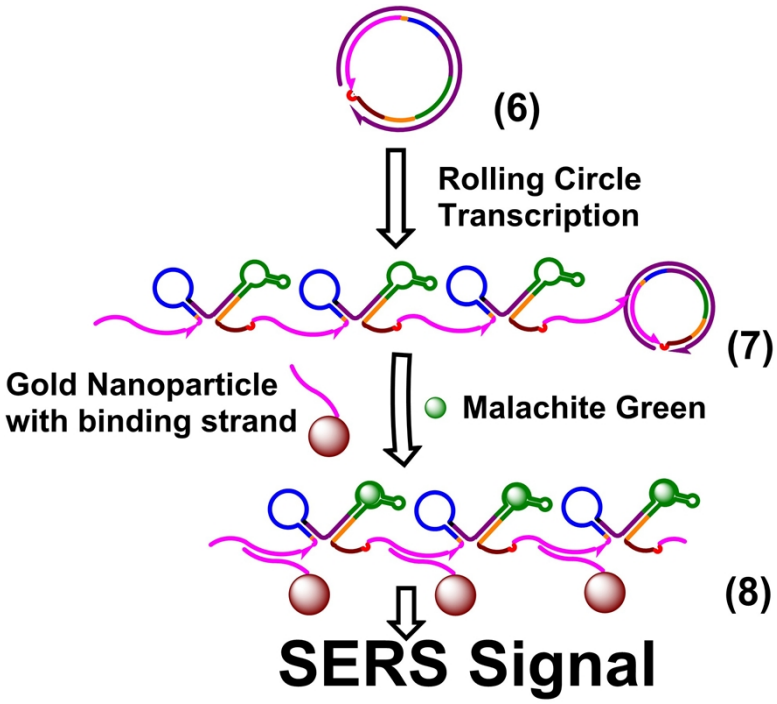


Scheme S3. Diagram for the Interlinking Stage

RCT and Signal-producing Stage (Scheme S4)

RCT was started based on the RCT template, in the presence of RNA polymerase, its buffer and NTPs mixture, producing RCT products, a long RNA strand with repetitive units, whose sequence were complementary to the circular probe.

Each unit of the RCT product contained a MG aptamer segment (transcribed based on the MG aptamer template in the circular probe) and can bind MG molecular in HEPES buffer; also, gold nanoparticles were attached on the transcripts to enhance the Raman scattering, by the hybridization of the attaching strands that were labeled on the AuNPs with the transcripts.



Scheme S4. Diagram for the RCT and Signal producing Stage

Typical experimental procedures (using SERS as signal production approach)

Prepared circular probe (10^{-8} M), RCP primer (0.10 μM), NEBuffer 2, Klenow (0.6 IU μL^{-1}), Nb.BbvCI (0.4 IU μL^{-1}), T7 RNA Polymerase (2.0 IU μL^{-1}), dNTP mixture (0.25 mM each), and NTP mixture (0.10 mM each) were mixed; the analyte was then added. The mixture was incubated at 37 °C for 3 h.

For the SERS measurements, MG of 1 μM and the AuNPs (labeled with attaching strand) were added into the resulting reaction mixture, and incubated at 37 °C for 1 h. Then, the mixture was sufficiently centrifuged in an EP tube, and washed with TAE buffer. Centrifugation and washing were repeated for three times. The residues were dispersed in water and then dropped on a thin gold layer supported by a glass slide. After air-drying, the residue were subjected to SERS measurements.

S4. PAGE and TIRF characterization of the network.

To verify whether the reaction system worked as planned as described above (or, as in Scheme 1), non-denaturing polyacrylamide gel electrophoresis (PAGE) was carried out to testify the DNA reaction part. The results are shown in Figure S3. And the generation of RCT products and its binding with MG molecules was testified with TIRF.

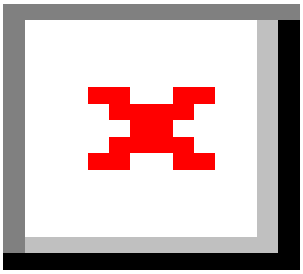


Figure S3. Polyacrylamide gel electrophoresis (PAGE) pattern. *m*: Marker; *a*: Circular probe strand 1; *b*: Circular probe strand 2; *c*: ligation product of Circular probe strand 1 and 2; *d*: primer; *e*: product of triggering stage; *f*: product after triggering and RCP stage; *g*: product after interlinking stage.

In the PAGE pattern, lanes a, b and c exhibited the result of the ligation of the two material strands (circular strands 1 and 2). PAGE of ligation product (lane c) indicated a nearly perfect ligation, verifying that the desired circular probe was produced.

Lanes d and e exhibited the result of the triggering stage. The product of the triggering stage, i.e. the combination of prepared circular probe (lane c), the target miRNA (not shown) and the

RCP primer (lane d), was shown in lane e. A single band with slightly lower migration rate than that of the circular probe was observed, verifying the generation of the initialization complex.

Lane f exhibited the result of RCP stage. A single band at the starting point was observed, indicating the generation of high-molecular-weight product. This was in accordance with the nature of the RCP product.

Lane g exhibits the result of interlinking stage. As a prominent contrast to other lanes, multiple bands were observed for this case. Comparison of the RCP product (lane f) with this lane clearly indicated that the RCP products had been degraded into fragments, as the result of nicking.

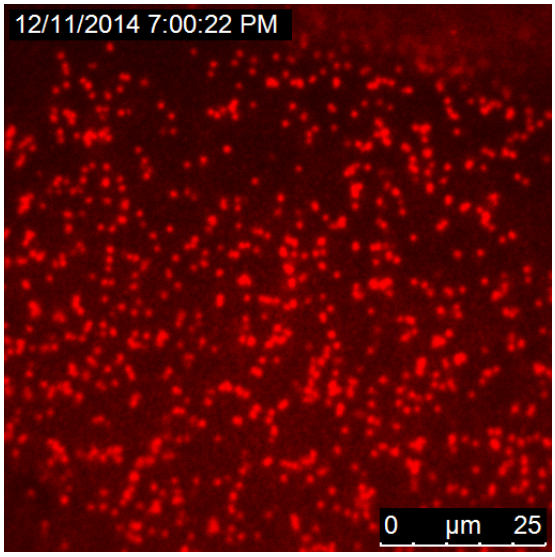


Figure S4. TIRF image of the product of the reaction system after binding with MG

The transcripts of RCT were imaged using TIRF (Figure S4). The reaction system was performed in a glass-bottom dish; MG was attached to the RCT products, while the gold nanoparticles were not added. The concentration of the target miRNA was 10^{-12} M. After the reactions, the reaction mixture was incubated with MG (10^{-9} M) for 30 min to allow the DNA and RNA molecules to absorb onto the glass bottom (based on attraction of opposite charges), and

then the fluorescence from the evanescent field close to the glass bottom was then imaged using an exciting wavelength of 635 nm.

Plenty of fluorescent dots and a few short string-shaped objects were observed in the emission range above 635 nm. This result was in accordance with the production of RCP transcript and its binding with MG.

S5. Optimization of reaction conditions

Various factors affected RCP, nicking and RCT processes, and thus affected the total amplification efficiency of the network. For these factors, the temperature and pH were predetermined by the user manual of the enzymes used in this study, *E. Coli* DNA Polymerase I Klenow fragment ("Klenow" for short), T7 RNA polymerase, and the nicking endonuclease, Nb.BbvCI. Fortunately, the three enzymes share the same optimal temperature (37°C) and the same optimal pH (8.0). Thus, the variable factors, i.e. the time duration and the content of the three enzymes, were examined as described below.

To investigate the influence of reaction time, the reaction system was carried out with a reaction mixture containing circular probe (10^{-8} M), RCP primer (0.10 μM), NEBuffer 2, Klenow (1.0 IU μL^{-1}), Nb.BbvCI (0.6 IU μL^{-1}), and T7 RNA Polymerase (3.0 IU μL^{-1}), dNTP mixture (0.25 mM each), and NTP mixture (0.10 mM each). The mixture was incubated at 37 °C for different reaction times ranging from 1 h to 5 h. The SERS response reached a platform after a reaction time of 3 h. This timespan was then chosen as the optimal time (Figure S5A).

The effects of the three enzymes on the output of the network were also examined.

To investigate the influence of RNA polymerase concentration, the reaction system was carried out with a reaction mixture containing circular probe (10^{-8} M), RCP primer (0.10 μM), NEBuffer 2, Klenow (1.0 IU μL^{-1}), Nb.BbvCI (0.6 IU μL^{-1}), and T7 RNA Polymerase (various concentration from 0 to 3.0 IU μL^{-1}), dNTP mixture (0.25 mM each), and NTP mixture (0.10 mM each). The mixture was incubated at 37 °C for 3 h. The SERS response reached a platform after the RNA polymerase concentration of 2.0 IU μL^{-1}. This concentration was then chosen as the optimal (Figure S5B).

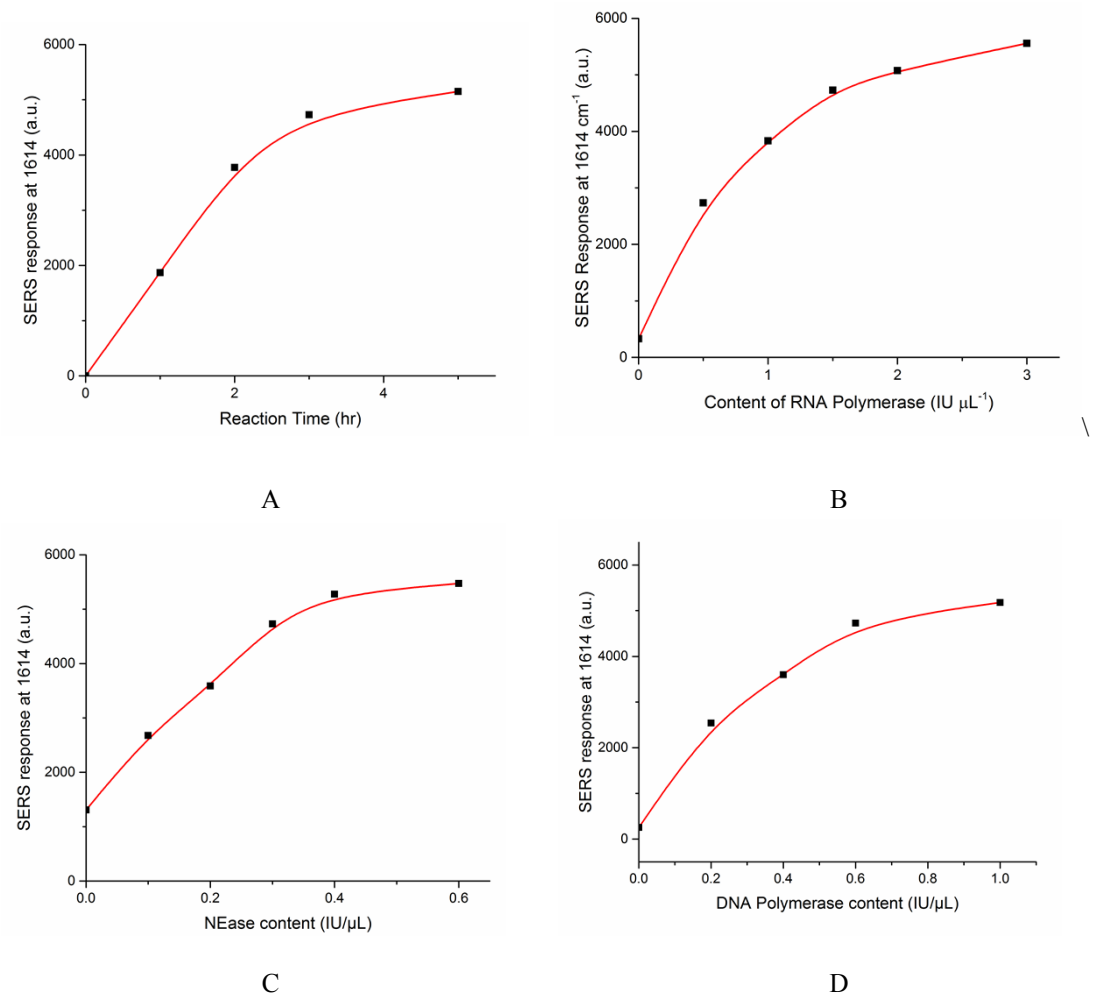


Figure S5. Optimization of reaction conditions. (A) Effect of reaction time; (B) Effect of T7 RNA Polymerase content; (C) Effect of Nb.BbvCI content; (D) Effect of DNA Polymerase Klenow fragment. Common reaction conditions: circular probe (10^{-8} M), RCP primer (0.10 μM), NEBuffer 2, Klenow (1.0 IU μL^{-1}), Nb.BbvCI (0.6 IU μL^{-1}), T7 RNA Polymerase (3.0 IU μL^{-1}), dNTP mixture (0.25 mM each), and NTP mixture (0.10 mM each), incubated at 37 °C for 3 h.

To investigate the influence of Nt.BbvCI concentration, the reaction system was carried out with a reaction mixture containing circular probe (10^{-8} M), RCP primer (0.10 μM), NEBuffer 2, Klenow (1.0 IU μL^{-1}), Nb.BbvCI (various concentration from 0 to 0.6 IU μL^{-1}), and T7 RNA Polymerase (2.0 IU μL^{-1}), dNTP mixture (0.25 mM each), and NTP mixture (0.10 mM each). The

mixture was incubated at 37 °C for 3 h. The SERS response reached a platform after the Nt.BbvCI concentration of 0.4 IU μL^{-1}. This concentration was then chosen as the optimal (Figure S5C).

To investigate the influence of DNA polymerase Klenow fragment concentration, the reaction system was carried out with a reaction mixture containing circular probe (10^{-8} M), RCP primer (0.10 μM), NEBuffer 2, Klenow (various concentration from 0 to 1.0 IU μL^{-1}), Nb.BbvCI (0.4 IU μL^{-1}), and T7 RNA Polymerase (2.0 IU μL^{-1}), dNTP mixture (0.25 mM each), and NTP mixture (0.10 mM each). The mixture was incubated at 37 °C for 3 h. The SERS response reached platform after the Klenow concentration of 0.6 IU μL^{-1}. This concentration was then chosen as the optimal (Figure S5D).

S6. Details of Different modes for the amplification comparison experiments

Mode "No amplification" (Basepoint for amplification comparison)

To provide a basepoint for the amplification, the SERS responses of MG and its aptamer of known concentrations were measured, i.e. in the case no amplification was applied. To carry out this measurement, MG of 1 μM and the AuNPs (labeled with attaching strand) were mixed with the simulated transcript of known concentrations. The incubated at 37 °C for 1 h. Then, the mixture was sufficiently centrifuged in an EP tube, and washed with TAE buffer. Centrifugation and washing were repeated for three times. The residues were dispersed in water and then dropped on a thin gold layer supported by a glass slide. After air-drying, the residue were subjected to SERS measurements. The SERS responses were shown in Figure S6.

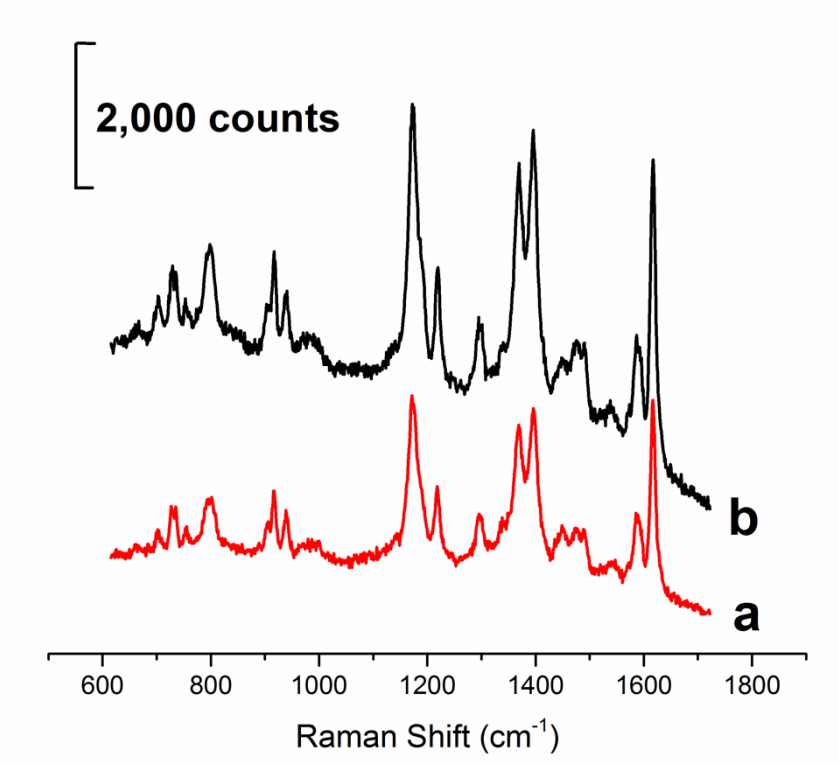


Figure S6. SERS responses in the "no amplification" mode.

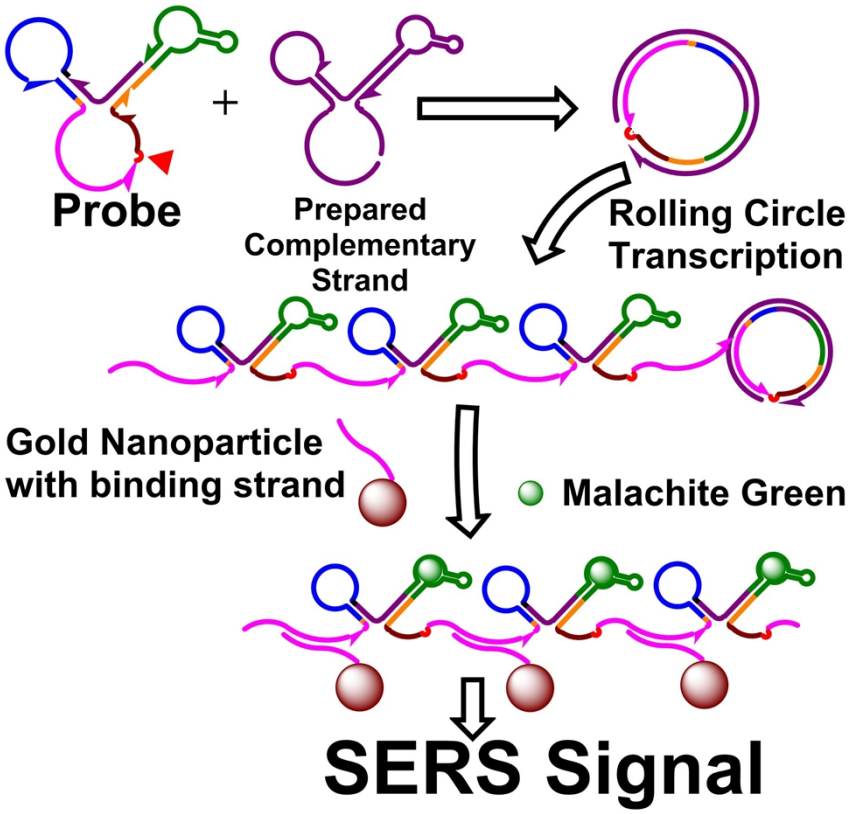
Simulated transcripts concentration: a) 10^{-9} M; b) 10^{-8} M.

Mode "RCT only"

The diagram of the mode "RCT only" is shown in Scheme S5. In this mode, only RCT stage and the signal producing stage were carried out with a simulated RCT template instead of generating the template in the reaction system.

Here the template was prepared by the hybridization of the circular probe and its complementary strands, i.e. the prepared complementary strands to circular probe strand 1 and 2. Experimentally, circular probe was prepared, and two complementary strands of excess concentration were mixed with the probe to allow the concentration of the latter to reach a certain concentration (tested from 10^{-13} M to 10^{-10} M in the mixture). The mixture was incubated at 37 °C for 30 min to allow a thorough hybridization. Then, the mixture was added with T7 RNA

Polymerase (2.0 IU μL^{-1}) and its buffer, and NTP mixture (0.10 mM each); the mixture was incubated at 37 °C for 3 h. The SERS responses were listed in Figure S7, and the calibration curve was shown in Figure 2 in the main text.



Scheme S5. Diagram of the mode “RCT only”

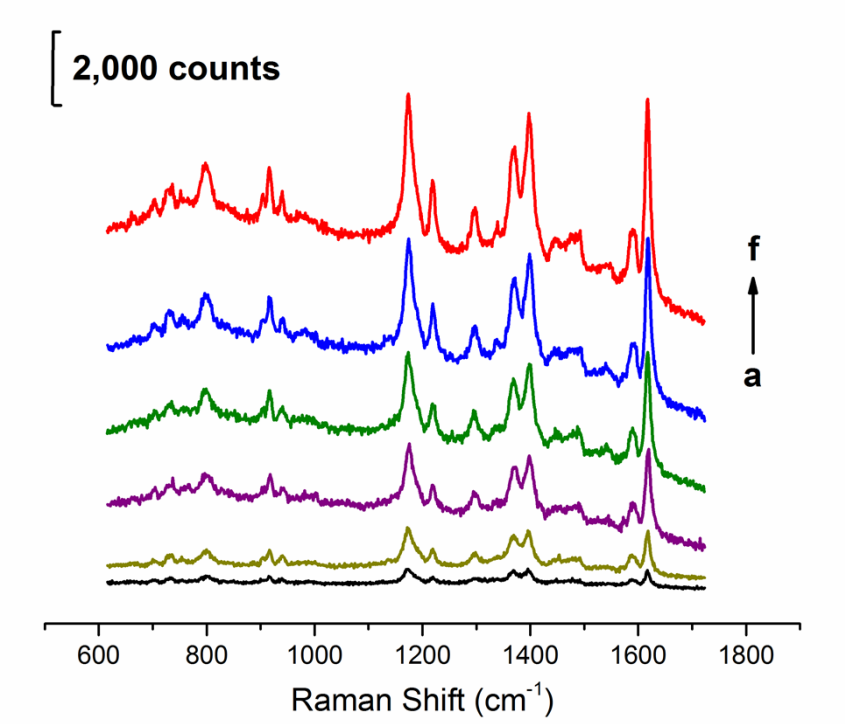
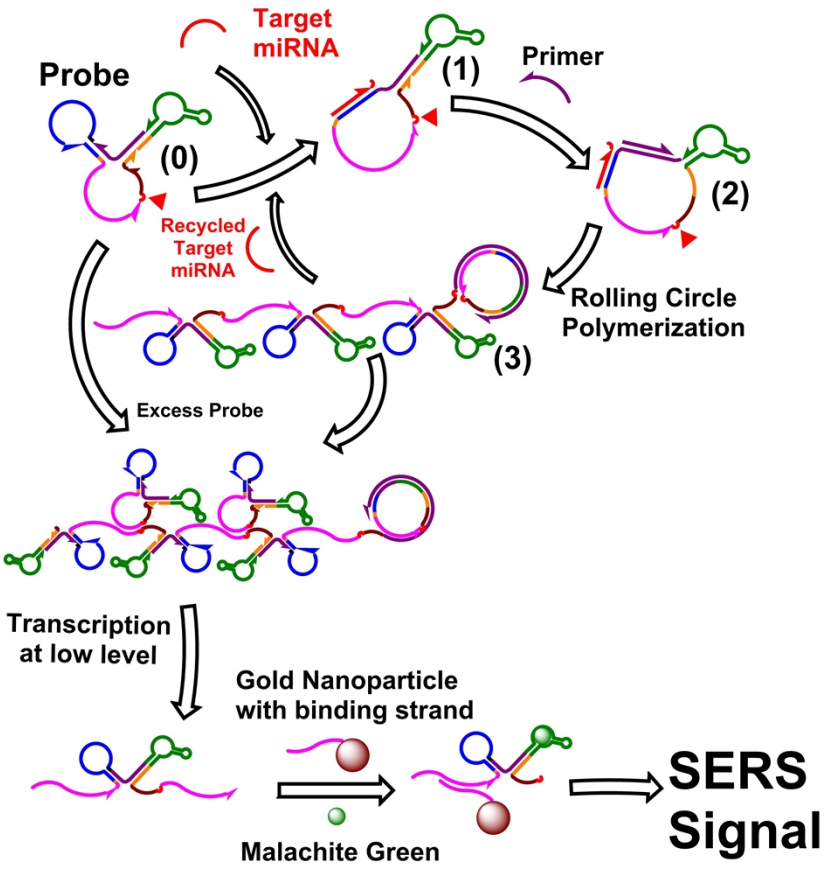


Figure S7. SERS responses in the RCT only mode.

Target miRNA concentrations: *a)* 1.0×10^{-13} M, *b)* 1.0×10^{-12} M, *c)* 1.0×10^{-11} M, *d)* 1.0×10^{-10} M.

Mode "without interlinking"

The diagram of the mode "without interlinking" is shown in Scheme S6. The difference of this mode from the complete reaction system was that nicking endonuclease was not added. Experimentally, a reaction mixture containing circular probe (10^{-8} M), RCP primer (0.10 μM), NEBuffer 2, Klenow (0.6 IU μL^{-1}), and T7 RNA Polymerase (2.0 IU μL^{-1}), dNTP mixture (0.25 mM each), and NTP mixture (0.10 mM each) was incubated at 37 °C for 3 h. The concentration of the target miRNA was tested in the range of from 10^{-13} M to 10^{-10} M. The SERS responses at different concentrations of target miRNA were shown in Figure S8. .



Scheme S6. Diagram of the mode “without nicking”

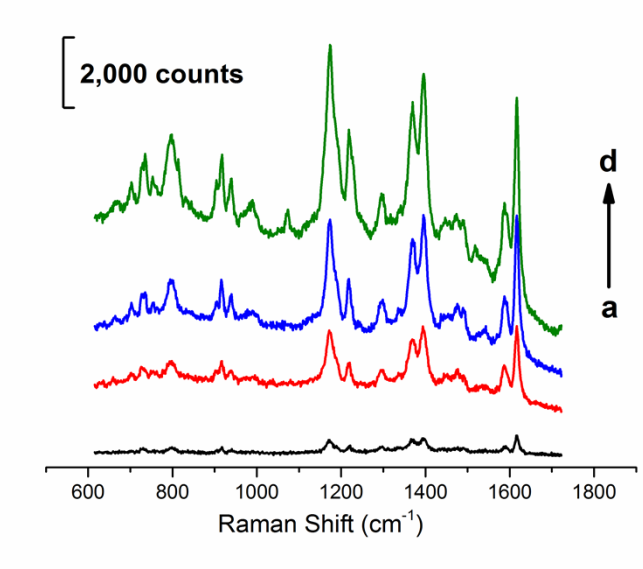


Figure S8. SERS responses in the “without nicking” mode.

Target miRNA concentrations: *a)* 1.0×10^{-13} M, *b)* 1.0×10^{-12} M, *c)* 1.0×10^{-11} M, *d)* 1.0×10^{-10} M.

The complete reaction system.

The SERS responses at different concentrations of target miRNA were shown in Figure S9. .

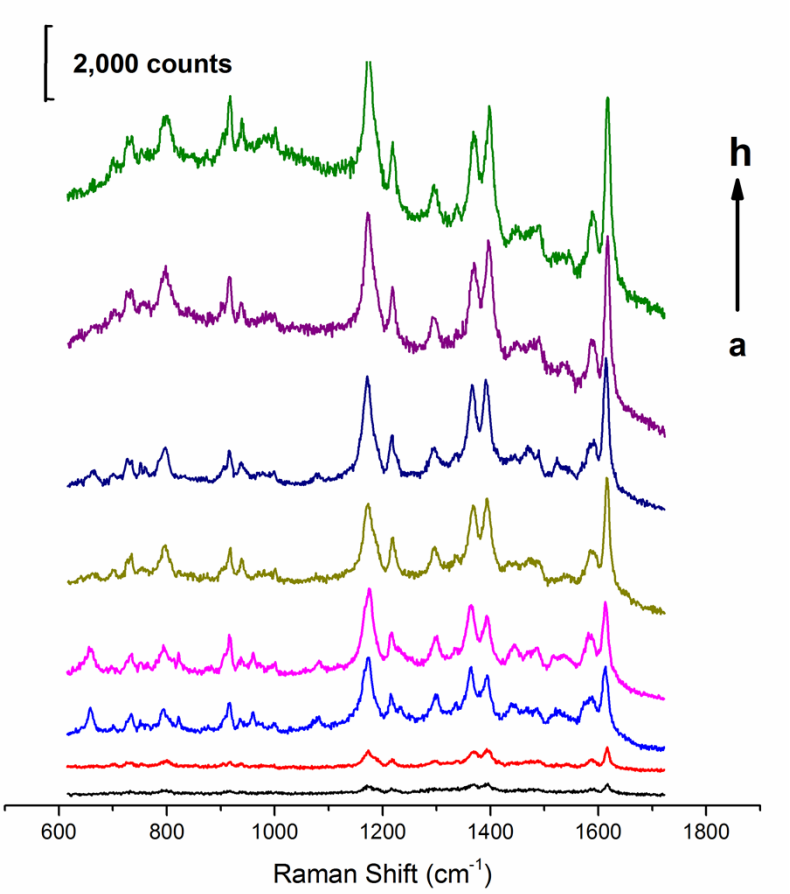


Figure S9. SERS responses for complete reaction system.

Target miRNA concentrations: *a)* blank, *b)* 1.0×10^{-16} M, *c)* 5.0×10^{-16} M, *d)* 1.0×10^{-15} M, *e)* 5.0×10^{-15} M, *f)* 1.0×10^{-14} M, *g)* 5.0×10^{-14} M, *h) 1*.0×10^{-14} M.

S7. Fluorescence Comparison Experiment

Given the well-established fact that the complex of MG and its RNA aptamer produced enhanced fluorescence, one might wonder why SERS was chosen for the signal in this study. Here a test was performed to address this issue: the signal stage was altered, namely, the attaching of the gold nanoparticle was omitted, and the fluorescence signal instead of SERS was recorded.

The fluorescence results were shown in Figure S10. The fluorescence signals of the products of the detection reaction system, when being excited by 618 nm wavelength, exhibited the

maximium emmission at circa 660 nm, which agreed with previous reports[S3]; furthermore, these signals discriminably differed from the fluorescence of MG itself in the HEPES buffer (curve a), both in the maximium emission wavelength and amplitude. These two facts verified the generation of the RCT products with MG aptamer, and its binding with MG molecules; and it was also indicated, indeed, to establish an assay based on the reported reaction system and fluorescence signal might be an option. However, as the performance of such proposed fluorescence-based assay was considered, it was discovered that this assay could only discriminate the target miRNA in the concentration range from 10^{-13} M to 10^{-11} M; in another word, its sensitivity was far lower than the SERS-based assay.

As a conclusion, SERS was obviously much more preferred than fluorescence as the signal-generating approach for this assay.

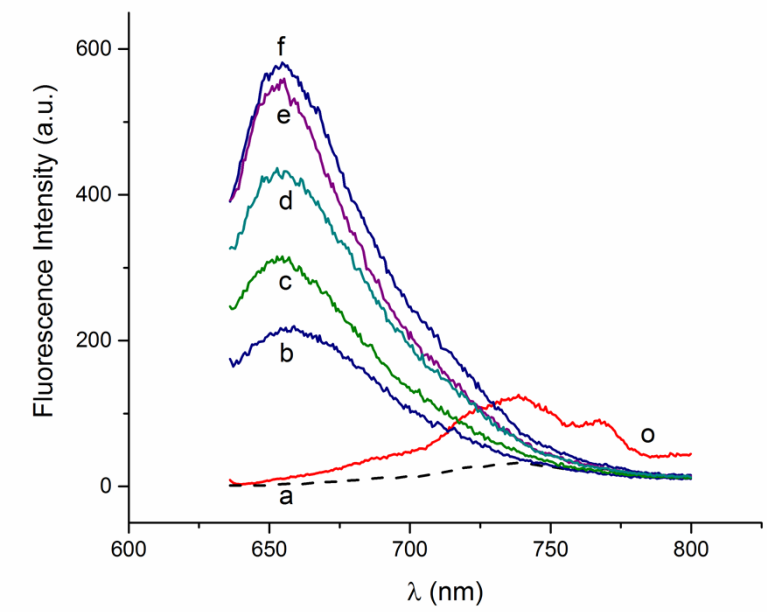


Figure S10. Responses in fluorescence comparison experiment. *o)* MG in HEPES, 10^{-8} M; *a)* blank; *b)* 10^{-13} M; *c)* 5×10^{-13} M; *d)* 10^{-12} M; *e)* 5×10^{-12} M; *f)* 10^{-11} M;

S8. Selectivity

The reaction system was initiated by the hybridization recognition of the target by the "trigger switch", providing this protocol with selectivity. Experiments were carried out to investigate whether there was cross interference. To demonstrate this, target miRNA (hsa-let-7a) of low concentration (1.0×10^{-13} M) was subjected to this reaction system along with the following

controls: the target's precursor (pre-has-let-7a), three mismatched miRNAs (hsa-let-7b, two mutants near 3'-end; hsa-let-7c, one mutant near 3'-end; and hsa-let-7d, two mutants, one on 5'-end; the sequences were shown in Table S2); and the negative control (a 22-nt miRNA with random and totally irrelevant sequence). These controls were applied at high concentration (the precursor at 1.0×10^{-11} M, and other subjects at 1.0×10^{-10} M) The circular probe, RCP primer, Klenow, Nb.BbvCI, T7 RNA polymerase were mixed in an Eppendorf tube, and target miRNA solution or a control analyte (10 μL) was added to form a reaction mixture containing circular probe (10^{-8} M), RCP primer (0.10 μM), NEBuffer 2, Klenow (0.6 IU μL^{-1}), Nb.BbvCI (0.4 IU μL^{-1}), and T7 RNA Polymerase (2.0 IU μL^{-1}), dNTP mixture (0.25 mM each), and NTP mixture (0.10 mM each). The mixture was incubated at 37 °C for 3 h.

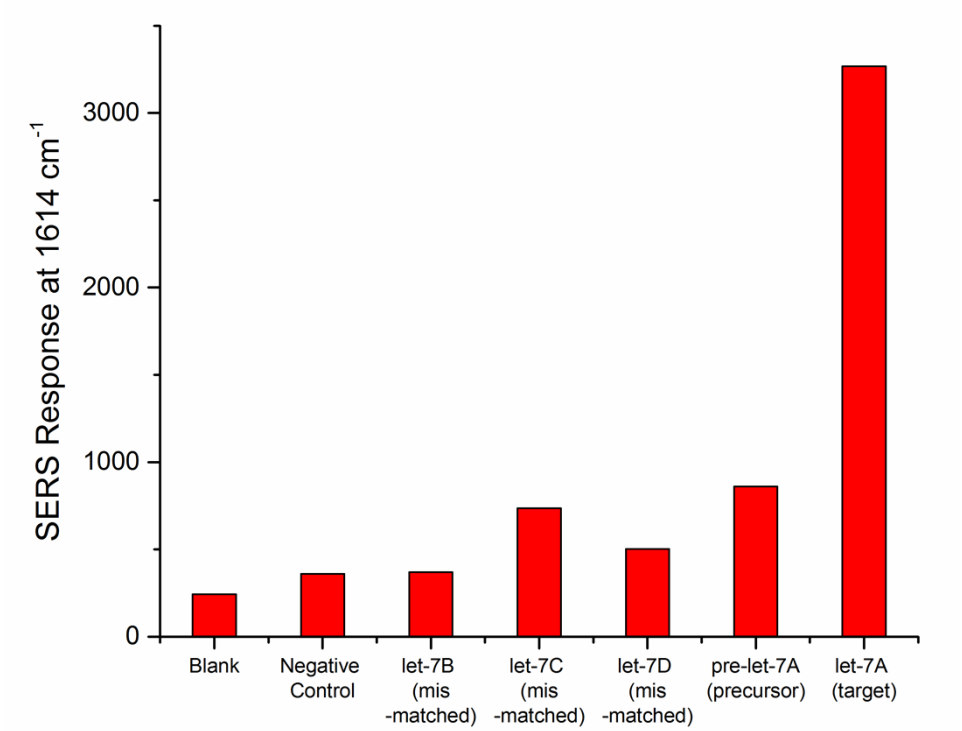


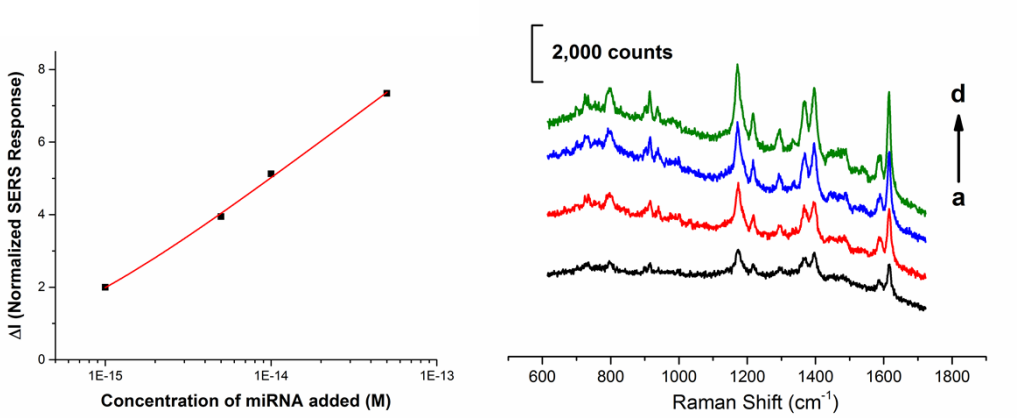
Figure S11. Selectivity for the detection of miRNA against the controls: the negative control; three mismatched miRNAs; and the precursor of the target. Concentrations the precursor, 1.0×10^{-11} M; concentration of the other controls, 1.0×10^{-10} M; target miRNA concentration, 10^{-13} M; with circular probe (10^{-8} M), RCP primer (0.10 μM), DNA polymerase Klenow fragment (0.60 IU μL^{-1}), Nb.BbvCI (0.4 IU μL^{-1}), and RNA polymerase (2.0 IU μL^{-1}).

The results (Figure S11) showed that the target miRNA yielded a prominently large SERS readout; the responses from mismatched hsa-let-7b and the negative control were barely

distinguishable from the blank response; the responses from hsa-let-7c, hsa-let-7d and the precursor (pre-hsa-let-7a) were higher than the blank response, but still prominently lower than that of the target. Considering the controls used were of the concentrations of 10^3 or 10^2 times of the target, it could be concluded that a good selectivity was thus verified.

S9. Assay in biological matrix and real samples

To test this assay's validity of detecting miRNA in biological matrix, human serum, instead of TAE buffer, was used to prepare the solution of target miRNA, which was used as the analyte and subjected to the assay in the optimal condition combination: circular probe (10^{-8} M), RCP primer (0.10 μM), NEBuffer 2, Klenow (0.60 IU μL^{-1}), Nb.BbvCI (0.40 IU μL^{-1}), and T7 RNA Polymerase (2.0 IU μL^{-1}), dNTP mixture (0.25 mM each), and NTP mixture (0.10 mM each). The mixture was incubated at 37 °C for 3 h. The added target miRNA concentrations were tested from 10^{-15} to 10^{-13} M. The SERS responses and the calibration curve were shown in Figure S12.



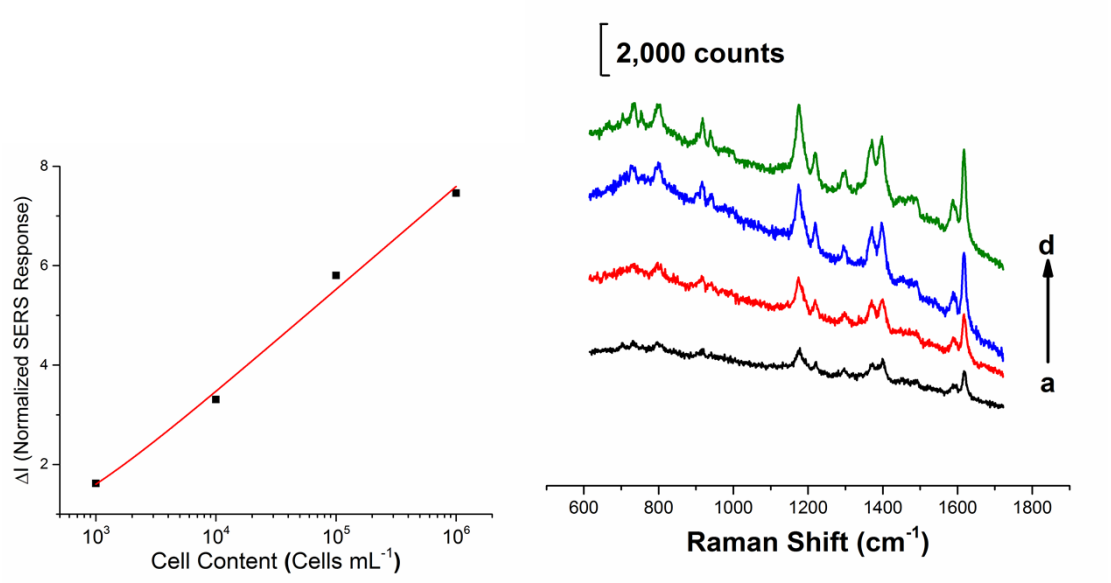
(A) (B)

Figure S12. Calibration curves of the detection of target miRNA added into human serum.

(A) Calibration curve; (B) SERS responses: *a)* 10^{-15} M; *b)* 5×10^{-15} M; *c)* 10^{-14} M; *d)* 5×10^{-14} M.

To test the validity of the method in the analysis of actual sample, RNA extract of HeLa cells was subjected to the reported protocol.

HeLa cells were cultured in RPMI-1640 with 10% Fetal Bovine Serum, under the atmosphere of 5% CO_2 at 37 °C. RNA was extracted from these cells using a Cwbio RNApure Tissue Kit. The RNA extracts were then subjected to the reported assay. Cell contents from 10^3 to 10^6 cells mL^{-1} were tested, and the SERS responses were shown in Figure S13.



(A) (B)

Figure S13. (A) Calibration curve of the assay for HeLa cells. ΔI was normalized to the same scale as in Fig. 1 and Fig. 2 in the main text. (B) SERS responses in the detection of miRNA in HeLa cells. Cell contents: *a)* 10^3 cells mL^{-1}; *b)* 10^4 cells mL^{-1}; *c)* 10^5 cells mL^{-1}; *d)* 10^6 cells mL^{-1}.

S10. Comparison with the recently reported miRNA assays

Table S3 Performances of some recently reported miRNA assays

Principle	Signal production modes	Detection limits
Toehold-initiated Rolling Circle Amplification [S4]	Fluorescence (FAM)	0.72 fM
Signal amplification by cycled reactions based on DSN (duplexspecific nuclease) [S5]	Fluorescence (FAM)	8 pM (in cells)
Rolling Circle Amplification on Encoded Gel Microparticles [S6]	Fluorescence (FAM)	0.3 fM
Hybridization chain reaction on graphene oxide [S7]	Fluorescence (FAM)	100 pM
Exponential Isothermal Amplification [S8]	Fluorescence (SYBR Green I)	0.1 fM
Hybridization and Intercalation labeling [S9]	TIRF (YOYO-1)	5 pM
Target-Primed and Branched Rolling-Circle Amplification [S10]	Fluorescence (SYBR Green I)	10 fM

Supplemented References

[S1] A. Kristoffersson, Rolling circle transcription on smallest size double stranded DNA minicircles, *Master Thesis of Uppsala University*, 2010

[S2] M. N. Stojanovic and D. M. Kolpashchikov, Modular Aptameric Sensors, *J. Am. Chem. Soc.*, 2004, **126**, 9266-9270

[S3] A. C. Bhasikuttan, J. Mohanty and H. Pal, Interaction of Malachite Green with Guanine-Rich Single-Stranded DNA: Preferential Binding to a G-Quadruplex, *Angew. Chem. Intl. Ed.*, 2007, **119**, 9465-9467

[S4] R. Deng, L. Tang, Q. Tian *et al.*, Toehold-initiated Rolling Circle Amplification for Visualizing Individual MicroRNAs in Situ in Single Cells, *Angew. Chem. Int. Ed.*, 2014, **53**, 2389 –2393

[S5] F. Degliangeli, P. Kshirsagar, V. Brunetti *et al.*, Absolute and Direct MicroRNA Quantification Using DNA−Gold Nanoparticle Probes, *J. Am. Chem. Soc.*, 2014, **136**, 2264–2267

[S6] S. C. Chapin and P. S. Doyle, Ultrasensitive Multiplexed MicroRNA Quantification on Encoded Gel Microparticles Using Rolling Circle Amplification, *Anal. Chem.*, 2011, **83**, 7179-7185

[S7] L. Yang, C. Liu, W. Ren et al., Graphene Surface-Anchored Fluorescence Sensor for Sensitive Detection of MicroRNA Coupled with Enzyme-Free Signal Amplification of Hybridization Chain Reaction, *Appl. Mater. Interfaces*, 2012, **4**, 6450-6453

[S8] H. Jia, Z. Li, C. Liu *et al.*, Ultrasensitive Detection of microRNAs by Exponential Isothermal Amplification, *Angew. Chem. Int. Ed.*, 2010, **49**, 5498 –5501

[S9] H.-M. Chan, L.-S. Chan, R. N.-S. Wong *et al.*, Direct Quantification of Single-Molecules of MicroRNA by Total Internal Reflection Fluorescence Microscopy, *Anal. Chem*., 2010, **82**, 6911–6918

[S10] Y. Cheng, X. Zhang, Z. Li *et al.*, Highly Sensitive Determination of microRNA Using Target-Primed and Branched Rolling-Circle Amplification, *Angew. Chem. Int. Ed.*, 2009, **48**, 3268 –3272