Supporting Information (SI) for

DNA-Mediated Rolling Circle Amplification for Ultrasensitive Detection of Thrombin Using MALDI-TOF Mass Spectrometry

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Experimental sections

Reagents and apparatus. Gold (III) chloride trihydrate ($HAuCl4{\cdot}4H_2O$) was purchased from Aladdin (Shanghai, China). Tris (2-carboxyethyl) phosphine hydrochloride (TCEP), human serum albumin (HSA), bovine serum albumin (BSA), immunoglobulin G (IgG), dithiothreitol (DTT), trifluoroacetic acid (TFA) and 3-Hydroxypicolinic Acid (3-HPA) were purchased from Sigma-Aldrich (USA). Thrombin was from Solarbio (Beijing, China). Streptavidin Magnetic Beads, Phi29 DNA polymerase, dNTPs and T4 DNA ligase were purchase from New England Biolabs (USA). NaCl, $MgCl_2$, KCl and sodium citrate were obtained from Beijing Chemical Reagent Co. (Beijing, China). All oligonucleotides used in this work were synthesized and purified by high performance liquid chromatography (HPLC) by Sangon Biotechnology (Shanghai, China). Their sequences were as follows: the thrombin primary aptamer, 5′ -Biotin-A15-GGTTGGTGTGGTTGG-3′ (Apt15), another thrombin aptamer, 5′ -Thiol (C6)-A15-GTCCGTGGTAGGGCAGGTTGGGGTGACT-3′ , padlock probe, 5′ -P-CTCAGCTGTGTAACAACATGAAGATTGTAGGTCAGAACTCACCTGTTAGAAACTGTGAAGATCGCTTATTATGTCCTATC-3′ (P= 5'-phosphate), the sequence used for amplification, 5′ -Thiol

(C6)-A15-CACAGCTGAGGATAGGACAT-3′ , the special sequence for mass spectrometry, 5′ -TGTCCTATCCTCAGC-3′ . All of the reagents were of analytical grade and used without further purification.

The X-ray photoelectron spectroscopy (XPS) wide and narrow scan spectra were acquired using PHI Quantera II (Ulvac-Phi, Japan). Transmission electron microscopy (TEM) images were collected on transmission electron microscope (H-7650B, Hitachi, Japan). Confocal image was collected from confocal microscope (LSM780, Zeiss, Germany). The fluorescence analysis was performed by fluorescence spectrophotometer (F-7000, Hitachi, Japan). UV−visible absorption spectra were recorded by an UV-3900s spectrophotometer (Hitachi, Japan). MALDI-TOF-MS analysis was performed on CLIN-TOF-II instrument (Bioyong, China). Data were acquired in a linear negative mode and evaluated by using the mass spectrometry software (Shimadzu Biotech., Japan).

Preparation of Bioconjugate of Apt29-AuNP-Primer. The apt29-AuNP-primer bioconjugate was prepared from sulfide-linkage between AuNPs and DNA sequences. The citrate-stabilized AuNPs, with an average diameter of 15 nm, were synthesized according to previously reported protocol with minor modifications.[1] Briefly, Aqueous gold (III) chloride trihydrate (HAuCl4; 25 mL, 1 mM) solution was heated to boil and then trisodium citrate (2.5 mL, 38,8 mM) was quickly added to reflux under stirring. After the color of the solution changed from pale yellow to deep red, the solution was refluxed for additional 30 min and then cooled to room temperature under stirring. The solution was finally filtered through a 200 nm filter, and then stored at 4 °C before use.

The experiment protocols to functionalize the aptamer29 (apt29) and the primer on AuNPs were described as follow. A solution composed of 10 μL of 10 μM apt29, 10 μL of 30 μM primer, and 10 μL of 10 mM TCEP were prepared. It was firstly incubated at room temperature for 1 h to activate the thiol-modified DNA. Then, synthesized AuNPs solution (500 μL) was added to the solution and they were aged overnight at room temperature. The solution was mixed with phosphate buffer (PB, 10 mM, pH 7.0, NaCl, 0.1 M) and aged for 12 h. Additional PB were added to adjust the reaction condition (PB, 10 mM, pH 7.0,

NaCl, 0.5 M) and aged for another 12 h. The resultant apt29-AuNPs-primer were then collected by centrifugation at 14000 rpm for 10 min, washed with PBS buffer, and then re-dispersed in PBS (500 μL).

Preparation of Magnetic Beads-Ap15. The MBs-apt bioconjugate was prepared by coupling the apt15 on streptavidin-coated MBs. A 200 μL of 5 mg/mL streptavidin-coated MBs suspension was washed twice with PBS and then re-dispersed in PBS (400 μL). A 75 μL of biotin-modified apt15 solution (10 μM) was added to the above solution and incubated at 37 °C for 2.5 h for the complete immobilization of apt15 on MBs. The resultant suspension (MBs-apt15) was magnetically separated and rinsed twice with PBS, and then redispersed in PBS (400 μL). The prepared MBs-apt15 was stored at 4 °C before use.

Construction of the MBs-apt15/Thrombin/Apt29-AuNP-Primer Sandwich Structure. A series of diluted thrombin solutions **(**20 μL) was mixed with 10 μL of MBs-apt15. They were incubated for 2 h to ensure the complete interaction and capture of thrombin. Afterward, the MBs-apt15 with the captured thrombin were magnetically separated and rinsed three times with 50 μL of PBS. The resultant MBs-apt15-thrombin were mixed with 50 μL of the apt29-AuNPs-primer solution and incubated at 37℃ for 90 min under gentle shaking, thereby forming the sandwich construction of MBs-apt15/thrombin/apt29-AuNPs-primer. The sandwich complex was then washing twice to remove unbound apt29-AuNPs-primer.

RCA Reaction. The resultant mixture was mixed with corresponding circular templates and T4 DNA ligase in 1× ligation buffer. This solution was incubated at 37 °C for 1 h. And RCA progress was carried out by adding the rolling circle amplification solution containing 1 mM dNTPs, and 0.2 U / μL phi29 polymerase in 1× polymerase buffer. The whole mixture solution was incubated for 90 min. Next, 1 μM special DNA probes was add to hybridization with RCA product for 30 min at 37 °C.

Purification of Target. After DNA probe assembly, DTT (100 mM) was used to release DNA molecules from the surface of AuNPs. Then, the supernatant was collected by magnetic separating while removing the MBs. Next, the resultant sample was operated by centrifugation using 100 KD ultra centrifugal filter (Pall, USA) at 5000 × g for 10 minutes (spun to dryness), and the retained DNA was recovered in 4 μL water after two washes of 400 μL water, which means molecule weight smaller than 100 KD would be

removed while larger kept (The molecular weight of our target DNA is much larger than 100 KD). These steps will make it possible to remove non-targets, including unassembled DNA probes, nanoparticles, and enzymes. The resulting product was then used for further MALDI-TOF MS detection.

MALDI-TOF-MS Analysis. The 3-hydroxypicolinic acid was chosen as MALDI matrix for DNA detection. The matrix solution was prepared by dissolving 20 mg 3-HPA and 45 mg dihydrogen ammonium citrate (DHAC) in 1 mL mixture solution of acetonitrile/water (1:1, v/v). MALDI-MS analysis was performed on CLIN-TOF-II instrument (Bioyong, China) using a standard stainless steel plate with manual pipetting. This instrument was equipped with a 337 nm nitrogen laser. Mass spectrum was acquired in a negative linear mode with acceleration voltage was 20 kV, and the vacuum pressure in the ion source was (3-5) $\times 10^{-6}$ Torr. Data evaluation was done by using the mass spectrometry software (Shimadzu Biotech., Japan).

Supplementary Figures

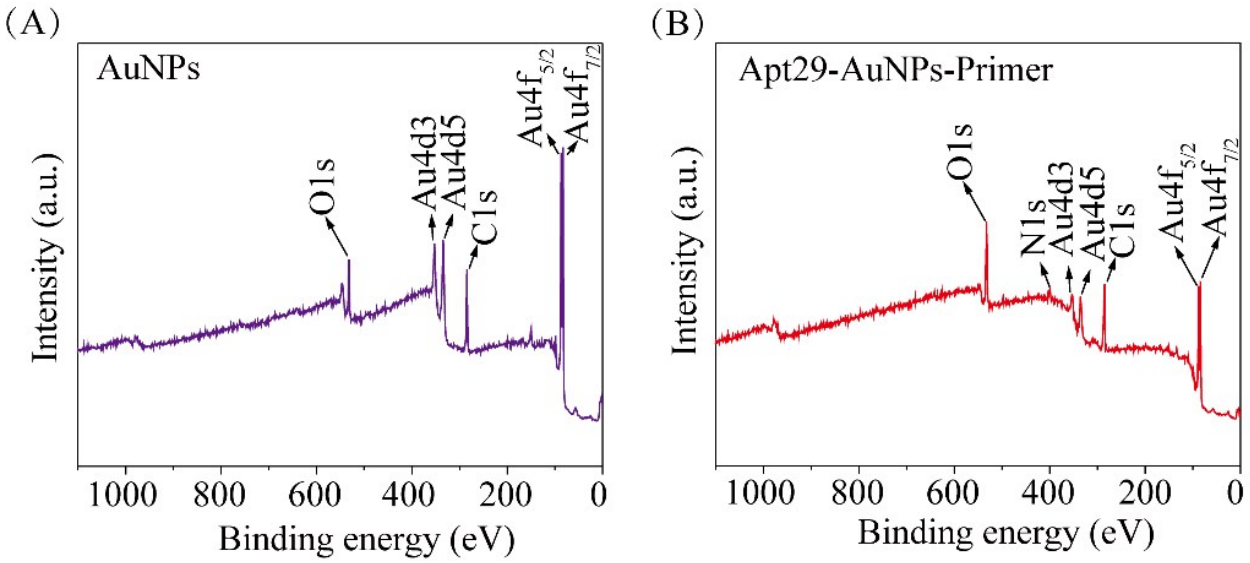

Fig. S1 XPS wide scan of (A) unmodified-AuNPS and (B) modified AuNPs.

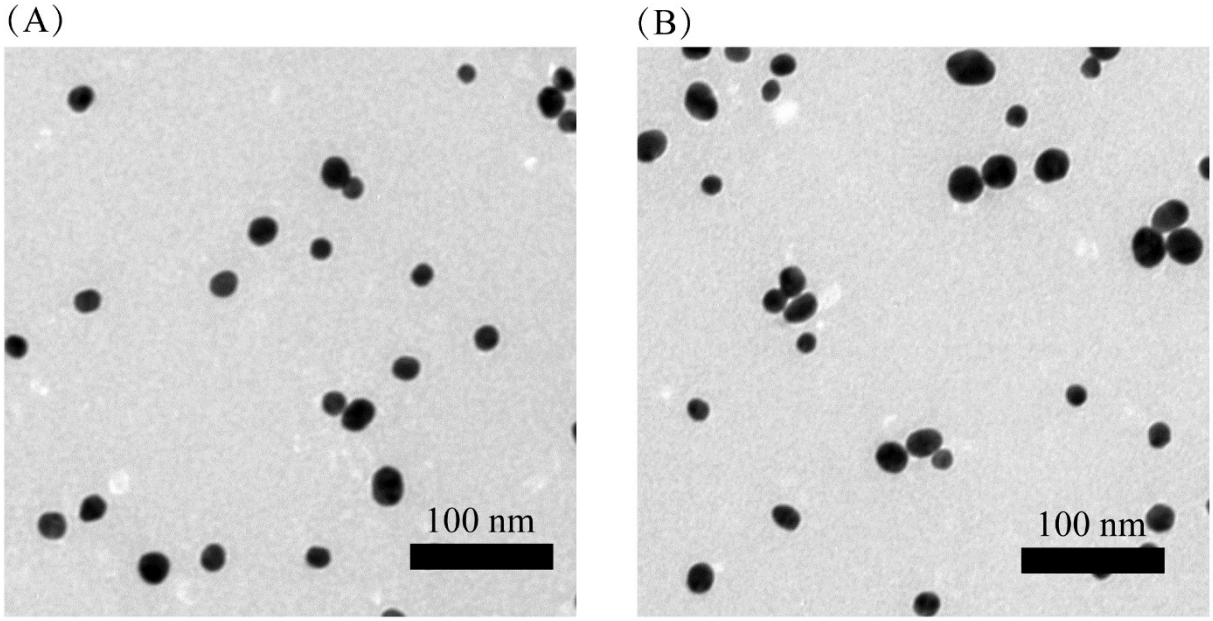

Fig. S2 TEM image of (A) unmodified AuNPs and (B) apt29-AuNPs-primer.

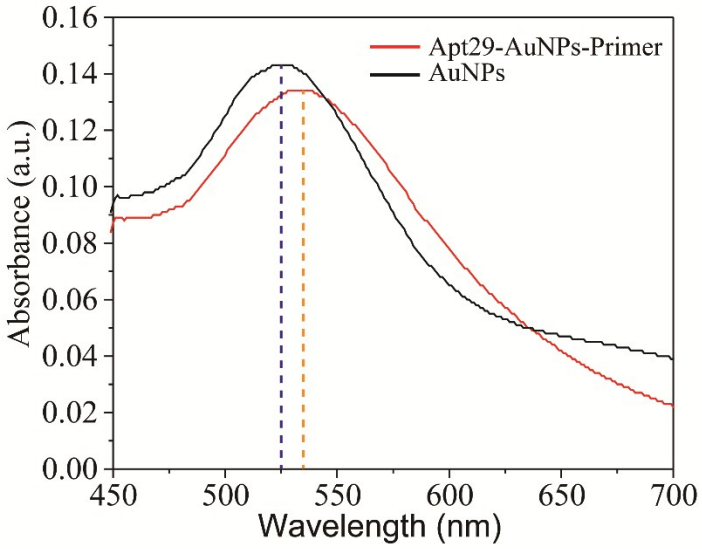

Fig. S3 UV-vis spectra of unmodified AuNPs (black line) and apt29-AuNPs-primer (red line).

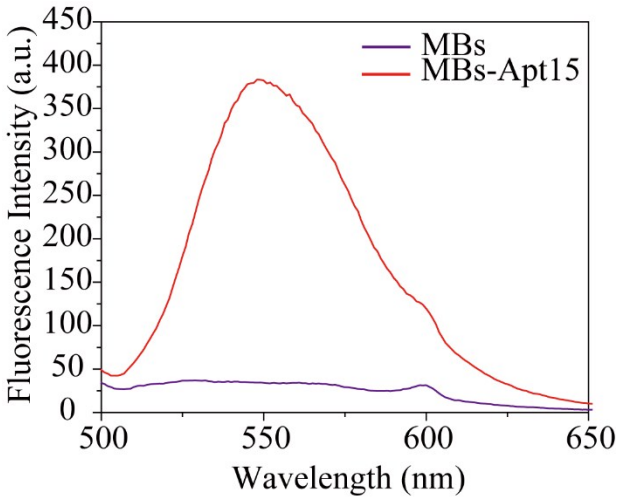

Fig. S4 Emission spectra of SYBR gold from unmodified MBs and MBs-apt15.

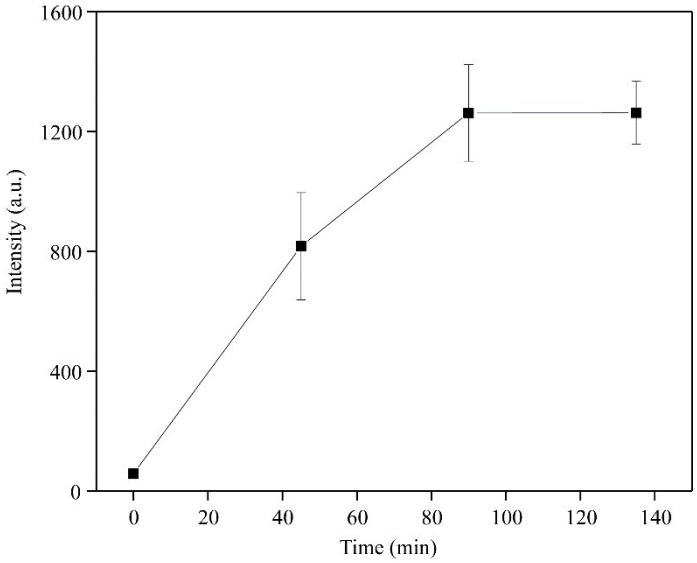

Fig. S5 Influence of RCA reaction time on the signal intensity responding to 1 pM of thrombin.

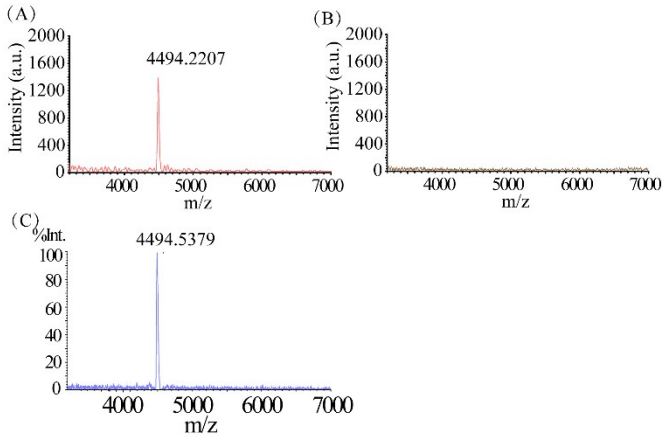

Fig. S6 MALDI-TOF MS response at 4494.2 m/z from special probes collected from the assay. (A) thrombin (1 pM), (B) thrombin-free sample. (C) Mass spectra of pure probes.

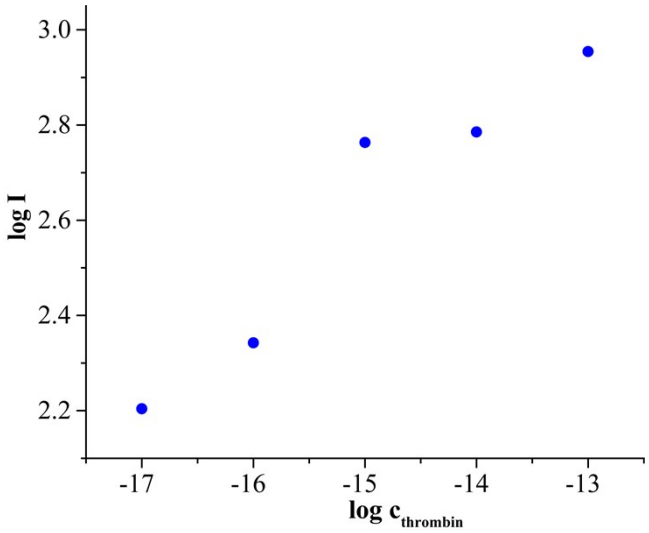

Fig. S7 The log-log scatters of the MS signal intensity versus the concentration of thrombin.

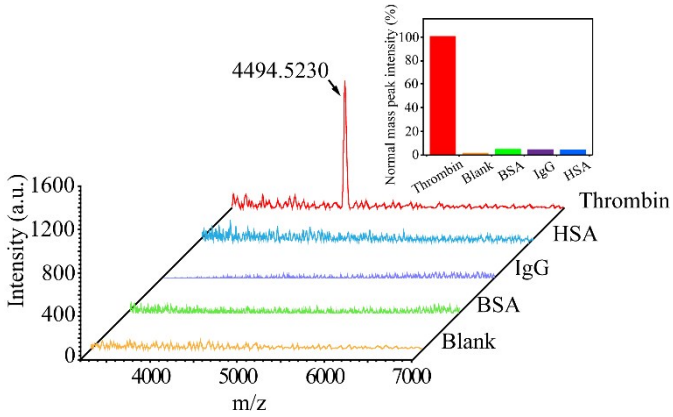

Fig. S8 Specific validation of the method achieved by MALDI-TOF MS response at 4494.5 m/z from special probes collected from target thrombin and other proteins. Water (blank), BSA (0.1 nM), IgG (0.1 nM), HSA (0.1 nM), and thrombin (1 pM).

Table S1 Atomic concentration obtained by XPS of AuNPs and midified AuNPs.

Sample	C (%)	N (%)	O (%)	Au (%)	Total (%)
AuNPs	53.80	0.00	29.18	17.02	100
Funtional AuNPs	56.99	7.65	28.03	7.32	100

Supplementary References

1. J. Liu and Y. Lu, *Nat. Protoc.*, 2006, **1**, 246-252.