Supplementary Information

Ultrasensitive SERS Assay of Lysozyme using a Novel and Unique Four-Way Helical Junction Molecule Probe for Signal Amplification

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S1 Experimental section

S1.1 Reagents and apparatus

Reagents: All oligonucletides used in the present study were purchased by Sangon Biotech Co., Ltd. (Shanghai China) and listed in Table S-1. The Klenow Fragment of DNA Polymerase I (KF polymerase), T4 DNA ligase (5 U/μL) and the deoxynucleotide solution mixture (dNTPs) were ordered from TaKaRa Biotechnology (Dalian) Co.,Ltd. Nb.BbvCI (10 U/μL) was obtained from New England Biolabs (Beijing). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was purchased from Sigma-Aldrich. $HAuCl_4 \bullet 4H_2O$, trisodium citrate and tri(2-carboxyethyl)phosphine hydrochloride (TCEP, 98%) were ordered from Shanghai Reagent Co., Ltd. (Shanghai, China). Carboxyl-modified magnetic beads (MBs, 0.5-

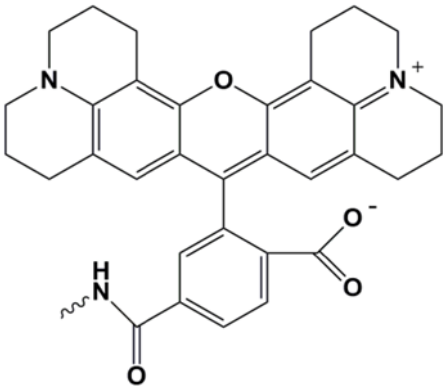
1.0 μm, 10 mg/mL) were purchased from BaseLine ChromTech Research Centre, China. The gold chip used for Raman detection was purchased from BioNavis Ltd. All the water used in the work was RNase-free. Phosphate buffered saline (PBS, 0.1 M, pH 7.4) was prepared by mixing the stock solutions of 0.1 M Na_2HPO_4 and 0.1 M NaH_2PO_4 and adjusting the pH with 0.1 M H_3PO_4 or 0.1 M NaOH. Unless otherwise mentioned, ultrapure water was used throughout the experiments. All other reagents employed in this work were analytical grade and were used without further purification.

Table S1. DNA Sequence Used in This Work.

Note	Sequences
DNA-1 (containing the lysozyme aptamer)	5'- ATC TAC GAA TTC ATC AGG GCT AAA GAG TGC AGA GTT ACT TAG GGA ACG ATC CCG ACT GTG CCA -3’
DNA-2 (the hairpin DNA template)	5'- AGA AAG GAG GAC TTT CAT ACC TCG AGG A CCT CA GC A GCT TCG AGC CTG GTG CGT GCT CAG TCA CTG AGC AAC TCA ACT CGC CAT CTG AGT CTG GAC ACG CTG CAG GCT CGA AGC TGC-3’
DNA-3 (conjugated on the surface MBs)	5’-NH_2-T_6-GGT CAC AGT CGG GAT CGT TCC TGA GTT GCT CAG TGA CTG AGC ACG CAC CAG GCT CGA AGC TGC TTT AAG CCT CAC GGT TCG CAT GAC GGG TCA ACC-3’
DNA-4 (linked with DNA-3)	5’-P-TGC GTC GAC GTG TCC CTG GAC TTT AAG AAA TCT GCA GCT TCG AGC CTG CAG CGT GTC CAG ACT CAG ATG GCG AGT CTA AGT AAC TCT -3’
DNA-5 (the linking bridge)	5’-CAG GGA CAC GTC GAC GCA GGT TGA CCC GTC ATG CGA-3’
DNA-6 (primer 1)	5'-AGA GTT ACT TAG -3’
DNA-7 (primer 2)	5'-TGG CAC AGT CGG GAT -3’
DNA-8 (Hairpin DNA)	5’-NH_2-T_6-AGA AAG GAG GAC TTT CAT ACC TCG AGG A CCT CA GC GTC CTC CTT TCT-3’
DNA-9 (Capture DNA)	5’-SH-AGA AAG GAG GAC GC -3’
DNA-10 (Signal DNA)	5'-ROX-TTT TTT CCT AGC GAC -SH-3’

* Underline means the lysozyme aptamer; The blue region means a Nt. BbvCI recognition sequence; The red region delegates the bases in the junction of the FHJM probe.

The structure of Rox



Apparatus: Raman measurements were performed on a Renisaw Invia Raman spectrometer (RamLab-010) at an excitation laser of 633 nm. A microscope equipped with a 50 × objective was used to focus the incident excitation laser. The laser power on the sample was 5 mW, and the accumulation time was 10 s. The Raman spectrometer was calibrated with WiRE Raman Software Version 2.0 (Renisaw Ltd.) Transmission electron microscopy (TEM) was measured on a JEOL JEM-2100 instrument. Ultraviolet spectra were conducted on a Cary 60 UV-Vis spectrometer (Agilent Technologies, USA).

S1.2 Synthesis of gold nanoparticles

Gold nanoparticles (AuNPs) were prepared by mingling tetrachloroauric acid ($HAuCl_4$) with trisodium citrate according to the references.[1] In detail, after boiling the $HAuCl_4$ solution (100 mL, 0.01%), trisodium citrate (2.6 mL, 1%)was rapidly added to the solution and stirred for 30 min at the boiling point. The color of the solution was changed from faint yellow to wine red, which could reveal the formation of AuNPs. The solution was cooled naturally to room temperature with continuous stirring, and the obtained AuNPs were characterized by TEM (Fig. S1). This synthesized gold colloidal solution was stored in brown glass at 4 °C for further use.

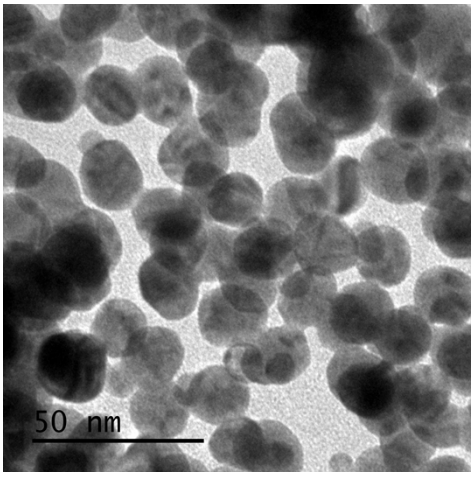


Fig. S1 TEM image of AuNPs synthesized (about 20 nm)

S1.3 Immobilization of DNA-3 and hairpin DNA onto MBs:

DNA-3 strands on the surface of MBs were achieved according to the literature.[2] Firstly, 70 μL of carboxylated MBs suspension was washed with 200 μL of 0.1 M imidazole-HCl solution (pH 6.8) three times. The MBs suspensions were separated from the solution on a magnetic rack. Secondly, to activate the carboxyl groups on the MBs, MBs were incubated at 37 °C for 30 min in imidazol-HCl solution (pH 6.8, 0.1 M, 200 μL) containing 0.1 M EDC. Then MBs were rinsed with 200 μL PBS (0.01 M, pH 7.4) three times. Thirdly, 200 μL 1.0 $\times 10^{-6}$ M DNA-3 modified amino group were added to this freshly activated MBs and incubated at 37 °C for 12 h. Finally, the excess DNA was moved away by magnetic separation. DNA-3 strands on MBs were washed three times by 200 μL PBS, then, dispersed in 200 μL PBS and stored at 4 °C for further use. Similarly, immobilization of hairpin DNA strands on the surface MBs was prepared following the procedure described above.

S1.4 Preparation of the FHJM probe linking on the surface of MBs.

A Four-Way Helical Junction Molecule probe was achieved according to the literatures with the ingenious modification.[3,4] The unique center can permit a correct assembly to occur without detectable competition by the Four-Way Helical Junction form, under stringent hybridization conditions.[5,6] Preparation process of the FHJM probe linking on the MBs was shown in Fig. S2. Firstly, DNA-3 strands (100 μL, 1.0 $\times 10^{-6}$ M) on the surface of MBs and DNA-4 (100 μL, 1.0 $\times 10^{-6}$ M) were linked by

DNA-5 (200 μL, 1.0 ×10^{-6} M) with T4 DNA ligase, after the double-stranded DNA despiralization and magnetic separation, DNA-b strands on the surface of MBs were acquired.[7,8] DNA-b strands on the surface of MBs was re-dispersed in 160 μL PBS. Secondly, 160 μL DNA-b strands on the surface of MBs, DNA-1 (10 μL 1.0 ×10^{-5} M), DNA-2 (10 μL, 1.0 ×10^{-5} M) and Tris-HCl (20 μL, 500 mM, pH 8.0) containing 50 mM magnesium chloride were mixed in a 1.5 mL Eppendorf tube to obtain the mixture sample-A. Thirdly, annealing reactions of the mixture sample-A were implemented at 80 ℃ in a hot water bath, and cooled slowly for 12 h in the insulation barrels of 2 L. Finally, after the excess DNA strands were removed by magnetic separation, the functional FHJM probe linking on the surface of MBs was successfully obtained.

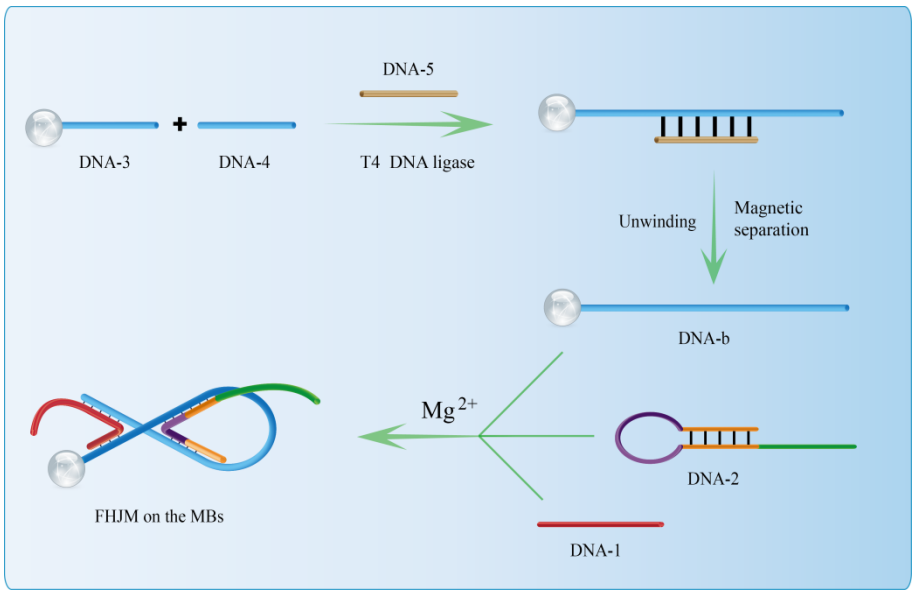


Fig. S2 Diagram for preparation of the FHJM probe linking on the MBs.

S1.5 Fabrication of the SERS signal probe

The SERS signal probe functionalized by AuNPs was fabricated according to the literatures.[9, 10] Firstly, signal DNA (3'-thiol, 5'-Rox, 100 μL, 1.0×10^{-6} M) and capture DNA (5'-thiol, 10 μL, 1.0×10^{-6} M)were activated with TCEP (30 μL, 10 mM) for 0.5 h to obtain the mixture sample-B. Then the mixture sample-B was dropwise added to 500 μL AuNPs, and shaken gently at 37 °C for 16 h to acquire the sample-C.

Secondly, the sample-C was aged in salts (0.1 M NaCl, 10 mM KH_2PO_4/ K_2HPO_4) for 18 h. Excess reagents were moved away by centrifuging at 10000 rpm for 30 min. The red precipitate was rinsed and centrifuged repeatedly for three times. The clear supernatant was carefully moved away, and the SERS signal probe was finally dispersed into 300 μL 0.01 M the buffer solution (10 mM KH_2PO_4/ K_2HPO_4, pH 7.4, 0.1 M NaCl) and stored at 4 °C.

S1.6 SERS Measurements

2 μL of measured suspension was casted on gold chip, and air-dried at room temperature ahead of SERS analysis. SERS spectra were acquired with an excitation laser of 633 nm, and the laser power was 5 mW by Raman spectrometer (Renishaw, U.K.). The acquisition time was 10 s for each SERS spectra. Line mapping was employed by a Streamline Raman mapping system, and SERS maps were fabricated by integrating the region within the characteristic peaks. Three spectra were collected from different sites of each sample, and averaged to express the SERS results. Error bars exhibited the standard deviation from three repeated experiments.

S2. UV-visible spectra of the Rox-DNA conjugates

The UV-visible spectra of signal DNA (Rox-DNA), capture DNA, AuNPs and SERS bio-bar codes (SERS signal probes) were recorded by a Cary 50 UV/Vis-NIR spectrophotometer. Their characteristic absorbance was displayed in Fig. S3, such as AuNPs (curve a) at about 520 nm, capture DNA (curve b) at about 260 nm, and Rox-DNA (curve c) at 260 nm and two characteristic absorbance at 500-600 nm in accord with the reference.[11] Curve d displayed both the characteristic absorbance of AuNPs and Rox-DNA, indicating that Rox-DNA and AuNPs have been successfully conjugated.

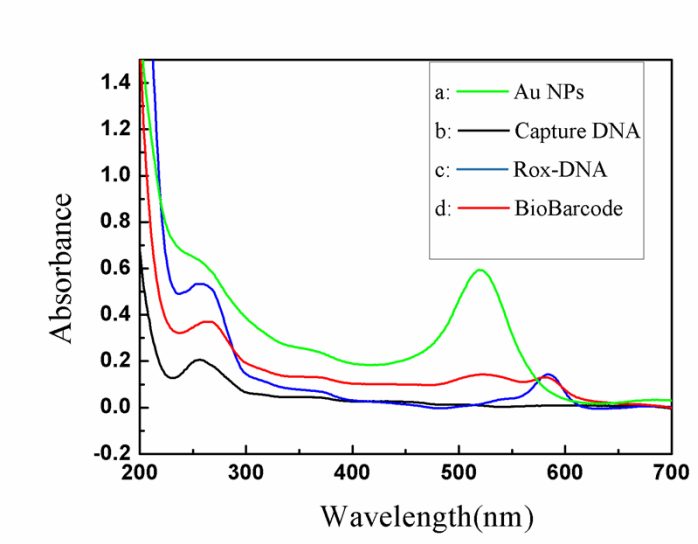


Fig. S3 UV spectra of AuNPs (a), DNA (b), Rox-DNA (c), and bio barcode DNA (d).

S3. Optimization of the experimental conditions

S3.1 Optimization of the reaction temperature and pH

The reaction temperature and pH of the reaction solution are two important factors affecting DNA hybridization and enzyme bioactivity. The intensity of Raman detection was investigated under different temperature and pH conditions. Fig. S4-A displayed the influence of pH on the Raman signal measured by 1.0×10^{-13} M lysozyme. The Raman intensity (ΔI) reached the maximum at pH 7.4. Accordingly, 0.01 M PBS at pH 7.4 was chosen for the following experiments. As shown in Figure S4-B, the Raman intensity (ΔI) changed under different temperature conditions from 20 ℃ to 37 ℃. A maximum was acquired at 37 ℃. So 37 ℃ was chosen for subsequent experiments.

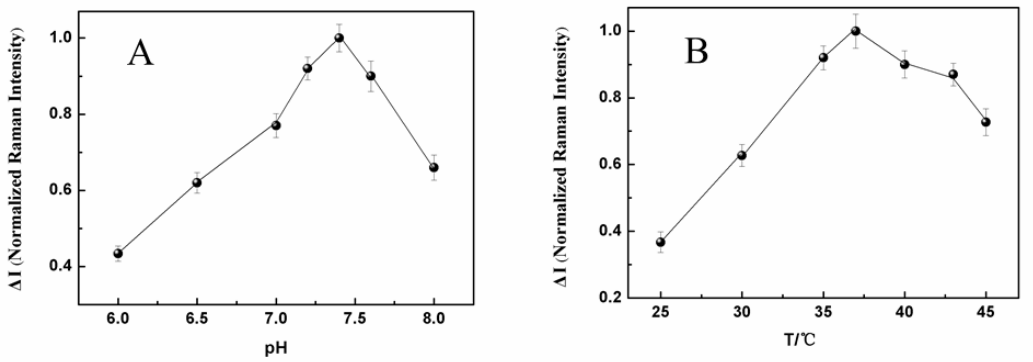


Fig. S4 (A) Effect of the reaction pH and (B) temperature, on the Raman intensity responding of 1.0×10^{-13} M lysozyme.

S3.2 Optimization of the amounts of KF polymerase and nicking endonuclease

The amounts of KF polymerase and nicking endonuclease were the important influencing factors for the Raman intensity. For achieving the best sensing performance, a series of control experiments were designed to optimize the amounts of KF polymerase and Nb.BbvCI. As shown in Fig. S5-A, the Raman intensities enhanced speedily when the amount of polymerase increased from 0.2 to 0.45 $U\mu L^{-1}$. But after 0.45 $U\mu L^{-1}$, the Raman intensity reduced slightly. So 0.45 $U\mu L^{-1}$ of KF polymerase was considered to be the optimum. Similarly, 0.5 $U\mu L^{-1}$ of Nb.BbvCI was chosen to be the optimal amount, as shown in Fig. S5-B.

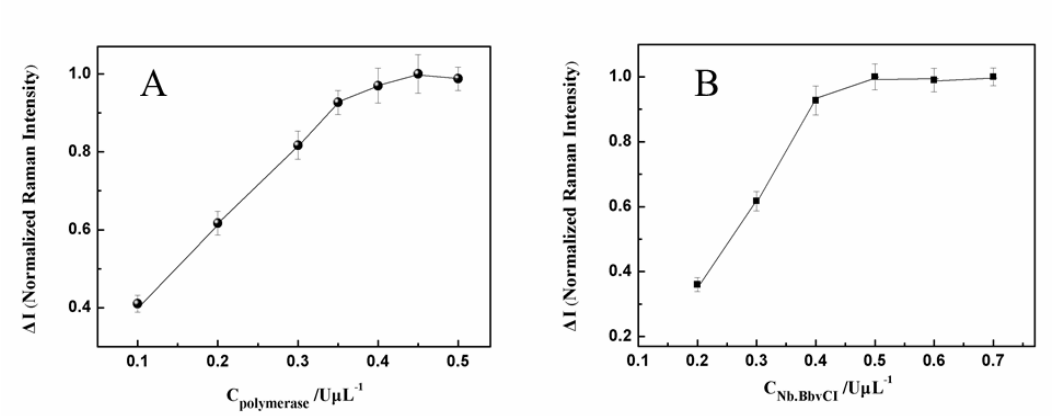


Fig. S5 Influence of the amount of KF polymerase (A) and Nb.BbvCI (B) on the Raman intensity respond of 1.0×10^{-13} M lysozyme.

S3.3 Optimization of the incubation time

To improve the sensitivity of Raman detection, the cycle reaction time was investigated by contrasting the experiments of different reaction time. As shown in Fig. S6, the Raman intensity enhanced speedily with the increase of incubation time, and obtained a plateau after 90 min. Thus, the cycle reaction time of the experiment was controlled at 90 min.

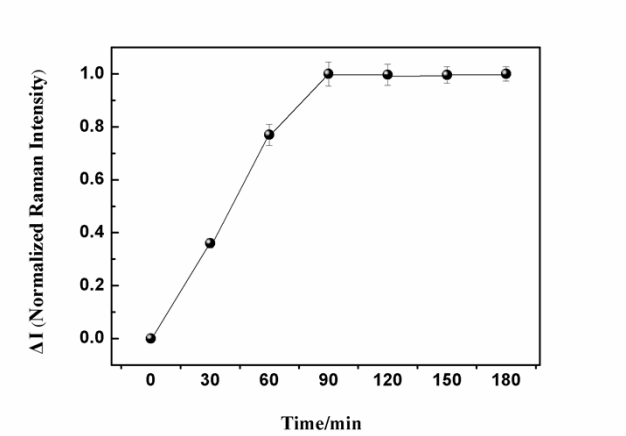


Fig. S6 Effect of the cycle reaction time, on the Raman intensity respond of 1.0×10^{-13} M lysozyme.

S4. Analysis of real samples

To assess its feasibility of the practical clinical analysis, this FHJM-SERS method was applied to determine lysozyme in human real serum samples by standard addition calibration. Owing to the high sensitivity and selectivity of this approach, human serum sample was centrifuged to remove any solid suspension, successively diluted before detection, then spiked with lysozyme at three different concentrations (1.0×10^{-13} M, 5.0×10^{-14} M, and 1.0×10^{-14} M). After SERS measurement, the results were summarized and listed in Table S3. The calculated content of lysozyme in human serum was 3.71×10^{-7} M, which was within the normal range according to the reported literature.[12] Each sample was measured by three parallel determinations, the value of recovery ranges from 87.6 to 106.9%, indicating that the FHJM-SERS strategy could be potentially applied for the detection of lysozyme in complex biological samples.

Table S2. Comparison of different methods for lysozyme detection.

Method	Transducer	Detection limits (μM)
The reduced aggregation of perylene probe	Fluorescence	70 pM [13]
Signal amplification on a Feed-forwar Network of DNA	Fluorescence	3.6 fM [14]
Conjugate Eu^{III} complex and aptamer-wrapped carbon nanotubes	Luminescence	0.9 nM [15]
Aptamer DNAzyme hairpin structures	Colorimetry	0.5 pM [16]
Cascade Signal amplification on a Triple-Helix Switch	SERS	0.54 fM [17]
Acidified-sulfate-induced aggregation	SERS	350 nM [18]
Selfassembly of DNAzyme aptamer conjugate	Chemiluminescence	0.1 pM [19]
The aggregation of quantum dots triggered by protein	Resonant light scattering	3 nM [20]
This work	FHJM-SERS	0.5 fM

Table S3. Determination of lysozyme in human serum samples by FHJM-SERS method. (n =3)

Samples	Lysozyme content added	Lysozyme content detected	Recovery (%)	RSD (%)
1	1.0×10^{-14} M	9.26×10^{-13} M	93.7 ± 0.21%	5.3%
2	5.0×10^{-14} M	4.50×10^{-14} M	96.2 ± 0.35%	4.7%
3	1.0×10^{-13} M	1.19×10^{-13} M	104.5 ± 0.23%	8.1%

S5. References

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