Supplementary Information

Sensitive SERS Detection of DNA and Lysozyme Based on Polymerase Assisted Cross Strand-Displacement Amplification

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S1 Experimental section

S1.1 Reagentss and apparatus

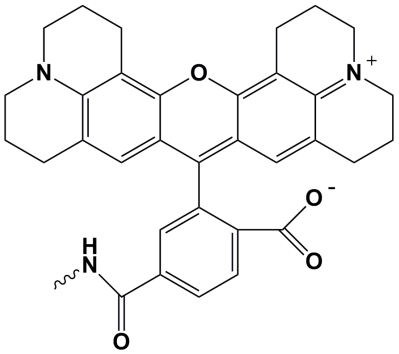
Reagents: All oligonucleotides used in the present study were synthesized and purified by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (China), and the sequences were listed in Table S1 (see supplementary materials). Klenow fragment of E. coli DNA polymerase I (5 IU μL-1, denoted as "polymerase" for short) and the mixture of four dNTPs (2.5 mM for each component) were purchased from TaKaRa Bio Inc. Hydrogen tetrachloroaurate(III) tetrhydrate ($HAuCl_4 \bullet 4H_2O$), trisodium citrate, tri(2-carboxyethyl)phosphine hydrochloride (TCEP, 98%) were ordered from Sigma-Aldrich. Other chemicals employed were of analytical reagent grade and were used without further purification. Doubly distilled water was used throughout the experiments. Microbeads coated with thiol groups (SH-MBs) were purchased from Tianjin BaseLine ChroTechResearch Centre (China) and the gold chip used for Raman detection was purchased from BioNavis Ltd.

Table S1. DNA Sequence Used in This Work

S0	5-GAT GAA TTC GTA GAT-3	Target DNA
S1	5-GCG CAT CAC TAG TTT TTT TGC AGA GTT ACT TAG TTT TTT ATC TAC GAA TTC ATC TTT TTT-Biotin-3	Template 1 immobilized on the streptavidin-coated 96 Wells microtiter plates
S2	5-CTA AGT AAC TCT GCA-3	Complementary with 3-end of Template 2 as a primer of SDA 2
S3	5-CTA GTG ATG CGC TTT TTT TTT TTT-SH-3	Complementary with 5-end of Template 1 and used to prepare the RCA primer probe

S4	5- SH-TTT TTT GAG TCA ACT GTG TGG TTA-3	Primer of RCA
S5	5-ATC TAC GAA TTC ATC AGG GCT AAA GAG TGC AGA GTT ACT TAG TTT TTT-NH2-3	Template 2 (aptamer of lysozyme)
S6	5-AGT TGA CTC CCC AAC CCG CCC TAC CCA AAA CCC AAC CCG CCC TAC CCA AAA CCC AAC CCG CCC TAC CCT AAC CAC AC-3	Circular DNA used for the template of RCA
S7	5-SH-TTT CCC AAC CCG CCC TAC CC-3	Capture DNA (Complementary with the product of RCA)
S8	5-SH-TTT CTA GTG ATG CGC-Rox-3	Rox modified Barcode DNA for Raman signal
S9	5-GAT GAA TT**A** GTA GAT-3	One base-mismatched DNA
S10	5-ACA CCT AAC AGC ATC-3	Noncomplementary DNA

The structure of ROX



Apparatus: SERS detection was performed on an inVia Raman Microscope (Renishaw, England). UV–vis absorption spectra were carried out on a Cary 50 UV–vis–NIR spectrophotometer (Varian). Fluorescence measurements were carried out at a F4500 fluorometer. Transmission electron microscopy (TEM) image was taken with JEOL JSM-6700F instrument (Hitachi).

S1.2 Preparation of AuNPs and AuNP-modified MBs

Gold nanoparticles (AuNPs) were prepared following the previously reported method by reduction of tetrachloroauric acid ($HAuCl_4$) with trisodium citrate[1]. Briefly, after boiling the $HAuCl_4$ solution (0.01%, 100 mL), 3.0 mL of 1% trisodium citrate was added dropwise to the solution and stirred for 20 min at the boiling point. When the color of the solution was turned from gray yellow to deep red, it could indicate the formation of AuNPs. With continuous stirring, the solution was cooled to room temperature and the preparated AuNPs were characterized by TEM (see Supporting Information, Figure S1).

AuNP-modified MBs (MB-AuNPs) was obtained by capping the synthesized AuNPs on the surface of SH-MBs through Au-S bonds[2]. Briefly, 2 mL of the AuNPs solution was added to 20 μL of 1% (w/v) SH-MBs, and shaken gently for 24 h. Then the obtained MB-AuNPs were washed with 500 μL of 0.01 M pH 7.4 phosphate buffer solution (PBS) containing 0.3 M NaCl for three times, and then redispersed in the same buffer solution. The preparated MB-AuNPs were characterized by TEM (see Supporting Information, Figure S1).

S1.3 Preparation of AuNP-Functionalized Raman probe

The AuNP-functionalized Raman probe was obtained by capping the capture DNA (S3, 5′-thiol) and signal DNA (S4, 5′-thiol and 3′-ROX) on the surface of AuNPs (Taton et al., 2000). Briefly, the mixture of 1.0×10^{-10} mol of S7 and 6.0×10^{-10} mol of S8 was added to 1 mL of freshly prepared gold nanoparticles and shaken gently for 16 h. After that, the solution was aged in salts (0.1 M NaCl, 10 mM acetate

buffer) for another 24 h and centrifuged for at least 30 min at 10,000 rpm to remove the excess oligonucleotides. The red precipitate was washed and centrifuged repeatedly for three times. The resulting Raman probe was dispersed into a buffer solution (0.01 mM PBS, pH 8.2, 0.3 M NaCl) and stored at 4 °C for further use.

S1.4 Preparation of RCA primer probe

The RCA primer probe was prepared by immobilizing the capture DNA (S3, 3′ -thiol) and primer DNA (S4, 5′-thiol) on the surface of MB-AuNPs. 100 μL of the prepared MB-AuNPs was added to the mixture of S3 and S4 with gently shaking for 16 h at room temperature, the MB-AuNPs/DNA conjugates were "aged" in the solution (0.1 M NaCl, 10 mM acetate buffer) for another 24 h. Excess reagents were removed by magnetic field. Following removal of the supernatant, the resulted RCA primer probe was washed with PBS, recentrifuged, and then redispersed in PBS for further use.

S1.5 Analysis of target DNA

The streptavidin-coated 96 Wells microtiter plates were washed with 100 μL of PBS twice before use. 0.1 nmol of biotin tagged template DNA (biotin-S1, in 50 μL PBS) was added into the wells and incubated for 1 h at room temperature. Unconjugated DNA was removed by discarding the supernatants, and the wells were washed three times with 100 μL of PBS buffer solution. Then 50 μL of the prepared RCA primer probe and SDA primer DNA (S4) were added to the wells. After incubating for 2 h at 25 °C, the supernatant was discarded and the substrate was washed three times with 100 μL of PBS.

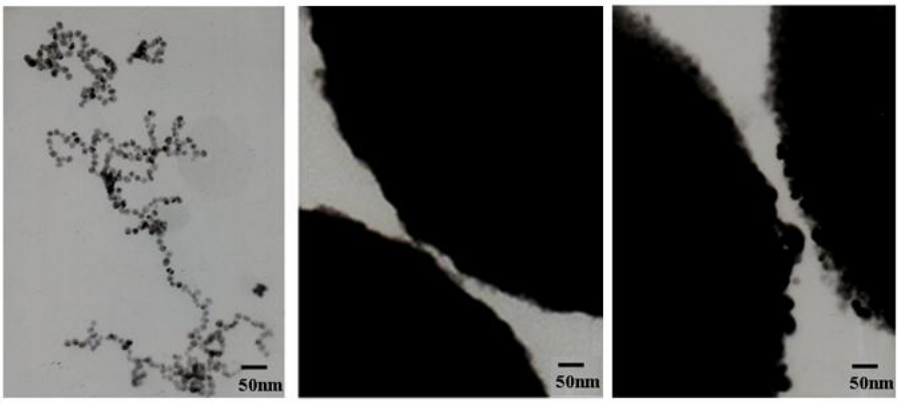
For target DNA detection, different concentrations of target DNA were added to the wells modified with Template 1, then 2.0 μL of Klenow polymerase, 10 μL MB-immobilized Template DNA (S5), 4.0×10^{-9} mol circular probe (S6), 1 μL T4 DNA ligase and 5 μL dNTPs (1 mM), and were added to perform the cross strand-displacement and RCA reaction reaction. After incubated at 37 °C for 2 h, the mixture was heated at 80 °C for 20 min to inactivate the Klenow polymerase. The supernatant was allowed to hybridize with Raman probes at room temperature for 1 h.

S1.6 Measurement of Raman spectrum

The MBs incorporated with Raman probes were washed with PBS for three times and redispersed in 100 μL of 0.01 M PBS buffer (pH 7.4). 2 μL of each mixture was pipetted onto the surface of the gold chip and the SERS spectra were measured by an inVia Raman microscope with a 633 nm laser. The laser power was 5 mW, and the acquisition time for each spectrum was 5 s. Three spectra from different sites were collected for each sample and calibrated with the WiRE Raman Software Version 3.3.

S2 Characterization of AuNPs and MB-AuNPs

The synthesized AuNPs for preparing MB-AuNPs and Raman dye modified AuNPs have an average diameter of approximately 15 nm as measured by TEM as shown in Figure S1 (A). The TEM of the final MB-AuNPs was shown in Figure S1 (C). Numerous AuNPs could be seen on the surface of the MB compared to the TEM of MB as shown in Figure S1 (B). The result showed that the MB-AuNPs were constructed as expected.



(A) (B) (C)

Figure S1 TEM images of (A) AuNPs, (B) thiol-modified MNPs with the average size of 3.0 μm, and (C) the prepared MB-AuNPs.

S3 Characterization of Raman probes

The prepared Raman probes were characterized by UV-visible spectra and fluorescence spectra. As could be seen from Figure S2 (A), curve a exhibited the characteristic absorbance of AuNPs at ~520 nm. Curve b was the UV-vis absorption spectra of ROX modified Barcode DNA (S8), which showed the characteristic absorbance of DNA at ~260 nm and the spectral property of rhodamine dye at 500-600 nm consistent with the literature[3]. Curve c exhibited both the characteristic absorbance of ROX-DNA and the characteristic absorbance of AuNPs, which indicated that the AuNPs had been successfully labeled with ROX modified DNA.

As further evidence, the fluorescence response was investigated and the result was shown in Figure S2 (B). It could be seen that the fluorescence intensity of the ROX-DNA solution at 600 nm was very high when excited at 530 nm (curve a). After being used to incorporate with AuNPs, the intensity of the supernatant was decreased

greatly (curve b), indicating that lots of the ROX-DNA were consumed to form the Raman probes.

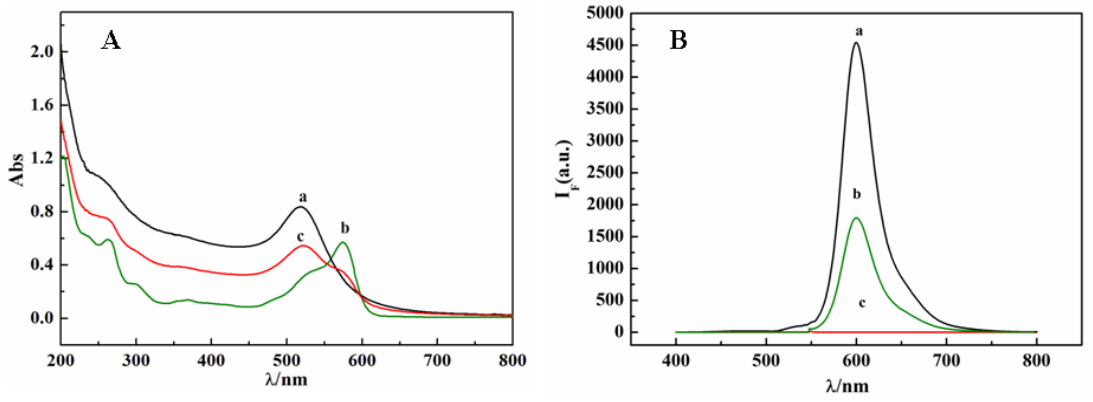


Figure S2. (A) UV spectra of AuNPs (a), ROX modified Barcode DNA (b) and AuNPs modified Raman probes (c); (B) Fluorescence spectra of ROX modified Barcode DNA (a), the supernatant separated from AuNPs modified Raman probes (b) and PBS (c).

S4 Optimization of the ratio of barcode DNA to signal DNA for Raman probe

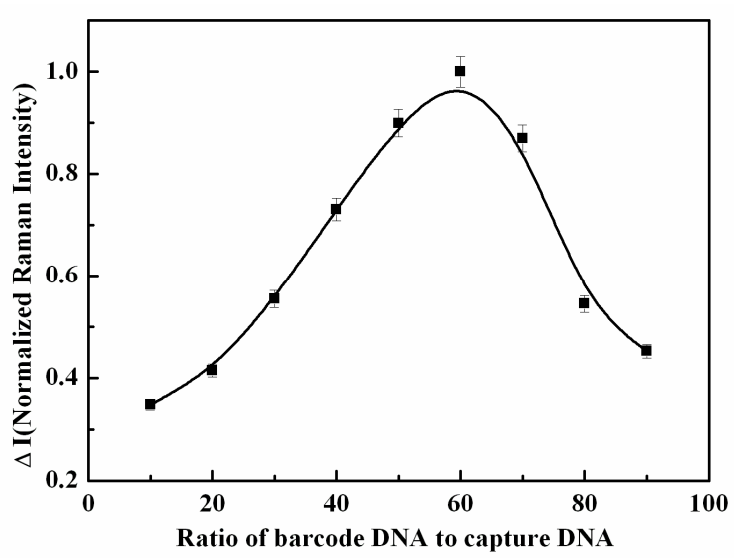


Figure S3. Raman signals for different ratio of barcode DNA to signal DNA. The concentration of DNA is 1.0×10^{-14} M.

S5 Optimization of the experimental conditions

S5.1 Influence of the amount of Klenow polymerase

To investigate the influence of the amount of Klenow polymerase used in the experiment of DNA detection, the Raman signal produced by 1.0×10^{-14} M target DNA was measured by using various amounts of polymerase. As shown in Figure S4, when the volume of polymerase increased from 0.2 to 0.5 U μL^{-1}, the Raman intensity increased gradually. But after that, the Raman intensity decreased slightly. Therefore, 0.4 U μL^{-1} of Klenow polymerase was considered to be optimum amount used in the amplification reaction.

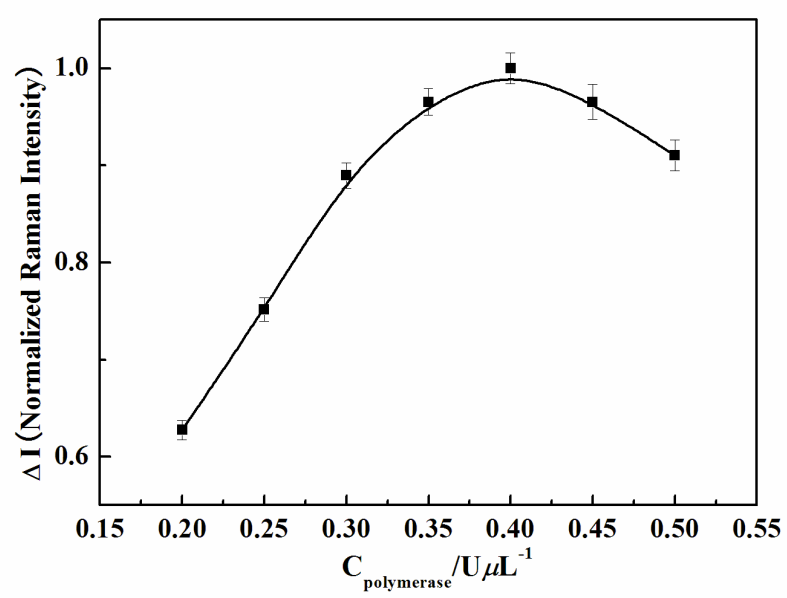


Figure S4. Influence of the amount of polymerase on the Raman intensity responding to 1.0×10^{-14} M target DNA.

S5.2 Optimization of the temperature

Figure S5 showed the influence of the temperature on the Raman intensity produced by 1.0×10^{-14} M target DNA. As it could be seen, a maximal Raman intensity was obtained when the reaction temperature of the system was at 37 °C. So we employed 37 °C as the optimal experimental temperature, which was in

accordance with the fact that enzymatic reactions are usually operated at 37 °C by virtue of the best bioactivity of enzymes.

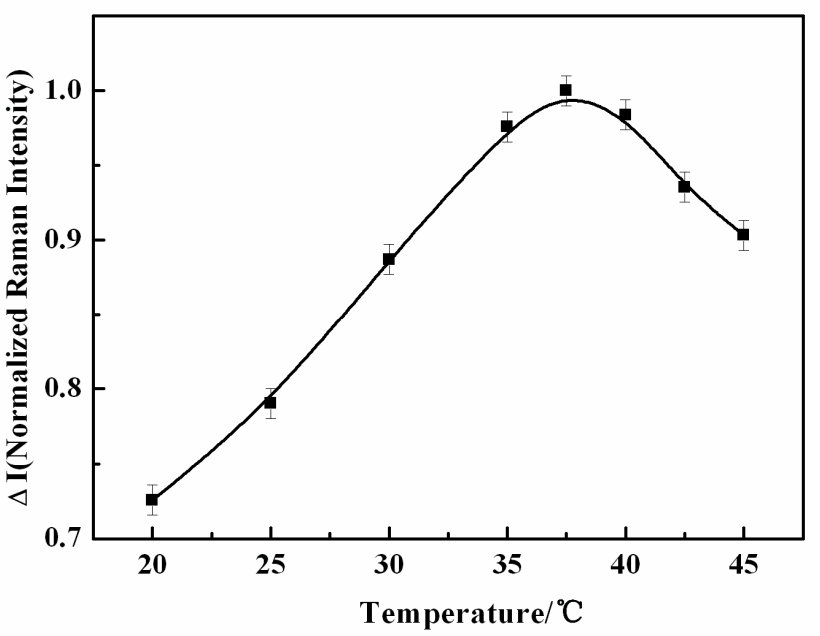


Figure S5. Influence of cycle reaction temperature on the Rman intensity responding to 1.0×10^{-14} M target DNA.

S5.3 Optimization of the reaction time

As a cross-strand displacement system, the reaction time is an important influencing factor. The final SERS signals were dependent on the amount of the Raman probes bound to the RCA products, the greater the number of RCA primer probes, the greater the amount of the RCA products. In order to obtain higher sensitivity, the time of the “one-pot” reaction was investigated and Figure 4 showed the changes of Raman signals generated by performing the experiment at different time intervals. The results showed that the Raman intensities increased rapidly with the increase of reaction time to 2 h, and a plateau effect was reached after this time. Therefore, the reaction time was controlled at 2 h all through the experiment.

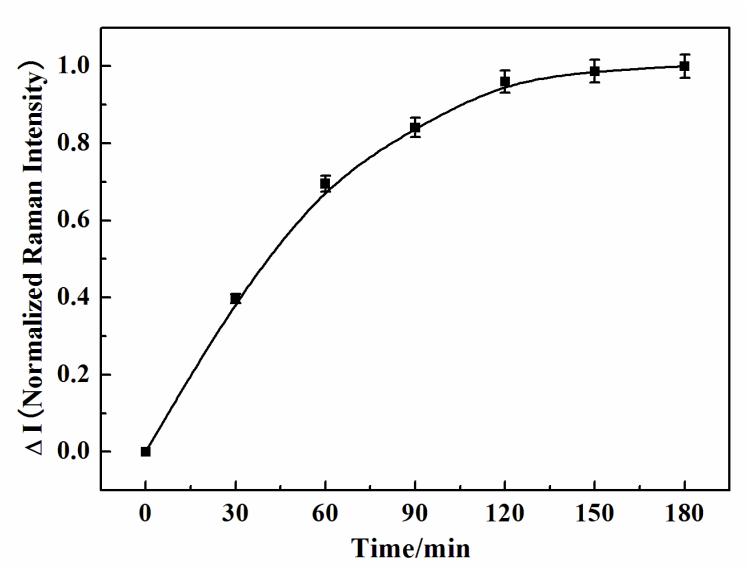


Figure S6. Influence of cycle reaction time on the Raman intensity responding to 1.0×10^{-14} M target DNA.

S6 Sensitivity of the method for DNA detection

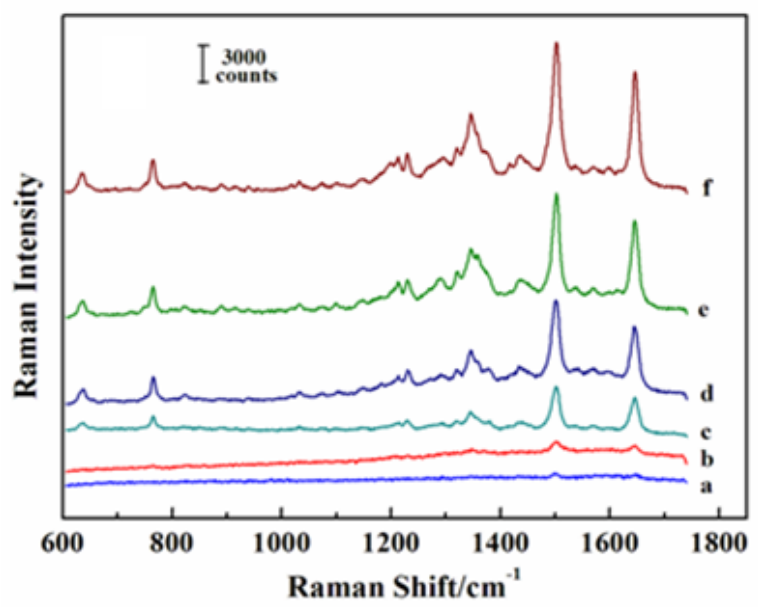
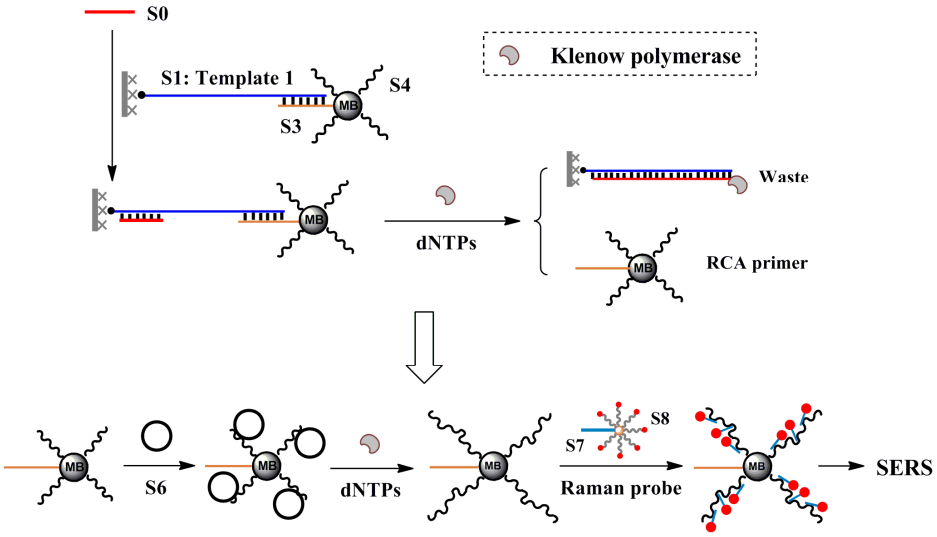


Figure S7. SERS spectra for increasing concentrations of target DNA: (a) 0 M, (b) 1.0×10^{-15} M, (c) 1.0×10^{-14} M, (d) 1.0×10^{-13} M, (e) 1.0×10^{-12} M and (f) 1.0×10^{-11} M.

S7 Verification of the amplification effect of CSDA

To verify the amplification effect of CSDA, another experiment was performed in the absence of S2 and Template 2. As shown in Scheme S1, in the presence of target DNA (S0), strand-displacement reaction could be initiated and the RCA primer probes could be released from the 96 Wells. Simultaneously, RCA was initiated to generate multiple long single-stranded DNAs with many repetitive sequence units for incorporating with the Raman probes. In the absence of S2 and Template 2, the target DNA could initiate the strand-displacement reaction and the RCA reaction. However, the CSDA could not be performed and it could be seen that the sensitivity of target detecting was significantly improved by CSDA.



Scheme S1. Schematic diagram for the detection of DNA based on Cross Strand-Displacement and Rolling Circle Amplification

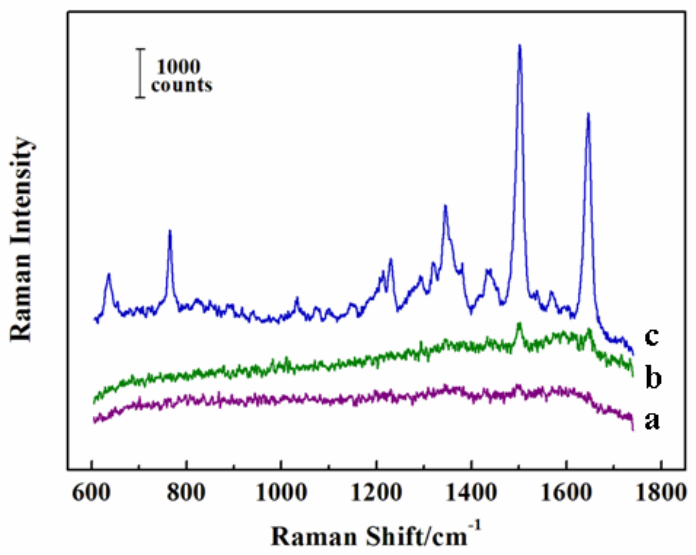


Figure S8. Verification of the amplification effect of CSDA by SERS: in the absence of target DNA (a), in the presence of 1.0×10^{-13} M target DNA without CSDA (b), in the presence of 1.0×10^{-13} M target DNA with CSDA (c).

S8 Selectivity of the method for DNA detection

The selectivity of this method was investigated by using the capture probe to hybridize with the same concentration of complete complementary target DNA sequence, the one-base mismatched DNA sequence and the noncomplementary DNA sequence, respectively, as shown in Figure S9. A well-defined Raman signal was obtained for the complementary sequence. The Raman intensity for one-base mismatched sequence was significantly weaker than that of the complementary sequences, and the noncomplementary sequence showed no response, suggesting the high selectivity of the SERS method for DNA detection.

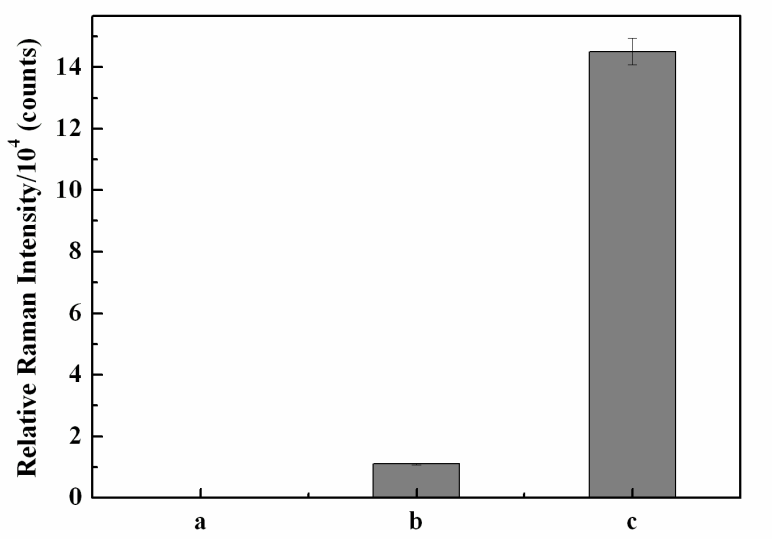


Figure S9. The Raman intensity in the presence of (a) noncomplementary sequence, (b) one-base mismatch sequence; (c) complementary sequence. All the concentrations of target DNA in (a), (b), and (c) were 1.0×10^{-13} M.

S9 Selectivity of the method for lysozyme detection

To assess the selectivity for lysozyme detection, bovine serum albumin (BSA) and thrombin were chosen as controls to assess the specificity of the strategy. As show in Figure S10, the presence of BSA and thrombin led to a SERS signal almost equivalent to the blank sample and the signal for lysozyme was much larger than those of BSA and thrombin. The experimental results demonstrate that the proposed method exhibits a good specificity for lysozyme detection, which is attributed to the specific binding between the target and its aptamer.

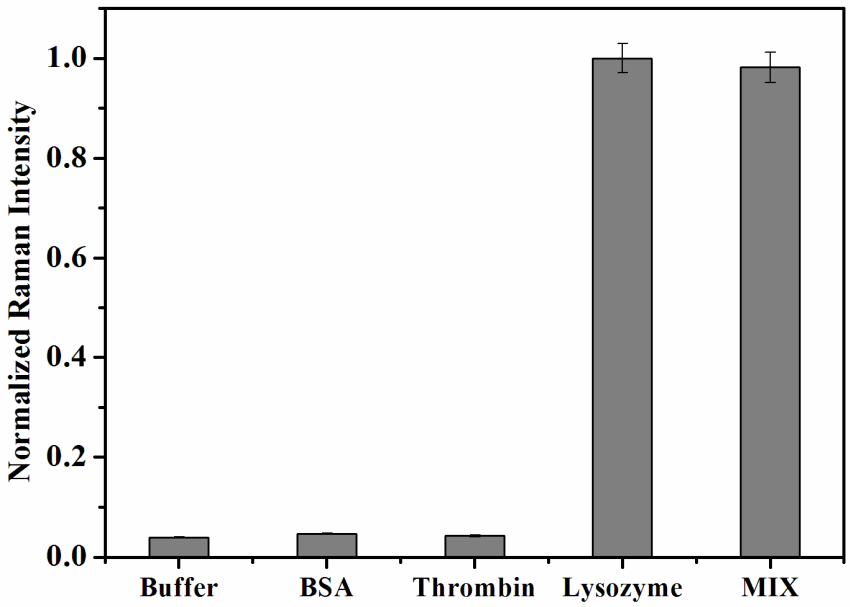


Figure S10 The Raman intensities after the addition of phosphate buffer solution or the proteins. The concentration of lysozyme: 1.0×10^{-13} M; The concentration of BSA and thrombin: 1.0×10^{-10} M.

S10 Determination of lysozyme in real samples

To verify the availability of the method for real samples, analysis of lysozyme from human serum was implemented. Because of the high sensitivity and selectivity of this method, the serum sample was diluted as the tested sample. The calculated content of lysozyme in human serum was 3.06×10^{-7} M, which was within the normal range of the previous reported values.[40] The recovery for the spiked lysozyme introduced at different concentrations was found to vary from 89.6 to 98.2% (see Supporting Information, Table S2). Three replicate determinations at different concentration levels exhibited RSDs ranging from 5.7 to 8.1% (n=3). The acceptable relative standard deviations and quantitative recoveries indicate the application potential of this assay for complex biological samples.

Table S2 Recovery ratio of the assay in the blood samples

	Lysozyme content added	Lysozyme content detected	Recovery (%)	RSD (%)
1	5×10^{-14} M	4.48×10^{-14} M	89.6%	5.7
2	1×10^{-13} M	9.20×10^{-12} M	92.0%	6.5
3	5×10^{-13} M	4.91×10^{-13} M	98.2%	8.1

a Each sample was repeated for three times and averaged to obtain the recovery and RSD values.

S11 Comparison of different methods for lysozyme detection

Table S3. Comparison between the proposed SERS method and other reported techniques based on aptamer recognition for the detection of lysozyme

principle	Label or probe	Detection modes	Detection limit
Probe aggregation[5]	perylene	Fluorescent	0.07 nM
Network of DNA-related reaction cycles[6]	TAMRA-DNA	Fluorescent	3.6 fM
Gold nanoparticle amplification[7]	$[Ru(bpy)_2(dcbpy)NHS]$	Electrochemiluminescence	1.0×10^{-13} M
Autonomous DNA machine[8]	CdS nanoparticles	Electrochemical	5.2×10^{-13} M
Electrostatic interaction[9]	$[Ru(NH_3)_6]^{3+}$	Electrochemical	35 nM
DNAzyme amplification[10]	DNAzyme	UV-vis absorption spectra	1.0×10^{-13} M
Polymerase and nicking enzyme amplification[11]	DNAzyme	UV-vis absorption spectra	0.1 fM
Silver colloid enhancement[12]	Label-Free	SERS	5 μg/mL
Proposed method	AuNP-Functionalized ROX-DNA	SERS	5.8×10^{-15} M

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