Electronic Supplementary Information

Multilayered Shell SERS Nanotags with Highly Uniform Single-Particle Raman Readout for Ultrasensitive Immunoassays

Renyong Liu, Bianhua Liu, Guijian Guan, Changlong Jiang and Zhongping Zhang*

Institute of Intelligent Machines, Chinese Academy of Sciences, Hefei, Anhui 230031, China.

E-mail: zpzhang@iim.ac.cn

EXPERIMENTAL SECTION

Chemicals and Materials. Human carcinoembryonic antigen (CEA), alpha fetoprotein (AFP), carbohydrate antigen 125 (CA 125) and the matched pairs of monoclonal antibodies mouse anti-human CEA (ML01C01) and CEA (ML01C02), mouse anti-human AFP (1001) and AFP (1002), mouse anti-human CA 125 (X306) and CA 125 (X52) were purchased from Shanghai Linc-Bio Science Co., Ltd. Normal human serum was purchased from Beijing Biosynthesis Biotechnology Co., Ltd. 4-aminobenzenethiol (4-ABT), 3-methoxybenzenethiol (3-MeOBT), 2-methoxybenzenethiol (2-MeOBT), 2-naphthalenethiol (2-NT) (from Sigma-Aldrich) were chosen as Raman tag molecules. Bovine serum albumin (BSA), poly(ethyleneimine) (PEI, Mw ≈ 25000) and sodium borohydride ($NaBH_4$) were obtained from Sigma-Aldrich. Tetraethylorthosilicate (TEOS), ammonium hydroxide (25%), silver nitrate ($AgNO_3$), poly(vinylpyrrolidone) (PVP, Mw ≈ 40000), trisodium citrate ($C_6H_5O_7Na_3 \cdot 2H_2O$), Tween-20, glutaraldehyde (GA) and ethanol were purchased from Shanghai Chemicals Ltd. 0.01 M phosphate-buffered saline (PBS, PH 7.4) solution was prepared by adding 1.22 g of K_2HPO_4, 0.136 g KH_2PO_4, and 0.85 g of NaCl in 1000 mL of ultrapure water. The clinical serum samples were gifted from the Second Affiliated Hospital of Anhui Medical University, China.

LBL Assembly of AgNPs at SiO_2 Particles. 12-nm AgNPs were synthesized in an aqueous solution by reducing $AgNO_3$ with $NaBH_4$ in the presence of PVP and $C_6H_5O_7Na_3$,[1] and uniform SiO_2 particles with a size of ~300 nm were prepared according to the Stöber method.[2] SiO_2 particles were first coated with PEI by suspended in 2 mg/mL positively charged PEI solution (containing 0.5 M NaCl). After centrifuged and washed with pure water, the PEI-coated SiO_2 particles were dispersed into AgNPs colloid and the mixture was stirred for 15 min. The particles attaching AgNPs at polyelectrolyte layer were separated and again dispersed in AgNPs colloid. After repeating four times, a dense layer of AgNPs was formed at the PEI layer. The SiO_2 particles were again suspended in the PEI solution for the formation of PEI electrolyte at the AgNPs layer. The assembled procedure was repeated until the desired number of bilayers was reached.

Preparation of SERS Nanotags and Conjugation of Antibody. 4-ABT, 3-MeOBT, 2-MeOBT and 2-NT were chosen as Raman tag molecules. 25 mg of multilayer-coated silica particles were separately suspended in 5 mL of ethanol solution containing 1 mM above molecules and incubated for 30 min at room temperature, which led to the permeation of tag molecules into the interlayers of the hybrid shell by the interactions with AgNPs. After centrifuged and washed with ethanol several times, the particles with different Raman tags were then suspended in 2.5% GA and shaken for 1 h at room temperature, in which the crosslinking reaction occurred between GA and amino groups of PEI layers. The nanotags with different Raman tags were thus produced. Subsequently, the antibody targeting to a specific analyte was further linked onto the surface of nanotags by the conjugation reaction with residual aldehyde groups. Typically, 125 μg of detection antibody in PBS buffer was

added into the nanotag suspension and the mixture was incubated for 1 h at room temperature. The antibody-modified nanotags were further suspended in 1% BSA and shaken for 1 h at 37 ºC and then washed three times with 0.05% Tween-20. The SERS nanotags with the ability of biological recognition for the immunoassay were finally obtained and dispersed in PBS with a final concentration of 25 mg/mL and stored at 4 ºC in a refrigerator.

Preparation of Paramagnetic Particles and Modification of Antibody. The paramagnetic Fe_3O_4 particles and core-shell Fe_3O_4@SiO_2 particles were prepared according to the previously reported method.[3] 15 mg of the core-shell Fe_3O_4@SiO_2 particles were first dispersed in 2 mg/mL PEI solution (containing 0.5 M NaCl). After centrifuged and washed with pure water, the PEI layer adsorbed at the SiO_2 shell was crosslinked by suspending the particles in 2.5% aqueous GA under shaking for 1 h at room temperature. The obtained particles were washed with PBS and isolated using a magnetic field, followed by the addition of 75 μg of capture antibody in PBS buffer for 1 h at room temperature. Under shaking, the capture antibody was chemically immobilized onto the PEI layer through the reaction with residual aldehyde groups. The products were thoroughly washed with 0.05% Tween-20, blocked with 1% BSA, dispersed in PBS buffer with a final concentration of 25 mg/mL and stored in a refrigerator prior to use.

Immunoassay Protocol. A 1:1 mixture of normal human serum and PBS buffer was used as the incubation solution for the SERS-based immunoassay. Antigen standard solutions were prepared by adding different amounts of antigens into the incubation solution. For each assay, 50 μL of antigen standard solutions at various concentrations were mixed with 50 μL of antibody-modified magnetic particles (25 mg/mL) and allowed to react for 1 h at 37 ºC. After

isolation and washing three times with PBS, the immunocomplexes were further reacted with 100 μL of SERS nanotags (25 mg/mL) for 1 h at 37 ºC. The sandwich-type immunocomplexes were separated under a magnetic field and washed three times with PBS buffer and redispersed in PBS buffer. The solution was sucked into a capillary tube and the sandwich-type immunocomplexes were then collected at one spot using an external magnetic field. The Raman spectra were recorded from this site.

Characterization and Instruments. The shape and structure of the particles were examined by FEI Sirion-200 field emission scanning electron microscopy (SEM) and JEOL 2010 transmission electron microscopy (TEM) operated at 200 kV accelerating voltage. UV-vis absorption spectra were recorded with a Shimadzu UV-2550 spectrometer. Single-particle light scattering images and Raman spectra were recorded on a silicon substrate using the DXR confocal microscopy Raman system (Thermo Fisher Scientific Inc.) equipped with a dark-field illumination. Raman spectra were collected with a 532-nm excitation laser and 50-μm slit for confocality. The laser power and accumulation time were 10 mW and 10 s, respectively. Raman measurements of single particles on the silicon substrate and SERS nanotags in solution were performed with 50× and 10×objective lens, respectively.

Calculation of Enhancement Factor (EF). The EF values of the single SiO_2@$(AgNPs/PEI)_n$ particles to 4-ABT were calculated by using the following equation:

$$EF = \frac{I_{SERS} / N_{SERS}}{I_{bulk} / N_{bulk}}$$

where I_{SERS} and I_{bulk} represent the intensities of the peak at 1434 cm^{-1} from single SiO_2@$(AgNPs/PEI)_n$ particles and solid powder, respectively. N_{SERS} is the number of molecules adsorbed on the $(AgNPs/PEI)_n$ layers, which is determined by measuring the

difference between the total 4-ABT amount and the residual amount in solution. As for SiO_2@(AgNPs/PEI)$_5$ particles, we used 5 mL 5×10^{-6} M of 4-ABT to label the particles in the EF calculation. The total number of tag molecules is 1.5×10^{16}. In our case, the Raman tag molecules with thiol group can strongly bind to AgNPs and can hardly adsorb onto other surfaces such as the hydrophilic glassware. After the incubation for 30 min, the tag molecules were not found in the residual solution by UV-vis spectral measurements, so it can be supposed that at least 95% of molecules were adsorbed into the hybrid shells. The number of SiO_2@(AgNPs/PEI)$_5$ particles in solution is about 9.5×10^{11}, so N_{SERS} can be calculated to be about 15,000. N_{bulk} is the molecule number of the neat 4-ABT in the laser illumination volume. We employed 532-nm laser and focused through objective lens (50×, NA = 0.5) to reduce instrumental noise. Assuming that the excitation volume as a cylinder, the diameter (d) and the height (h) were determined by the following equations:[4]

$$d = \frac{1.22\lambda_{laser}}{NA} \qquad h = \frac{2.2n\lambda_{laser}}{\pi(NA)^2}$$

where n is the refractive index of 4-ABT (n ≈ 1.6). N_{bulk} can be calculated to be about 1.7×10^{10}. The EFs of individual SiO_2@(AgNPs/PEI)$_5$ nanotags distributed in a very narrow range of 1.1×10^{7}~2.5×10^{7} were obtained.

References for Electronic Supplementary Information

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2 W. Stöber, A. Finker and E. Bohn, *J. Colloid Interface Sci.*, 1968, **26**, 62.

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4 W. R. Zhang, C. Lowe and R. Smith, *Progress in Organic Coatings*, 2009, **66**, 141.

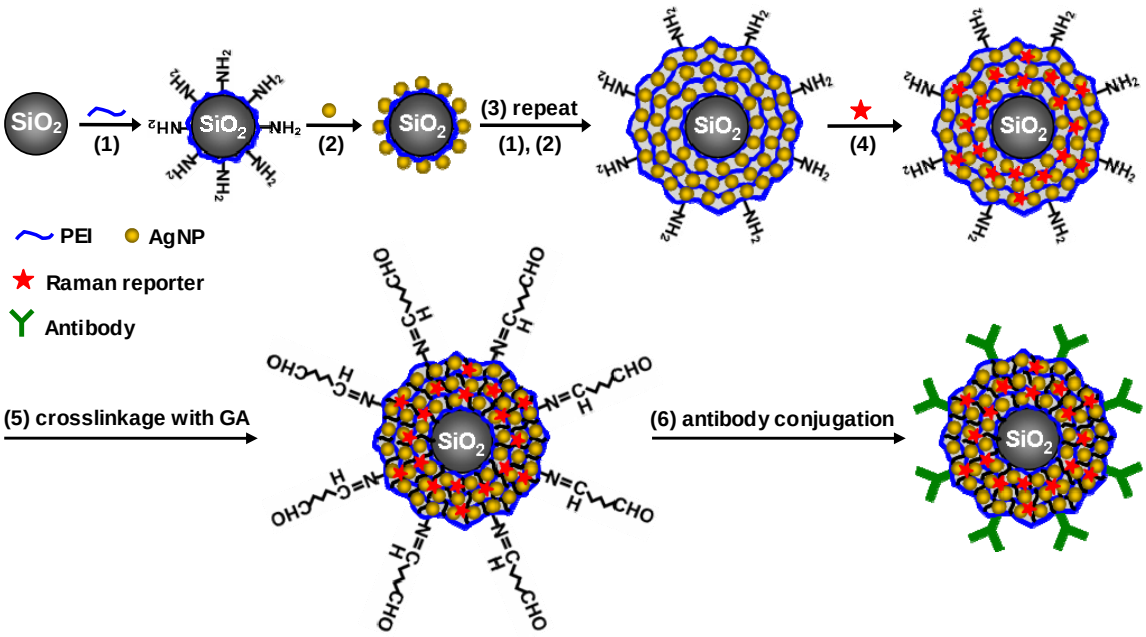

Fig. S1 Procedure for the assembly of SERS nanotags: (1) the coating of PEI at silica particles, (2) the assembly of AgNPs onto the PEI layer, (3) the alternate assembly of PEI and AgNPs onto the silica particles, (4) the incorporation of Raman tags, (5) the crosslinkage of interlayers using glutaraldehyde (GA) and (6) the conjugation of antibodies.

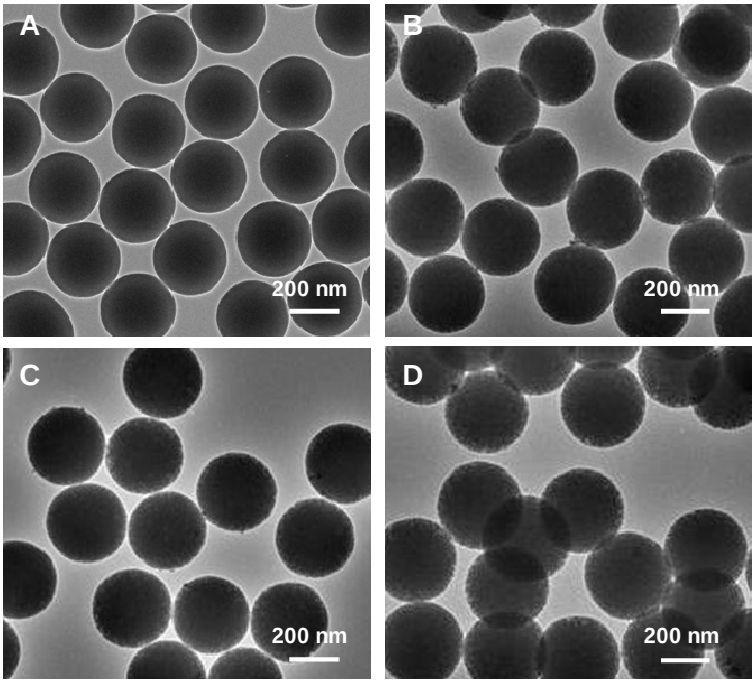

Fig. S2 The evolution of the multilayered structure of AgNPs. (A) TEM images of SiO_2 particles. (B-D) TEM images of SiO_2@$(AgNPs/PEI)_n$ particles with n = 1, 3 and 5.

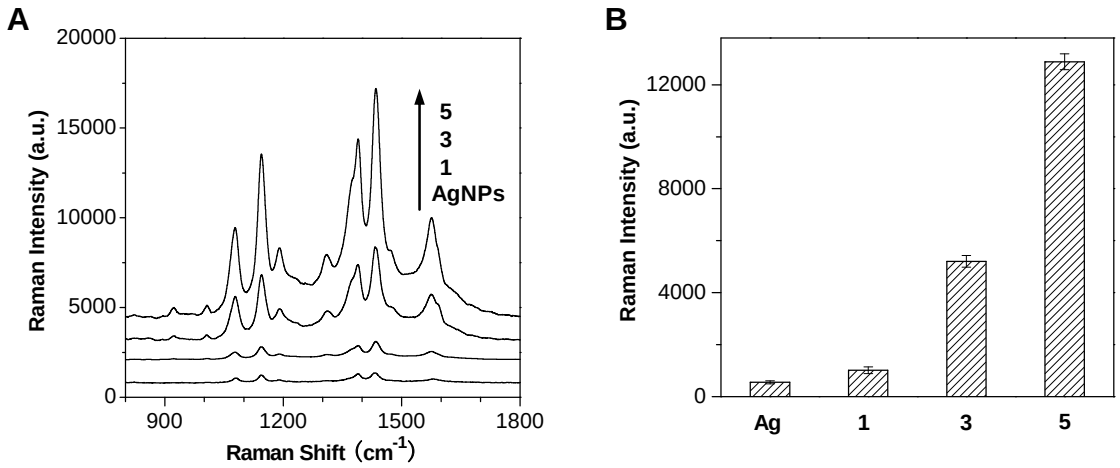

Fig. S3 Comparison of Raman signal intensity as a function of the number of assembled layers in solution. (A) SERS spectra of 4-ABT in original AgNPs and SiO_2@(AgNPs/PEI)$_n$ particles with n = 1, 3 and 5. (B) The comparison of corresponding SERS intensity at 1434 cm^{-1}. The concentration of 4-ABT (1 mM) in colloidal AgNPs was the same as that used in the preparation of nanotags.

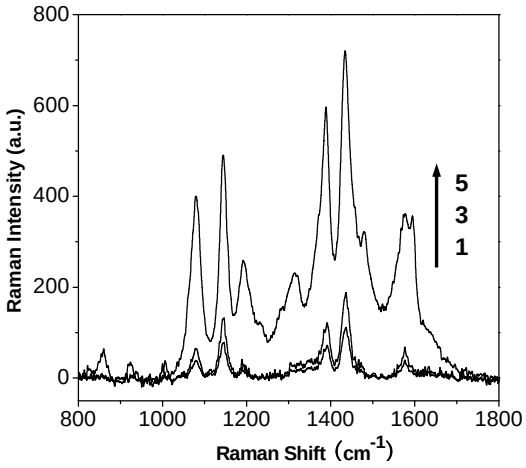

Fig. S4 SERS spectra of 4-ABT from SiO_2@(AgNPs/PEI)$_n$ particles with n = 1, 3 and 5. The SERS particles with different layer number were individually incubated with very low number of moles of 4-ABT (0.1 nmol). In this case, the surface of AgNPs was far from the saturation by molecules.

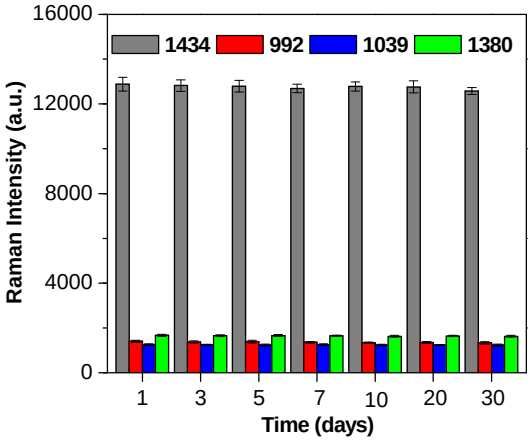

Fig. S5 The stability of the SERS nanotags. The Raman intensities of the represented peaks **(**1434, 992, 1039 and 1380 cm^{-1}, respectively) of nanotag-(4-ABT), (3-MeOBT), -(2-MeOBT) and -(2-NT) kept almost identical after they were suspended in water for one month.

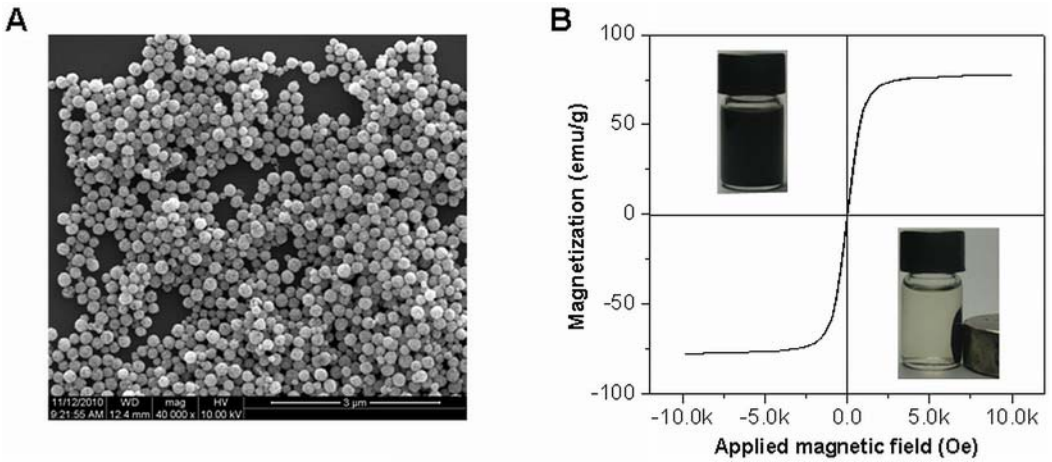

Fig. S6 The characterization of superparamagnetic particles. (A) SEM image of the prepared Fe_3O_4@SiO_2 particles. (B) Magnetization curve of the prepared Fe_3O_4@SiO_2 particles. The saturation magnetization value was 78 emu/g. The inset photographs were of the suspension assay before and after magnetic separation. The color of solution changed into transparent from black after the particles were highly concentrated under an external field.

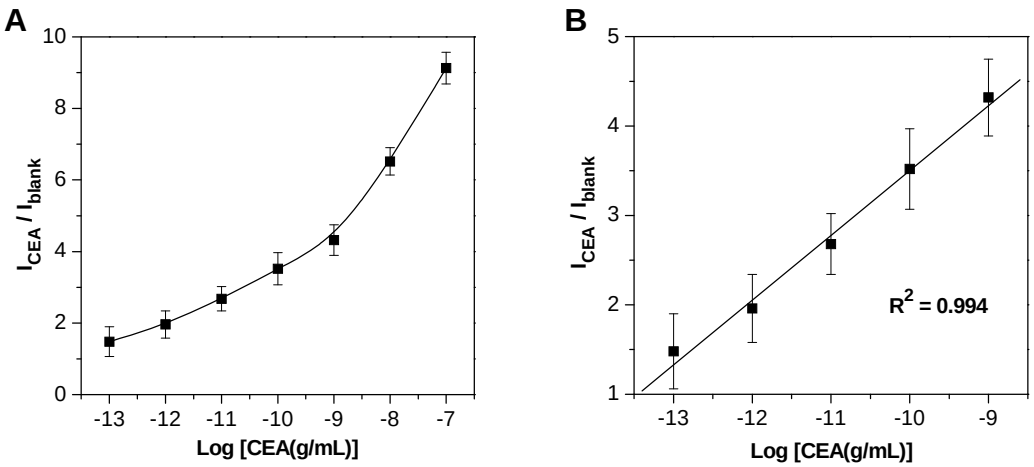

Fig. S7 The Raman intensity plots for the detection of CEA. (A) The Raman intensity ratio (I_{CEA}/I_{blank}) of 4-ABT at 1434 cm^{-1} with the logarithm of CEA concentrations. (B) The linear correlation of Raman intensity (at 1434 cm^{-1}) with the logarithm of CEA concentrations from 0.1 pg/mL to 1.0 ng/mL. The linear regression equation is $y = 0.724x + 10.8$ ($R^2 = 0.994$).

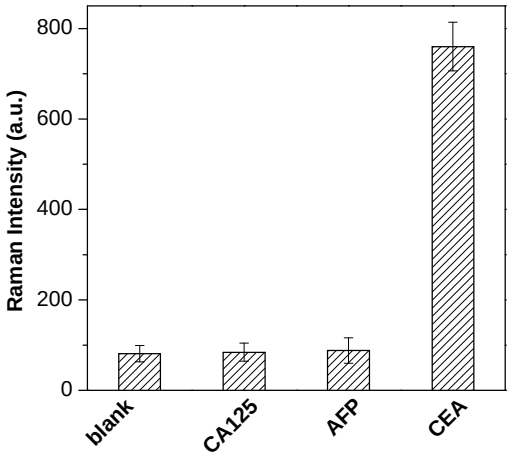

Fig. S8 The selectivity of the immunoassay of CEA (10 ng/mL) to AFP (10 ng/mL) and CA125 (10 U/mL). The SERS intensity of the peak at 1434 cm^{-1} was used for the evaluation of Raman readout. It can be seen that the Raman readouts are similar to that of the blank sample, revealing the high selectivity of this immunoassay method.

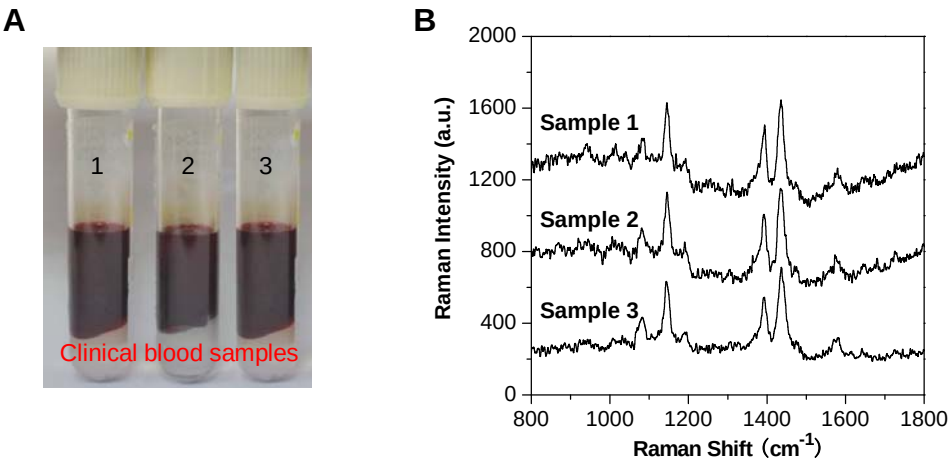

Fig. S9 The real detection of clinical blood samples with nanotag-(4-ABT). (A) The photograph of the three patient blood samples. (B) The corresponding SERS spectra obtained by nanotag-based immunoassay method after diluted by 10, 10 and 1000 times with PBS for sample 1, 2 and 3, respectively. All blood samples were handled in accordance with approved Institutional Review Board (IRB) protocols at the Second Affiliated Hospital of Anhui Medical University, China. Peripheral venous blood was collected into the serum separation tube and allowed to clot at room temperature for 30 minutes. After centrifuged at 2000 g for 15 minutes, the serum was separated and stored at -80 ºC before analysis.

Table S1. Real detection and recovery test of CEA in clinical serum samples.

Sample No.	Referenced ECLIA method (ng/mL)[a]	Proposed SERS method (ng/mL)[b]	Spiked CEA (ng/mL)	Measured CEA (ng/mL)	Recovery (%)
1	3.50	0.39	0.10	0.51	104.08
2	3.09	0.34	0.10	0.38	86.36
3	183.60	0.16	0.10	0.25	96.15

[a]The data represent natural CEA levels in the three clinical serum samples detected by the standard electrochemiluminescent immunoassay (ECLIA) method in clinical diagnostics. [b]The data represent the CEA concentrations in the three clinical serum samples obtained by SERS nanotag-based immunoassay method after 10, 10 and 1000 times dilution with PBS for sample 1, 2 and 3, respectively.

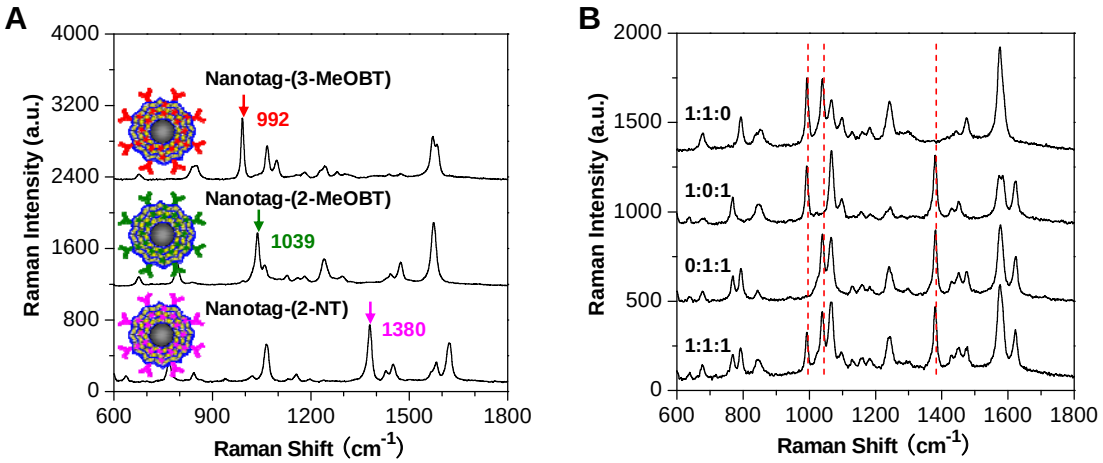

Fig. S10 (A) SERS spectra of nanotag-(3-MeOBT), -(2-MeOBT) and -(2-NT). The three strong peaks of the nanotags appeared at 992, 1039 and 1380 cm^{-1}, respectively. (B) SERS spectra of a ternary mixture of nanotag-(3-MeOBT), -(2-MeOBT) and -(2-NT) with different concentration ratios. The existence of each nanotag in the mixture could clearly be indicated by the resolved peaks, as noted with red lines.

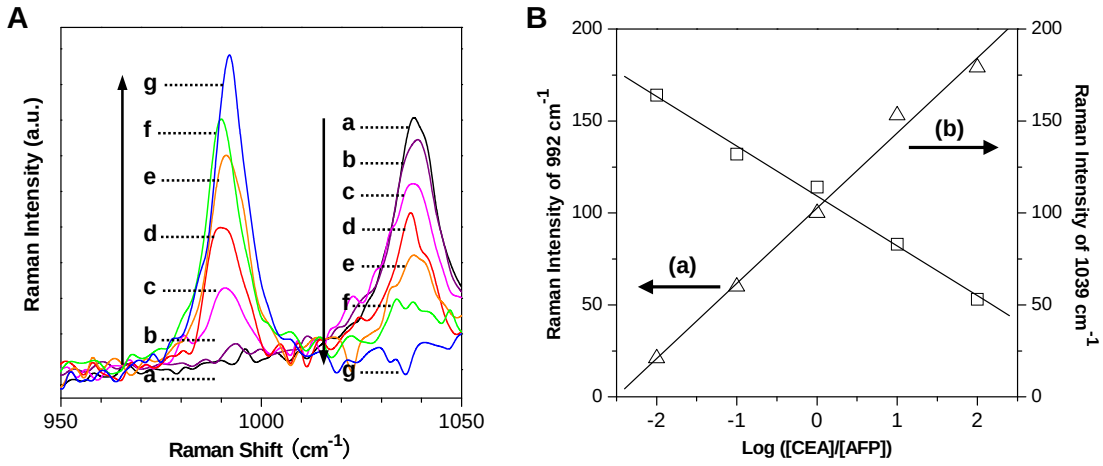

Fig. S11 (A) SERS spectra from different ratios of CEA and AFP in human serum: (a) 0:1; (b) 1:100; (c) 1:10; (d) 1:1; (e) 10:1; (f) 100:1; (g) 1:0. (B) The corresponding Raman intensity at 992 cm^{-1} (a) and 1039 cm^{-1} (b) versus the logarithmic ratio of CEA and AFP. The above results show that the intensity of each peak exhibits a very good linear behavior versus the corresponding analyte concentrations.

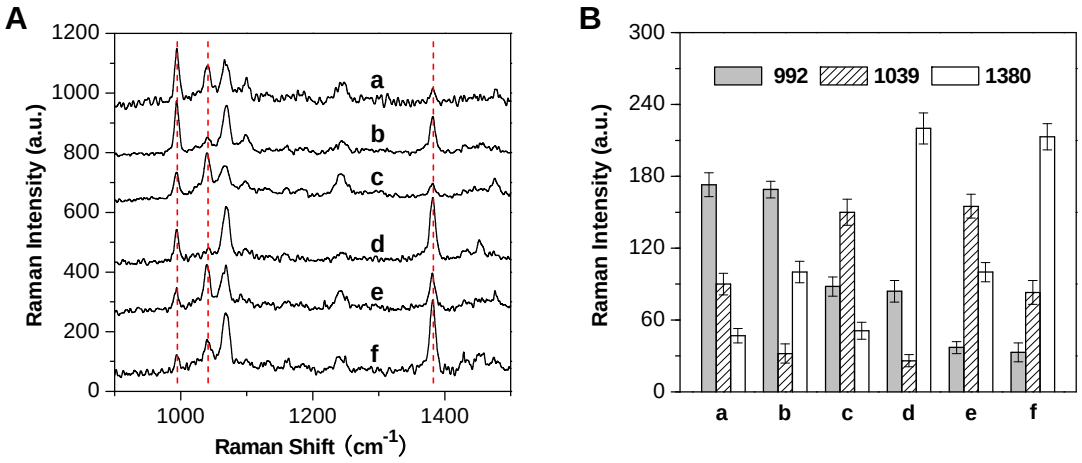

Fig. S12 (A) SERS nanotag immunoassays of CEA, AFP and CA 125 in six human serum samples with different concentration ratios, respectivley: (a) 1.0 ng/mL, 0.1 ng/mL, 0.01 U/mL; (b) 1.0 ng/mL, 0.01 ng/mL, 0.1 U/mL; (c) 0.1 ng/mL, 1.0 ng/mL, 0.01 U/mL; (d) 0.1 ng/mL, 0.01 ng/mL, 1.0 U/mL; (e) 0.01 ng/mL, 1.0 ng/mL, 0.1 U/mL; (f) 0.01 ng/mL, 0.1 ng/mL, 1.0 U/mL. (B) The bar graphs of the corresponding Raman intensity at 992, 1039 and 1380 cm^{-1}.