Supporting Information

Wavelength and shape dependent SERS study to develop ultrasensitive

nanotags for imaging of cancer cells

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Experimental descriptions

Materials and methods

All the reagents and solvents were purchased from Sigma Aldrich, Fluka, Alfa Aesar, Acros and BBI solutions, and used without further purification. EGFR-IgG_{2a} (sc-120), HER2 (sc-71667, Neu 0.N.211) were supplied by Santa Cruz Biotechnology, Inc and BioLegend. Normal phase purifications were carried out using Merck Silica Gel 60 (particle size: 0.040-0.063mm, 230-400 mesh). Analytical characterization was performed on a HPLC-MS (Agilent-1200 series) with a DAD detector and a single quadruple mass spectrometer (6130 series) with an ESI probe. Raman spectra were measured Renishaw in Via Raman Microscope in Singapore Bioimaging Consortium, Agency for Science, Technology and Research (A*STAR), Singapore. SERS measurements were carried out in a Renishaw InVia Raman (UK) microscope with a laser beam directed to the sample through $50 \times$ and $20 \times$ objective lens and a Peltier cooled CCD detector. Samples were excited with a 633 nm and 785 nm excitation wavelength laser and Stokes shifted Raman spectra were collected in the range of 400 to 2000 cm^{-1} with 1 cm^{-1} resolution. Prior to the measurement, a calibration with a silicon standard (Raman peak centered at 520 cm^{-1}) was performed. WiRE 3.2 software package was used for data acquisition. Surface plasmon resonance (SPR) was recorded Hitachi U2090 spectrophotometer and SpectraMax M2 spectrophotometer (Molecular Devices), UV-Vis and the data analysis was performed using Origin 8.0 and Microsoft Excel 2010.

Synthesis of Nanorods:

Nanorods (NR) were synthesized according to the well-documented seed-mediated growth method. Specifically, for the synthesis of seeds for the nanorods, CTAB aqueous solution (5 ml, 0.20 M) was mixed with 5.0 ml of 0.5 mM HAuCl₄. Then 0.60 ml of ice-cold

10 mM NaBH₄ solution was added. After vigorous stirring, the seed solution was kept at 25 °C. For the nanorods, a separate growth solution was prepared. CTAB (5 ml, 0.20 M) was first added to 0.15 ml of 4 mM AgNO₃ solution at 25 °C, followed by 5.0 ml of 1 mM HAuCl₄ and 70 μ l of 78.8 mM ascorbic acid. The final step was the addition of 12 μ l of the previously prepared seed solution to the growth solution at 27–30 °C. The temperature of the growth medium was kept constant at 27–30 °C in all preparations. The reaction was allowed to last for 4-5 h. Gold seed solution ([Au] \approx 4 mM) with constant stirring at room temperature for 8 h.

Generally after synthesis of Au nanorod, the plasmon peak at NIR was shift towards lower wavelength after aging few days. To stop blue shift of NIR plasmon peak, we have added 50 μ l of 0.01 mM solution of Na₂S solution after the complete synthesis of Au nanorod and stirring for 10 min. The as prepared Au nanorod collected by centrifuge and redispersed in deionised water for further experiment.

Synthesis of Nanostars:

To synthesize the nanostars, HAuCl₄.3H₂O aqueous solution (1 ml, 1 wt%) was first diluted with 90 ml of water, followed by the injection of trisodium citrate (2 ml, 38.8 mM). Subsequently, freshly prepared NaBH₄ solution (1 ml, 0.075 wt% in 38.8 mM trisodium citrate solution) was added and then the reaction mixture was kept at room temperature overnight with constant stirring to form the seed solution. Subsequently, 50 ml of the gold seed solution was mixed with PVP (10 mM) at room temperature for 24 h to prepare the PVP-coated gold seed solution. Finally, 82 μ L of 50 mM HAuCl₄ aqueous solution was mixed with 15 ml of 10 mM PVP in DMF, followed by the rapid addition of 43 μ l of the PVP-coated

SERS measurements of Cy3LA, Cy5LA, Cy7LA, Cy7.5LA and CyNAMLA-381 with different geometry of gold colloids

For measurement of SERS intensity we first prepared 1 ml of 50 μ M of **Cy3LA**, **Cy5LA**, **Cy7LA**, **Cy7.5LA and CyNAMLA-381** stock in deionized water. From the stock solution, we used the 5 μ L of each dye for different substrate. We varied the concentration of substrate to get the saturation point. The concentration of substrate and details experiment is in table below (Table S1). Briefly 5 μ L dye stock was mixed with different concentration gold substrate at a certain volume to keep the all volume fixed and incubated for 1hr. 20 μ L of the reporter-gold colloid mixture solutions were placed on a glass slide with cover slip, and their Raman spectra (range: 400 to 2000 cm⁻¹, resolution: 1 cm⁻¹, acquisition time: 10 s) were measured in a Renishaw InVia Raman microscope under excitation with a 633 nm and 785 nm excitation wavelength lase using same power. The results are plotted as average intensities of three independent experiments.

[∎] Dye(50uM)	AuNR 700 8.6 nM (Stock)	AuNR 800 9.3 nm (Stock)	AuNR 900 9.8 nM (Stock)	Au nanostar 2 nM (Stock)	AuNPs-8 Au sphere 0.004 nM (Stock)
	50 μL	50 μL	50 μL	50 μL	50 μL
Cy-5LA (5μL),	25 μL	25 μL	25 μL	25 μL	25 μL
Cy-	12.5 μL	12.5 μL	12.5 μL	12.5 μL	12.5 μL
7LA(5μL)	10 μL	10 μL	10 μL	10 μL	10 μL
Cy- 7.5LA(5μL)	5 μL	5 uL	5 uL	5 uL	5 uL
Cv-NAMLA-	2.5 μL	2.5 μL	2.5 μL	2.5 μL	2.5 μL
381 (5μL)	1.25 μL	1.25 μL	1.25 μL	1.25 μL	1.25 μL
	0.75 μL	0.75 μL	0.75 μL	0.75 μL	0.75 μL

Table S1. Details experimental procedure of SERS measurement where the dye concentration fixed and substrate concentration varied. The green highlighted part is the saturation point for the corresponding substrate. We keep here the total volume is 55 μ L for all cases. For certain cases we added 5 mL dye stock, mentioned amount of Au substrate and reaming volume we filled with deionized water.



Figure S1. Absorbance spectra of five different Raman reporters (Cy3LA (blue), Cy5LA (pink), Cy7LA (light blue), Cy7.5LA (cyan), and CyNAMLA-381 (red) in deionized water. The maxima absorbance of the Raman reporters are 540 nm, 645 nm, 750 nm, 740-790 nm and 790 nm for Cy3LA, Cy5LA, Cy7LA, Cy7.5LA and CyNAMLA-381 respectively.



Figure S2. Representative SERS spectra of five different Raman reporters (Cy3LA, Cy5LA, Cy7LA, Cy7LA, Cy7.5LA and CyNAMLA-381 which were adsorbed on the surface of gold nanosphere (GNSp) and excited at 785 nm laser source. The spectra were recorded from 400-800 cm⁻¹ in a Raman microscope (785 nm laser excitation, acquisition time: 10 s, laser power vary from 1 mW to 50 mW).



Figure S3. Concentration (50 uM, of different Raman reporters (Cy5LA, Cy7LA, Cy7.5LA and CyNAMLA-381) was fixed with respect to a variable concentrate of gold substrates (GNSp, GNR-700, GNR-800, GNR-900). As SERS intensity of Cy3LA is very poor, so we performed Cy-3LA separately and Cy-3LA was directly applied to measure EF factor in which concentration of dyes 8 μ M and substrates concentration is ~9 μ M.

Mixed Thiolated PEG encapsulation:

The reporter molecule (Cy7LA 10 μ M) was incubated with individual gold substrates such as citrate-stabilized Au-colloid, CTAB capped Au nanorod and PVP-stabilized AuNanostar for 10 min to 15 min before the hetero functional linker HS-PEG-CO2H (10 µM) was added to all solutions (volume: 2.6 mL). After shaking for 15 min, the Au-colloid was exposed to excess PEG-SH (1.8 mL, 10 µM) and similarly, Au-nanostar was exposed excess PEG-SH (2 mL, 10 µL) after 30 min shaking to maximize the surface coverage and stabilize the PEG and the chemisorbed reporter molecule. After 4 h continuous shaking, free PEG-SH was removed by 3 rounds of centrifugation (6000 rpm, 5 min), and the nanoparticles were resuspended in phosphate buffered saline (PBS) for bioconjugation. N-(3-(dimethylamino)propyl)-N'-ethylcarbodiimide (EDC) (125 nmol) and N-hydroxysuccinimide (NHS) (125 nmol) were applied to activate the acid functional groups on the surface of gold colloids. After 30 min incubation, excess of EDC and NHS was removed by 3 rounds of centrifugation (8000 rpm, 6 min), and re-suspended in PBS using Amicon Ultra 3K centrifuge filters (Milipore). The activated particles Cy7LA@GNSp@PEG and Cy7LA@GNSt@PEG were then reacted with two different antibodies (a mouse monoclonal anti-EGFR IgG2a (12 nM), and a mouse monoclonal anti-HER2) at 25 °C for 2 h and then overnight at 4 °C. Further nonspecific binding chemicals and antibodies were removed by centrifugation (8000 rpm, 10 min) and the final nanotags were re-suspended in PBS and stored at 4 °C.



Figure S4. (a) Surface plasmon resonance of Cy7LA@GNSp@PEG nanotags before and after antibody conjugation. (b) Surface plasmon resonance of Cy7LA@GNSt@PEG nanotags before and after antibody conjugation. Grey color represents before anti-EGFR conjugation and black color represents after anti-EGFR conjugation.

Transmission Electron Microscopy

Gold nanoparticles were visualized using 200 keV TEM (JEOL 2010, Japan). Ten microliter droplets of the sample were drop casted onto a piece of ultrathin Formvar-coated 300-mesh copper grid (Electron Microscopy Sciences, Inc.) and left to dry in air. TEM images of the prepared colloidal gold nanoparticles were used for the size distribution measurements. For each sample, the size of at least 100 particles was measured and the average size and the standard distribution were obtained. In all cases, mean size and standard distribution measured by TEM were used for the calculations.



Figure S5. Transmission Electron Microscopy (TEM) images of the gold nanoparticles after pegylation(a. sphere, b. NR 800, c. nanostar and d, e, f is their corresponding high resolution TEM images), as the molecular weight of PEG is less we could not find the coating, but we get the SERS signal after antibody conjugation also. We have tested only nanorod -800 for further biological conjugation, for that we present only NR-800 after pegylation.

OSCC cell culture

OSCC cells were grown in DMEM medium supplemented with 10% (v/v) fetal bovine serum (FBS) and antibiotics (100 U mL⁻¹ antibiotic /100 mg mL⁻¹ antimycotic) in a humidified atmosphere at 37 °C with 5% (v/v) CO₂. Approximately 5000 cells per well were seeded on 8-well glass slide chamber the day before SERS measurements.

SERS mapping

OSCC, cells were grown as mentioned above in 8-well glass slide chambers. For cell mapping experiments, OSCC cells were plated in each well 24 h before the SERS measurements. Antibody-conjugated Cy7LA@GNSp@PEG-anti-EGFR, Cy7LA@GNSt@PEG-anti-EGFR and Cy7LA@GNR@PAA-anti-EGFR (final concentration 10 pM) were incubated with OSCC cells for 3 h at 33 °C. Afterwards, the cells were washed with cold PBS (×3), gently scrapped and re-suspended in PBS (every well of a 8 well-glass slide chamber. Raman experiments were performed in a Renishaw InVia Raman microscope with a laser beam directed to the sample through 20× objective lens and a Peltier cooled CCD detector. Samples were excited with a 785 nm excitation wavelength laser, and Raman mapping experiments were performed at maximum peak at 503 cm⁻¹ or 554 cm⁻¹ for nanorods. These measurements were carried out as raster scans in 2 mm steps over OSCC and SKBR-3 cells with a computer-controlled *xy*-stage. Acquisition time for all spectra was 10 s.



Figure S6. A negative cell SERS mapping experiment was performed in OSCC cells. Cy7LA@GNSp@anti-HER2 and Cy7LA@GNR-800@anti-HER2 nanotags were incubated in OSCC cells for 2 h and washed perfectly to remove unbounded proteins and scanned at 503 cm⁻¹. There is very weak signal which is due to nanoparticle aggregation or some artefact.

Cytotoxicity Measurement:

To study the cytotoxicity, we dispensed 100 μ l of cell suspension (~5000 cells/ well) in a 96well plate. The cells were pre-incubated for 24 h in high glucose media (DMEM) with 9 % fetal bovine serum (FBS) and 1% Anti-Anti with in a humidified incubator (37 °C, 5% CO₂). Next, 10 μ l of various concentrations of dyes and nano-conjugates (GNSp@Cy7.5LA@PEG; GNSp@Cy-7.5LA@PEG; Cy-7LA; Cy-7LA; GNSp@Cy7LA@PEG; GNSp@Cy-7LA@PEG; GNSt@Cy7LA@PEG; GNSt@Cy-7LA@PEG; Cy-7.5LA; Cy-7.5LA; GNR@Cy-7LA@PAA ; GNR@Cy-7LA@PAA were added into the culture media. This 96 well plate was incubated for 3 h before addition of 10 μ L of CCK-8 solution in each well. After 4 h incubation, absorption values were recorded at 450 nm wavelength. The cytotoxicity results were evaluated from these measured values.