Surface-enhanced Raman spectroscopy (SERS) nanoprobes for ratiometric detection of cancer cells†

Linhu Li, Mengling Liao, Yingfan Chen, Beibei Shan and Ming Li *

We report a ratiometric strategy for detection of different types of breast cancer cells by surface-enhanced Raman spectroscopy (SERS), which simultaneously quantifies the levels of dual biomarkers distinctly expressed on cancer cells to consequently achieve their expression ratio. Two SERS nanoprobes that are encoded with distinct SERS signatures are conjugated with urokinase plasminogen activation receptor (uPAR)- and epidermal growth factor receptor (EGFR)-targeting peptides. The SERS imaging of single live cells can accurately quantify the cellular biomarker expression difference from the SERS intensity ratio and is further employed for cancer cell screening. The results show that MDA-MB-231 and MCF-7 exhibit distinct expression of the uPAR and EGFR and they can be respectively discriminated by the intensity ratio of SERS signals from uPAR- and EGFR-targeting SERS nanoprobes. The ratiometric strategy permits background-free SERS detection of cancer cells and dramatically improves the signal-to-noise ratio of targeted cellular SERS imaging, thus enabling accurate cancer cell screening without the need for additional references. It is believed that the present ratiometric method should be a promising avenue for breast cancer diagnostics and screening, which can be easily extended for detection of other cancer cell types.

1. Introduction

Current efforts in several cancer types have shown that distinct subtypes of cancer cells have significant implications of cancer progression, prognostics and therapeutic efficacy. Rapid identification and analysis of cancer cells in tissues or body fluids can transform our understanding of cancer biology, and permit a potential route for cancer diagnostics and real-time monitoring of therapeutic efficacy, eventually benefiting the clinical management of cancer. However, the clinical method for detection of cancer cells as cancer biomarkers is yet to be established partly due to the biological complexity of cancer. Considerable studies have shown that each cancer cell line has unique cellular biomarker (i.e., genomic and proteomic) expression, distinguishing them from each other. The utility of the exclusive features of these biomarkers is a quite effective route to identification of cancer cells. However, accurate identification of cancer cells requires development of methods that enable sensitive and selective cancer cell detection through precise molecular recognition of their expressed biomarkers. Endogenous proteomic biomarkers have been extensively exploited for distinguishing and screening various cancer cells. Conventional approaches for cancer cell detection include reverse transcription-polymerase chain reaction (RT-PCR), optical methods, negative selection, cell-size filtration, CellSearch™ and microfluidic techniques. Fluorescence-assisted cell sorting (FACS) is the most commonly used method for separating and identifying cancer cells. Cancer cells can be made visible using fluorescent labels such as organic dye molecules and tiny quantum dots. The most advanced flow cytometers using fluorescent labels can accommodate a flow rate up to 50 000 cells per second. Despite the high sensitivity and spatial resolution, fluorescence-based approaches usually suffer from drawbacks in terms of limited multiplexing, photobleaching, phototoxicity, and autofluorescence interference from a biological background. In addition, detection of only a single biomarker per assay is usually performed, limiting the accuracy of cancer cell identification.

Herein, we introduce a ratiometric approach to discriminate between cancer cells using surface-enhanced Raman spectroscopy (SERS) through targeting extracellular biomarkers, characteristic of cancer cells. SERS has recently become the most sensitive technique among optical sensing/imaging modalities as the signal intensity of molecular vibration is enhanced by $10^8$–$10^{14}$ fold.
compared to spontaneous Raman signals.\textsuperscript{22–28} The SERS enhancement is mainly due to the strong localized surface plasmon resonance (LSPR) whenever Raman molecules come in close proximity to the surface of metal nanostructures.\textsuperscript{22,29} Benefits of SERS nanoprobes over existing methods include the great spectral multiplexing capability for simultaneous detection of multiple targets owing to narrow vibrational Raman bands, quantification capacity with the help of molecular signatures, the requirement of only a single laser source having a single excitation wavelength, and high photostability.\textsuperscript{30–33} SERS has been demonstrated to be valuable for live-cell imaging based on the specific targeting of endogenous biomarkers.\textsuperscript{34,35} Furthermore, we have previously developed a novel class of SERS nanoprobes where Raman-active molecules were incorporated between a near-infrared (NIR) plasmonic gold nanostar (GNS) and a thin silica protective layer.\textsuperscript{33,36–38} This SERS nanoprobe has multiple merits for chemical analysis and biological imaging, including (i) high SERS signals due to encapsulation of a large number of Raman molecules, (ii) flexible encoding capacity with desirable SERS signatures, (iii) powerful multiplexing capability, (iv) easy modification with biological functionalities, (v) high stability in robust biological media, etc.\textsuperscript{31,36,39,40} Our previous studies have demonstrated the drastically high, both in vitro and in vivo detection sensitivity of SERS nanoprobes compared to conventional fluorophores (i.e., organic dyes and quantum dots).

To exemplify the ratiometric detection of cancer cells of different types, two breast cancer cell lines, MDA-MB-231 and MCF-7, were chosen. MDA-MB-231 is a highly aggressive breast cancer cell line belonging to the Basal-like subtype with a significantly over-expressing urokinase plasminogen activation receptor (uPAR), and MCF-7 is a breast cancer cell line of the Luminal A subtype with low uPAR expression.\textsuperscript{41,42} In addition, both MDA-MB-231 and MCF-7 cell lines possess similar expression levels of the epidermal growth factor receptor (EGFR).\textsuperscript{42} Thus, we are in principle able to distinguish MDA-MB-231 cells from MCF-7 cells by quantifying the uPAR : EGFR ratio. Based on the synthetic methodology for SERS nanoprobes developed in our previous work,\textsuperscript{31,36,46} To create SERS nanoprobes encoded with NTP or DATT, a freshly prepared reaction solution was adjusted to around 9–10 by addition of 5 mM freshly prepared APTMS in ethanol. After 30 min, and the reaction continued for one day with magnetic stirring. 5 mL anhydrous ethanol was subsequently added, and the reaction solution was allowed to stand for one more day to generate a condensed silica layer. The reaction solution was then centrifuged and washed with anhydrous ethanol and deionized water, respectively. The pellets were SERS nanoprobes encoded with NTP or DATT, and re-dispersed in 1 × PBS for further use.

2. Experimental section

2.1 Chemicals and materials

Chloroauric acid (HAuCl₄·xH₂O, 99.999% trace metals basis), trisodium citrate dihydrate (HOC(COONa)(CH₂COONa)₂·2H₂O, ≥ 99%), poly(vinylpyrrolidone) (PVP, (C₉H₈N₄O₅)n, molecular weight = 10 kg mol⁻¹), sodium borohydride (NaBH₄, ≥ 99%), N,N-dimethylformamide (DMF, anhydrous 99.8%), sodium hydride (pellets, 99.99% trace metals basis), (3-aminopropyl)-trimethoxysilane (APTMOS, 97%), sodium silicate (Na₄O(SiO₂)₃·xH₂O, reagent grade), 4-nitrothiophenol (NTP, technical grade 80%), N-hydroxysuccinimide (NHS, 98%), N-ethyl-N’-(3-dimethylaminopropyl)carbodiimide (EDC, ≥ 97.0%), RPMI 1640, fetal bovine serum (FBS), penicillin–streptomycin, and WST-1 reagent were purchased from Sigma-Aldrich (St. Louis, MO). Diamino-1,3,5-triazine-2-thiol (DATT, 95%) was purchased from Enamine LLC (Cincinnati, OH). MDA-MB-231 and MCF-7 breast cancer cell lines were purchased from the American Type Culture Collection (ATCC) (Manassas, VA). Methoxy-poly(ethylene glycol)-silane (mPEG-silane, molecular weight = 2 kg mol⁻¹) was obtained from Laysan Bio Inc. (Arab, AL). Phosphate buffered saline (1 × PBS, pH 7.4) solution was purchased from Quality Biology (Gaithersburg, MD). Pyrex\textsuperscript{48} Petri dish was purchased from Corning Incorporated (Corning, NY), and quartz coverslips were purchased from Alfa Aesar (Ward Hill, MA). (3-Triethoxysilyl)propylsuccinic anhydride (TEPSA, C₁₃H₂₅O₆Si, > 95%) was purchased from Gelest Inc. (Morrisville, PA). Both uPAR-targeting peptide (YSNKYSNIHWWG) and EGFR-targeting peptide (VRPMPLQ) with C-terminal amidation were obtained from GenScript USA Inc. (Piscataway, NJ). All other reagents or solvents used in this study were of analytical grade and used without further purification.

2.2 Preparation of SERS nanoprobes

Gold nanostars (GNSs) were synthesized by a seed-mediated method detailed in our previous work.\textsuperscript{31,36,46} To create SERS nanoprobes encoded with NTP or DATT, a freshly prepared solution of Raman-active molecules (NTP or DATT, 10 μM) was added to the GNS colloids and kept stirring for 30 min followed by addition of 5 mM freshly prepared APTMS in ethanol. After another 30 min of continuous stirring, the pH value of the reaction solution was adjusted to around 9–10 by addition of NaOH aqueous solution. Then, 200 μL of freshly prepared 0.54 wt% sodium silicate solution was added dropwise within 30 min, and the reaction continued for one day with magnetic stirring. 5 mL anhydrous ethanol was subsequently added, and the reaction solution was allowed to stand for one more day to generate a condensated silica layer. The reaction solution was then centrifuged and washed with anhydrous ethanol and deionized water, respectively. The pellets were SERS nanoprobes encoded with NTP or DATT, and re-dispersed in 1 × PBS for further use.

2.3 Functionalization of SERS nanoprobes with peptides

uPAR or EGFR targeting-SERS nanoprobes were prepared using an established method with a slight modification.\textsuperscript{47,48} First, SERS nanoprobes were co-modified with mPEG-silane and TEPSA to improve the biocompatibility, and reduce aggregation and nonspecific binding of proteins when SERS nanoprobes were used in biological settings.\textsuperscript{48–50} Typically, a mixture of 5.65 μM mPEG-silane and 11.3 μM TEPSA was added to an ethanolic solution of 50 pM SERS nanoprobes. After the reaction
continued for 12 h with magnetic stirring, the solution was successively centrifuged and washed with ethanol and deionized water, respectively. The resulting solids were carboxyl-terminated, PEGylated SERS nanoprobes and diluted to a concentration of 50 pM with 1× PBS.

After successful modification with PEG and TEPSA, the modified SERS nanoprobes were functionalized with uPAR- and EGFR-targeting peptides by the EDC/NHS coupling chemistry. Both peptides were customized with C-terminal amidation. To prepare uPAR- or EGFR-SERS nanoprobes, the carboxyl-terminated, PEGylated SERS nanoprobes were first activated by NHS/EDC. Briefly, the co-modified SERS nanoprobes were diluted to 5 pM with 1× PBS followed by addition of an aqueous solution of 25 mM NHS and 100 mM EDC. After kept stirring for 30 min, uPAR- or EGFR-targeting peptides were added to reach a final concentration of 6 mM, and the solution was allowed to incubate for 20 h under magnetic stirring at room temperature. After washing at least 4 times with 1× PBS, the SERS nanoprobes were cleaned via successive centrifugation and washing with 1× PBS. Finally, the uPAR- or EGFR-SERS nanoprobes were re-dispersed in 1× PBS with a concentration of 50 pM.

2.4 Cell culture

Human breast cancer cell lines, MDA-MB-231 and MCF-7, were acquired from ATCC and incubated in RPMI 1640 supplemented with 10% v/v FBS and 1% penicillin–streptomycin in a humidified incubator at 37 °C/5% CO₂.

For the SERS study, the cells (1 × 10⁶ cells per mL) were grown on a 60 mm Petri dish with complete culture medium at 37 °C for 24 h. The culture medium contains uPAR- and (or) EGFR-targeting SERS nanoprobes, and the cells were incubated for 1 h followed by replacement of culture medium with freshly prepared RPMI 1640 culture medium and allowed to stand for 12 h. After centrifugation, the pellets were added to a quartz-bottomed Petri dish containing new RPMI 1640 culture medium supplemented with 10% v/v FBS and 1% penicillin–streptomycin, and then SERS measurements were subsequently performed.

2.5 Cell viability measurement

The cellular cytotoxicity of the SERS nanoprobes was evaluated by a WST-1 assay. MDA-MB-231 cells or MCF-7 cells were seeded into a 96-well plate at a density of 1 × 10⁴ cells per well and incubated for 24 h in RPMI 1640 supplemented with 10% PBS and 1% penicillin–streptomycin, followed by addition of 50 pM uPAR- or EGFR-SERS nanoprobes. After incubation for 4 days, the cells were subject to the WST-1 assay.

2.6 Characterization

Transmission electron microscopy (TEM) images were collected using a FEI Tecnai G² Spirit TWIN transmission electron microscope at an accelerating voltage of 120 kV. The sample was added dropwise onto ultrathin Formvar-coated 200 mesh copper grids (Ted Pella, Inc.) and allowed to dry in air. Extinction spectra were recorded on an Aviv Model 14DS UV-vis spectrophotometer (Aviv Biomedical, Lakewood, NJ).

2.7 SERS measurements

All SERS measurements were performed using a home-built, inverted high-speed confocal Raman microscope in our laboratory. A compact LM series solid laser of 785 nm emission wavelength (Ondax) mounted in front of a filter (LL01-785-12.5, Semrock) was used as the excitation source. High-speed XY scanning was controlled using galvanometer mirrors (GVS112, Thorlabs). A 0.65–1.25 NA, 60× oil immersion objective lens (RMS60X-PFOD, Olympus) was used to focus the laser beam and collect the Raman-scattering photons from the sample. The backscattered photons were collected using a 50 μm multimode fiber (M14L01, Thorlabs), delivered to a HoloSpec f/1.8 spectrograph (Kaiser Optical Systems, Andor) and the dispersed light was finally detected using an iDus CCD camera (DU420A-BEX2-DD, Andor). LabView 2013 (National Instruments) and MATLAB 2013 (Mathworks) were used to control the system, acquire the data, and analyze the data. Raman and SERS spectra were recorded using a laser power of 5 mW and an integration time of 1 s.

2.8 Statistical analysis

Statistical analyses of the experimental values were conveyed as mean ± standard errors of at least three independent experiments. Statistical significance was determined using the two-tailed t-test. A difference is considered significant at p < 0.05.

3. Results and discussion

3.1 SERS nanoprobes and their functionalization with peptides

SERS nanoprobes were prepared using highly SERS-active GNSs with a near-infrared (NIR) LSPR maximum of 738 nm, and show optimal SERS enhancement under the NIR 785 nm laser excitation, as demonstrated in our previous work. The overall size (core plus protruding tips) of GNSs is 44.4 ± 5.8 nm (Fig. S1, ESI†). NTP and DATT were respectively used as Raman molecules to encode SERS nanoprobes of unique spectral-molecular signatures because of their non-overlapping Raman signatures and well-understood Au–S chemistry (Fig. 1 and Fig. S2, ESI†). In SERS nanoprobes, Raman-active (NTP or DATT) molecules were sandwiched between the GNS core and the silica outer layer (Fig. S3, ESI†). To incorporate the targeting functionality into SERS nanoprobes and improve the biocompatibility, SERS nanoprobes were first co-modified with TEPSA and mPEG-silane at a molar ratio of 2:1. PEGylation of the SERS nanoprobes can improve the biocompatibility, minimize the cytotoxicity in biological environments and inhibit nonspecific protein adsorption to their surfaces (Fig. 1b). It has been shown that both the pH value and NaCl concentration have negligible effects on the SERS intensity, indicating excellent stability against various robust environments (Fig. S4, ESI†).

It has been reported that uPAR is significantly over-expressed in MDA-MB-231, which promotes an aggressive phenotype in breast cancer. Thus, uPAR is of biological significance as a molecular target for breast cancer because of its accessibility on the surface of cancer cells. In contrast, MCF-7 has a low
expression level of uPAR while both MDA-MB-231 and MCF-7 have similar expression to the EGFR. To target uPAR and EGFR biomarkers, the uPAR-targeting peptide (VSNKYFSNIHWGC) and the EGFR-targeting peptide (VRPMPLQ) were exploited for targeting molecules and conjugated onto the SERS nanoprobes through the well-established EDC/NHS coupling chemistry (Fig. 1a). These results in the uPAR- and EGFR-targeting SERS nanoprobes characteristic of SERS signatures of NTP and DATT, respectively (Fig. 1b). The molecular structures of NTP and DATT as well as the schematic geometry of the SERS nanoprobes are also shown (left). (c) Extinction spectra of gold nanostars and uPAR-/EGFR-targeting SERS nanoprobes. (d) Representative TEM image of DATT-encoded SERS nanoprobes.

3.2 Ratiometric SERS response of mixed SERS nanoprobes

The SERS spectra of the mixed SERS nanoprobes of various molar ratios were collected at a constant total (uPAR-plus EGFR-SERS nanoprobes) concentration (Fig. 2a). It can be clearly seen that the SERS intensity of the uPAR-SERS nanoprobes (i.e., 1340 cm⁻¹) increases and the SERS intensity (i.e., 633 cm⁻¹ and 913 cm⁻¹) of the EGFR-SERS nanoprobes decreases with the increasing uPAR-nanoprobe : EGFR-nanoprobe ratio. We plot the intensity ratio \( I_{1340} / I_{913} \) as a function of the concentration percentage of SERS nanoprobes, and the calibration curve is determined to be \( y = 3.00(±0.29)x - 0.056(±0.16) \) through linear fitting \( (R^2 = 0.96) \), where \( y \) is the SERS intensity ratio \( I_{1340} / I_{913} \), and \( x \) is the uPAR concentration percentage (Fig. 2b).

3.3 Targeted cellular SERS imaging of live cells

We first performed the in vitro SERS imaging of live cells using uPAR- or EGFR-SERS nanoprobes to examine their targeting ability. The cells investigated are breast cancer cell lines, MDA-MB-231 and MCF-7, with distinct expression of the uPAR and EGFR. Rapid, accurate identification of the two cell lines is able to uncover the breast cancer progression and cancer biology. It has been demonstrated that there is no observable cytotoxicity toward both cancer cell lines in the presence of SERS probes even when exceeding the concentration used here (Fig. S6, ESI†). Since the SERS intensity ratio only relies on the biomarker expression ratio, a total SERS probe concentration of 50 pM was used to avoid the complete binding of biomarkers as well as
mitigate the cytotoxicity. Fig. 3a illustrates the detection principle of both SERS nanoprobes for the targeted cellular imaging for simultaneous detection of the uPAR and EGFR. When the cells are incubated with a mixture of uPAR- and EGFR-SERS nanoprobes, the two SERS nanoprobes are respectively bound to the uPAR and EGFR expressed on the cell surface through a specific peptide–biomarker interaction, similar to the antibody–antigen interaction. Ideally, the SERS intensity ratio from the SERS nanoprobes rationally reflects the relative amount of uPAR and EGFR biomarkers on live cells (Fig. 4). As expected, the uPAR-SERS nanoprobes bind to the uPAR expressed on the cell surface while the EGFR-SERS nanoprobes to the EGFR through their respective targeting peptides. There are more uPAR-SERS nanoprobes appearing around the MDA-MB-231 cells than EGFR-SERS nanoprobes. On the other hand, we can see a few EGFR-SERS nanoprobes but negligible uPAR-SERS nanoprobes appearing around MCF-7 cells. MDA-MB-231 expresses more uPAR receptors than MCF-7 while they have a similar EGFR expression, so that both cell lines have significantly different uPAR:EGFR ratios. The percentage of individual SERS nanoprobes around the cells can be derived from the SERS intensity ratio over the whole cell based on the calibration curve (Fig. 2b).

It is reasoned that the amount of SERS nanoprobes targeted to the cells is proportional to the expression levels of the uPAR and EGFR. The statistical results obtained from 19 cells show that the \( \frac{I_{uPAR}}{I_{EGFR}} \) ratio of the MDA-MB-231 cells is a much higher middle value around 1.8 while it is around 0.48 for MCF-7 cells (Fig. 4b). Thus, we are able to quantitatively discriminate between MDA-MB-231 and MCF-7 by quantifying the uPAR:EGFR expression ratio using the encoded SERS nanoprobes which target to the uPAR and EGFR biomarkers on the cell surface.

Identification of different types of breast cancer cells has prognostic and therapeutic implications for patients with breast cancer. Numerous efforts have been made to distinguish between different types of cancer cells or between cancer cells and normal cells based on cell surface biomarker expression. The present SERS approach exploits the difference in expression levels of the uPAR and EGFR to ratiometrically identify MDA-MB-231 and MCF-7 cells. The uPAR/EGFR expression ratio is derived from the SERS intensity ratio of uPAR- to EGFR-targeting nanoprobes. The ratiometric strategy excludes the background interference and improves the detection accuracy of cancer cells with high sensitivity and excellent specificity. Unlike the conventional fluorescence method, SERS has no photobleaching, less autofluorescence interference, and requires only a single laser wavelength for multiplexing. The unique features associated with SERS make it ideal as a screening tool for live cancer cells.

3.4 Ratiometric detection of live cells

Taking great advantages of high sensitivity and powerful multiplexing capability using a single laser source, we employed SERS to perform simultaneous imaging detection of uPAR and EGFR biomarkers on live cells (Fig. 4). As expected, the uPAR-SERS nanoprobes bind to the uPAR expressed on the cell surface while the EGFR-SERS nanoprobes to the EGFR through their respective targeting peptides. There are more uPAR-SERS nanoprobes appearing around the MDA-MB-231 cells than EGFR-SERS nanoprobes. On the other hand, we can see a few EGFR-SERS nanoprobes but negligible uPAR-SERS nanoprobes appearing around MCF-7 cells. MDA-MB-231 expresses more uPAR receptors than MCF-7 while they have a similar EGFR expression, so that both cell lines have significantly different uPAR:EGFR ratios. The percentage of individual SERS nanoprobes around the cells can be derived from the SERS intensity ratio over the whole cell based on the calibration curve (Fig. 2b).

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Our SERS approach utilizes the intrinsic cell-resident marker, EGFR, as the internal reference, which would be particularly advantageous for accurate identification of cancer cells because expression of a biomarker may occur in both cancer cells and non-cancer cells to a different degree. The ratiometric strategy exploits the ratio of biomarker expression levels to reduce the interference from other cells. In addition, the ratiometric strategy developed in this work permits identification of cancer cells, independent of the variations in cell concentration, measurement conditions and nanoprobe concentration, and is superior to conventional approaches for cancer cell detection based on the evaluation of the absolute expression level of biomarkers. Few studies have focused on the development of ratiometric SERS biosensors for detection of biomarkers of pathological significance, but our current work further moves forward the ratiometric SERS strategy for cell discrimination, taking advantages of plasmonic GNSs with highly SERS activity, unique design of SERS nanoprobes and intrinsic extracellular biomarker ratios.

4. Conclusions

In summary, we have reported a SERS-based strategy for quantitative ratiometric discrimination of two different types of breast cancer cell lines, MDA-MB-231 and MCF-7. SERS nanoprobes are encoded with unique SERS signatures and conjugated with uPAR- or EGFR-targeting peptides, which target cell-surface proteins, uPAR and EGFR, distinctly expressed on MDA-MB-231 and MCF-7. We demonstrated that simultaneous quantitative detection of both protein biomarkers on single live cells enables ratiometric discrimination between MDA-MB-231 and MCF-7. MDA-MB-231
showed significant aggregation of uPAR-SERS nanoprobes but the aggregation is low for MCF-7 cells. Both cell lines have similar and relatively weak SERS signals when EGFR-SERS nanoprobes were used. The two cell lines show an uPAR: EGFR expression ratio of ca. 1.8 and 0.48 for MDA-MB-231 and MCF-7 cells, respectively. In ratiometric detection, the EGFR biomarker is used as the internal reference, and the SERS intensity ratio only depends on the biomarker expression, regardless of the cell concentration, measurement conditions and nanoprobe concentrations. The present method excludes the background interference from normal cells or other cancer cells that may have biomarker expressions as well. Taking into account the merits of high sensitivity and powerful multiplexing capability of SERS, and accuracy due to the ratiometric discrimination without any external reference, the ratiometric SERS method holds great potential for cancer cell screening and diagnostic applications of cancer along with the development of cellular biology for biomarker expression profiling.

Conflict of interest

There are no conflicts to declare.

Acknowledgements

M. L. acknowledges financial support from the National Thousand Young Talents Program of China, the National Natural Science Foundation of China (No. 2017X2146), the Innovation-Driven Project of Central South University (No. 2018CX002) and the Hunan Provincial Science & Technology Program (No. 2017XX2027).

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Supporting Information

Surface-enhanced Raman Spectroscopy (SERS) Nanoprobes for Ratiometric Detection of Cancer Cells

Linhu Li, Mengling Liao, Yingfan Chen, Beibei Shan and Ming Li*
Figure S1. TEM image of gold nanostars used for preparation of SERS nanoprobes and size distribution statistically obtained from TEM image of GNSs using the ImageJ software. More than 150 GNSs were counted. The size of GNSs is called the overall size including the core and protruding tips, the model of which is schematically illustrated above. It can be seen that the overall size of GNSs is about 41.4±5.8 nm.
Figure S2. Spontaneous Raman spectra of NTP and DATT powder. The spectra were collected under the excitation of 785 nm with 5 mW power and 1 s integration time.
Figure S3. Schematic illustration of NTP or DATT encoded SERS tags. (a) Molecular structures of Raman reporters, 4-nitrothiophenol (NTP) and diamino-1,3,5-triazine-2-thiol (DATT), (b) Au-S interaction to form NTP (or DATT)-GNS complexes, and (c) synthesis progress of NTP or DATT encoded SERS tags. Raman reporters (NTP or DATT) are chemically bound onto the Au surface through the strong Au-S bond and then a SiO$_2$ layer are coated to encapsulate the encoded SERS tags, forming the sandwich structure, Au@Ramam-reporters@SiO$_2$. The SiO$_2$ layer possesses excellent biocompatible and can be used for flexible bioconjugation with biomolecules such as proteins, peptides and nucleic acids.
Figure S4. Stability of SERS nanoprobes without peptides conjugation. SERS spectra of SERS nanoprobes in the solution of (A) pH=2.5 and pH=11.6, (B) 1×PBS solution with 1.0 mM and 1.0 M NaCl. All results show that the present SERS nanoprobes have excellent stability against robust environments (e.g., pH, NaCl concentration).
Figure S5. TEM image of (A) uPAR-SERS nanoprobes and (B) EGFR SERS nanoprobes. It can be seen that 3-5 nm silica layer was coated onto the GNS surface.
Table S1. Peak assignments of normal Raman bands and SERS bands of NTP, DATT, NTP-encoded SERS nanoprobes and DATT-encoded SERS nanoprobes $^{1-4}$

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Figure S6. Cell viability of MDA-MB-231 cells and MCF-7 cells incubated with uPAR or EGFR-SERS nanoprobes. MDA-MB-231 cells were incubated with uPAR-SERS nanoprobes (200 pM), and MCF-7 cells were incubated with EGFR-SERS nanoprobes (200 pM). It can be clearly seen that no significant cytotoxicity was observed for both SERS nanoprobes used in this work.
Figure S7. SERS images overlaid with bright-field images of uPAR-SERS nanoprobe-pretreated MDA-MB-231 cells and EGFR-SERS nanoprobe-pretreated MCF-7 cells incubated with unconjugated NTP-encoded SERS nanoprobes (50 pM). It is clearly seen that there is no significant SERS signal observed in both cell lines, indicating high specificity of both SERS nanoprobes toward to uPAR and EGFR expressed on the cell surface.