Electronic Supplementary Information

A Chemically Reactive Raman Probe for Ultrasensitively Monitoring and Imaging the *in vivo* Generation of Femtomolar Oxidative Species as Induced by Anti-Tumor Drugs in Living Cells

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EXPERIMENTAL SECTION

Chemicals and materials. All chemicals were of analytical degrade and used without further purification. Dihydrorhodamine 123 (DHR-123), O-(2-mercaptoethyl)-O'-(2-carboxyethy)heptaethylene glycol (PEG thiol acid), N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodimide hydrochloride (EDC), 1,7-bis[4-hy-droxy-3-methoxyphenyl]-1,6-heptadiene-3,5-dione (curcumin) and 3,4,5-triphydroxyl-benzoic acid (gallic acid) were obtained from Sigma-Aldrich. Silver nitrate ($AgNO_3$), sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$), sodium hydroxide (NaOH), hydrogen peroxide (H_2O_2) (30%, aqueous solution), sodium hypochlorite (NaClO, 5% aqueous solution), dimethyl sulfoxide (DMSO, 99.9%), $CoCl_2 \cdot 6H_2O$, $CuCl_2 \cdot 2H_2O$, $FeCl_3 \cdot 6H_2O$, $ZnSO_4 \cdot 7H_2O$, threonine, cysteine, glycine, and glucose were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Ultrapure water (18.2MΩ cm) was produced using Millipore water purification system.

Preparation of Ag nanoparticles. Ag nanoparticles (AgNPs) were prepared by the reduction of $AgNO_3$ with $Na_3C_6H_5O_7 \cdot 2H_2O$ in water. Typically, 100 mg of $AgNO_3$ was dissolved in 250 mL of ultrapure water and brought to boiling. 10 mL of 1% $Na_3C_6H_5O_7 \cdot 2H_2O$ was rapidly injected into the above boiling solution. The mixture was kept on boiling for 1 h. The resulting yellow silver colloids were further stirred for 30 min. After cooled to room temperature, the AgNPs colloid was obtained and stored at 4 ^oC for

following experiments. The size of AgNPs is about 30 nm, and the concentration of the AgNPs is 0.60 nM which is calculated using Beer's law and the extinction coefficients (ε) (30-nm AgNPs are 2.3×10^{10} $M^{-1}cm^{-1}$).[1]

Synthesis of AgNPs-PEG-(DHR-123) probes. O-(2-mercaptoethyl)-O'-(2-carboxyethy)heptaethylene glycol (PEG thiol acid, HS-PEG-COOH) was first chemically attached to AgNPs. Briefly, 10 μL of 10 μM PEG thiol acid was added into 1.0 mL of AgNPs colloid solution in a polypropylene tube with rapid mixing for 3 h, and the free PEG was removed by centrifugation at 4000 rpm for 15 min and washing with water three times, in which AgNPs-S-PEG-COOH was produced by the formation of Ag-S bond. Then, the end carboxyl groups of PEG chain at the modified AgNPs were further activated using 1-ethyl-3-(3-dimethylaminopropyl) carbodimide hydrochloride (EDC) in aqueous solution. 5.0 μL of 25 mM EDC and 5.0 μL of 25 mM N-hydroxysuccinimide (NHS) were sequentially added into 1.0 mL of AgNPs-S-PEG-COOH solution, and allowed to react for 1 h, and the excess EDC and NHS were removed from the AgNPs solution by centrifugation**.** Finally, the active AgNPs were dispersed in PBS buffer and mixed with 10 μL of 1.0 mM dihydrorhodamine 123 (DHR-123) in DMSO. After incubation at room temperature for 3 h, the resultant AgNPs-PEG-(DHR-123) probes were separated by centrifugation at 6000 rpm and re-suspended in 1.0 mL of PBS buffer. The probe solution was stored at 4 ^{o}C refrigerator for further use.

Oxidative species and their preparations. H_2O_2 and ^{-}OCl were directly used in the aqueous solution with different concentrations. Singlet oxygen ($^1O_2$) and hydroxyl radical ($^{\cdot}OH$) were obtained by the classical chemical reactions.[2] 1 μM $^1O_2$ aqueous solution was first generated by the reaction of 1 μM H_2O_2 with 10 μM NaClO, and the resultant solution was then diluted with water to get the different concentrations of $^1O_2$. Similarly, 1 μM $^{\cdot}OH$ aqueous solution was produced through the Fenton reaction of 1 μM H_2O_2 with 10 μM Co^{2+}, and further diluted into various concentrations. All the above stock

solutions were stored at 4^{o}C refrigerator for further use.

Cell cultures. The human lung adenocarcinoma cell line (A549 cells) and the human cervix adenocarcinoma cell line (HeLa cells) were purchased from Institute of Biochemistry and Cell Biology of Chinese Academy of Sciences, and were cultured for amplification with high glucose Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum (FBS, Invitrogen), penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37 ^{o}C in a humidified incubator (MCO-18AC, Sanyo, Japan) containing 5% CO_2 /95% air. At harvesting, the media was first removed, and the cells were then washed with PBS solution (8.1 mM Na_2HPO_4, 1.2 mM KH_2PO_4, 138 mM NaCl, 2.7 mM KCl, pH 7.4) three times and finally were detached from the wall of culture dish with 0.5 mL trypsin solution (Invitrogen). To stop the trypsin reaction, 2 mL of DMEM media was added.

Raman measurements of ROS in solution. The Raman responses of AgNPs-PEG-(DHR-123) probes to various reactive oxidative species (ROS) including $^{\cdot}OH$, ^{-}OCl, $^{1}O_2$ and H_2O_2 were first performed in solution**.** Typically, 40 μL of ROS standard solutions at various concentrations were mixed with 40 μL of the probes and allowed to react at room temperature. The resulting solution was sucked into a capillary tube and the Raman spectra were recorded from randomly selected sites. Raman measurements were performed with a 532 nm laser and 50× objective (1 μm^2 spots), and the laser power and the acquisition time were1 mW and 2 s, respectively.

Raman measurements of external additions of ROS into living cells. Cells (A549 and HeLa cells) were seeded at a density of 1.0 × 10^6 cells mL^{-1} for the detection of external additions of ROS ($^{\cdot}OH$, ^{-}OCl, $^{1}O_2$ and H_2O_2). The cells were first incubated with 100 μL of AgNPs-PEG-(DHR-123) probes dispersed in cell culture media (in 6 cm cell culture dishes) for 1 h at 37 ^{o}C in a humidified incubator containing 5% CO_2 /95% air. ROS aqueous solution with different concentrations of were then added into the culture

media, and incubated at 37 ^{o}C for 30 min. Finally, the culture media was removed and the cells were washed with PBS buffer three times. Raman measurements were performed with a 532 nm laser and 50× objective (1 μm^2 spots), and the laser power and the acquisition time were1 mW and 2 s, respectively.

Raman measurements of endogenous ROS in cells by the inducements of anti-tumor drugs. The cells were first incubated with 100 μL of AgNPs-PEG-(DHR-123) probes dispersed in cell culture media for 1 h at 37 ^{o}C. The anti-tumor drugs, gallic acid and curcumin, were selected to induce the endogenous generation of ROS in living cells. Typically, gallic acid (200 mM in ethanol) and curcumin (10 mM in DMSO) were appropriately diluted with DMEM medium and were added into the culture media, respectively. After incubation with the cells at 37 ^{o}C for 30 min, the culture media was removed and the cells were washed with PBS buffer three times. Raman measurements were performed with a 532 nm laser and 50× objective (1 μm^2 spots), and the laser power and the acquisition time were1 mW and 2 s, respectively.

Characterization and Instruments. UV-vis absorption spectra were recorded with a Shimadzu UV-2550 spectrometer. The morphologies of AgNPs were examined using a JEOL 2010 transmission electron microscope. The photos of the cells were taken on a Leica DMI6000 inverted fluorescence microscope. Raman measurements were conducted with a confocal microscopy Raman spectrometer (ThermoFisher DXR Raman Microscope) equipped with CCD detector.

References

[1] Kanjanawarut, R.; Su, X. D. *Anal. Chem.* **2009**, *81*, 6122.

[2] Gao, J. J.; Xu, K. H.; Tang, B.; Yin, L. L. ; Yang, G. W. ; An, L. G. *FEBS J.* **2007**, *274*, 1725.

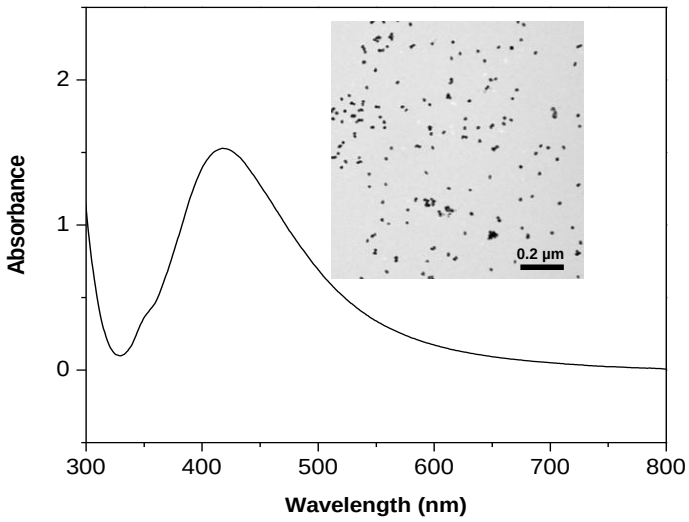

Fig. S1. UV-vis absorption spectrum of the AgNPs (the inset is the TEM image of the AgNPs with the diameter of 30 nm).

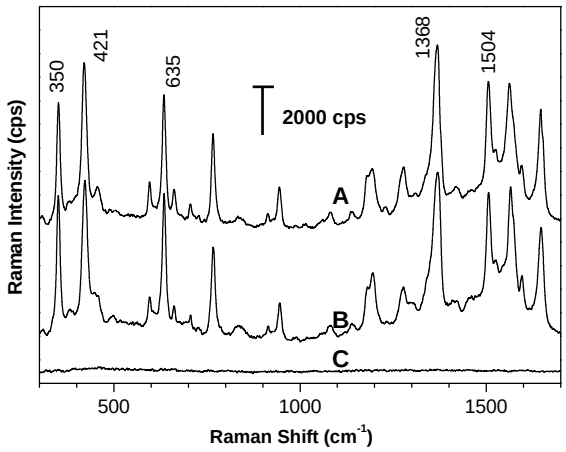

Fig. S2. (A) SERS spectra of the AgNPs-PEG-(DHR-123) probes after reacted with 1.0×10^{-6} M H_2O_2 (the strong Raman peaks at 350, 421, 635, 1368 and 1504 cm^{-1} are the same as that of pure Rd-123). (B) SERS spectra of the pure Rd-123. (C) SERS spectra of AgNPs-PEG-(DHR-123) probes.

Table S1. The SERS bands of the Rd-123 and their vibrational mode assignments

SERS bands	vibrational modes
350	xanthene ring stretching
421	xanthene ring and NH_2 oscillation
447	C-H out of plane bending of xanthene ring
597	NH_2 wagging
635	xanthene ring and phenyl ring stretching
671	C-H out of plane bending of xanthene ring
711	C-H out of plane bending of phenyl ring
768	C-H out of plane bending of xanthene ring
920	xanthene ring and phenyl ring stretching
946	xanthene ring and phenyl ring stretching
1081	phenyl ring stretching
1121	C-H bending of xanthene ring
1181	C-H bending of xanthene ring
1193	C-H bending of xanthene ring
1237	C-H bending of xanthene ring
1273	C-H bending of xanthene ring
1368	xanthene ring stretching
1504	xanthene ring stretching
1548	xanthene ring stretching
1650	xanthene ring stretching

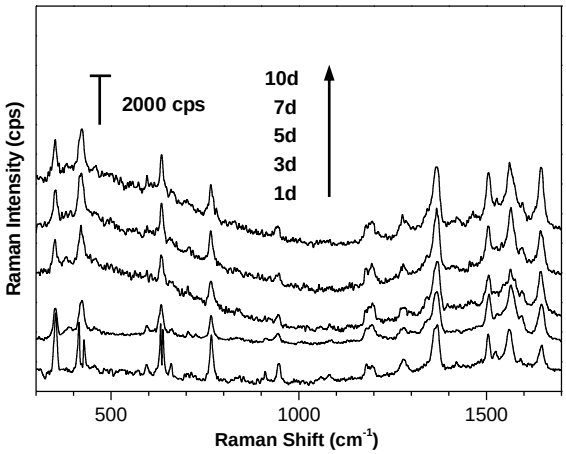

Fig. S3. The probes were kept in aqueous solution at room temperature for 1, 3, 5, 7 and 10 days and then reacted with H_2O_2 (1.0×10^{-6} M) for 30 min, respectively. It can be seen that they still effectively react with H_2O_2 and lead to the nearly identical intense Raman readout, indicating that the probes highly respond to ROS, but are stable at solution in the presence of soluble oxygen in aqueous solutions.

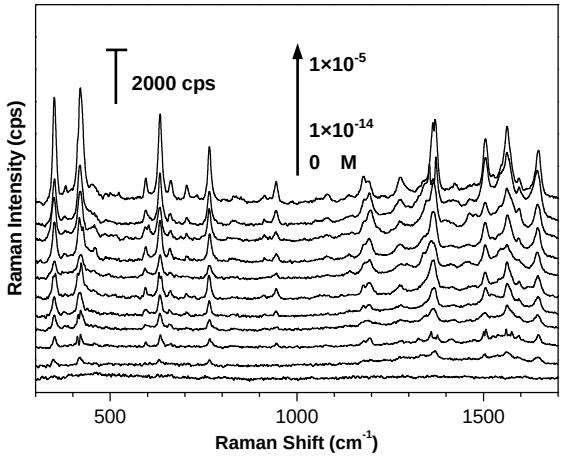

Fig. S4. SERS spectra of the probes after reacted with $^{\bullet}$OH with the increment of concentration from 1×10^{-14} to 10^{-5} M by a factor of 10 (the bottom line represents the blank).

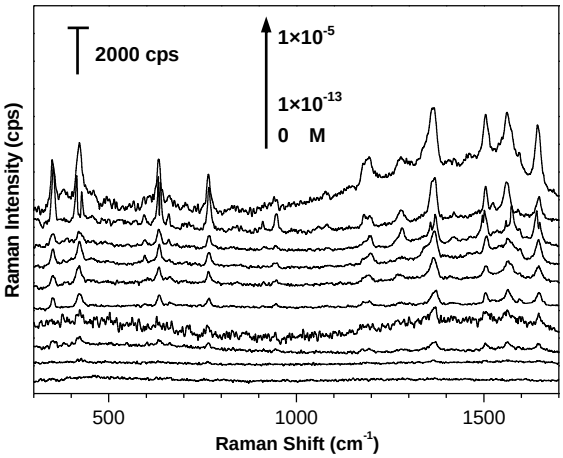

Fig. S5. SERS spectra of the probes after reacted with $^1O_2$ with the increment of concentration from 1×10^{-13} to 10^{-5} M by a factor of 10 (the bottom line represents the blank).

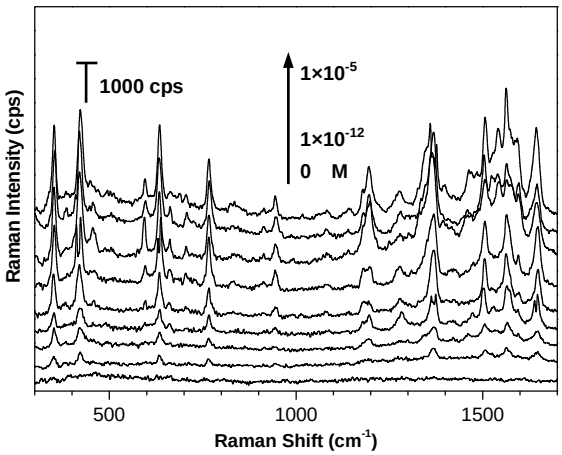

Fig. S6. SERS spectra of the probes after reacted with H_2O_2 with the increment of concentration from 1×10^{-12} to 10^{-5} M by a factor of 10 (the bottom line represents the blank).

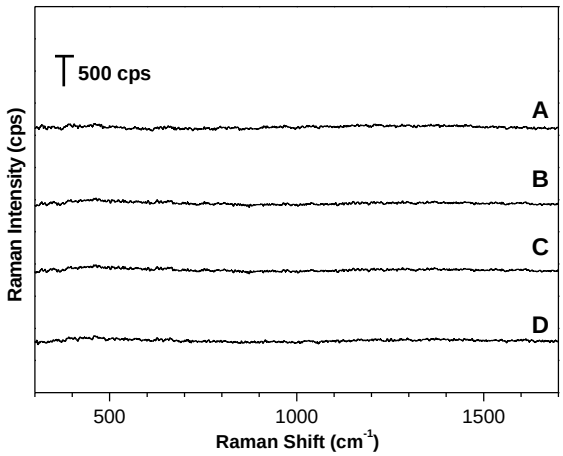

Fig. S7. SERS spectra of the probes after treated with various metal ions (1×10^{-4} M): (A) Cu^{2+}, (B) Zn^{2+}, (C) Fe^{3+} and (D) Co^{2+}. It can be seen that no Raman signal was detected, indicating that the probes can not react with those metal ions.

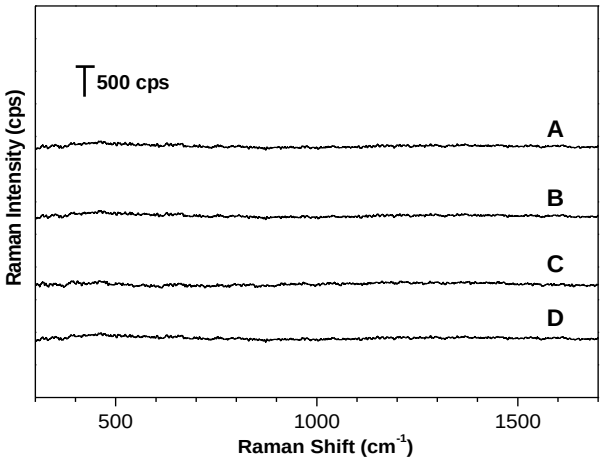

Fig. S8. SERS spectra of the probes after treated with various biomolecules (1×10^{-4} M) (A) glucose, (B) cysteine, (C) glycine and (D) threonine. It can be seen that no Raman signal was detected, indicating that the probes can not react with those biomolecules.

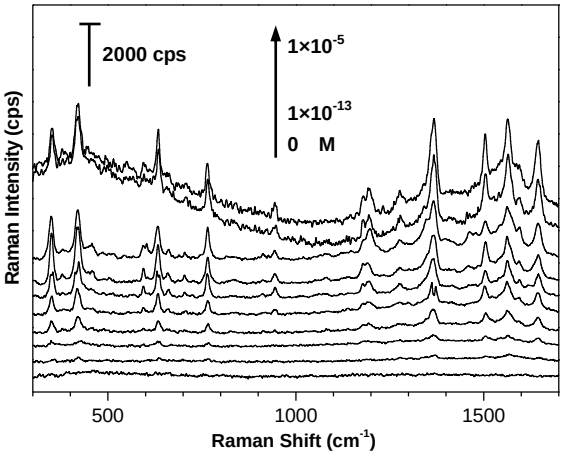

Fig. S9. SERS spectra obtained from the probe-loaded A549 cells after further incubated with H_2O_2 solution with a concentration increment from 1×10^{-13} to 10^{-5} M by a factor of 10 (the bottom line represents the blank).

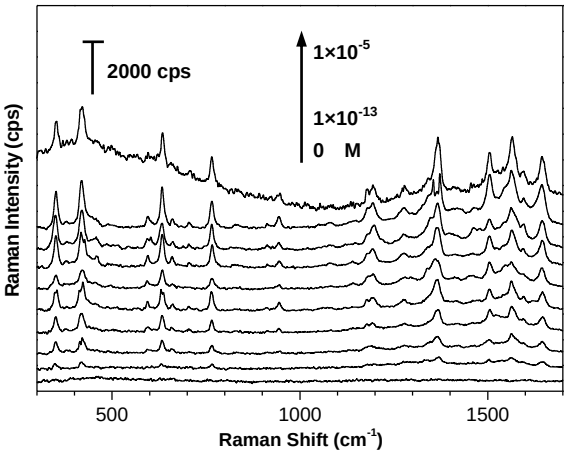

Fig. S10. SERS spectra obtained from the probe-loaded A549 cells after further incubated with $^{\bullet}OH$ solution with a concentration increment from 1×10^{-13} to 10^{-5} M by a factor of 10 (the bottom line represents the blank).

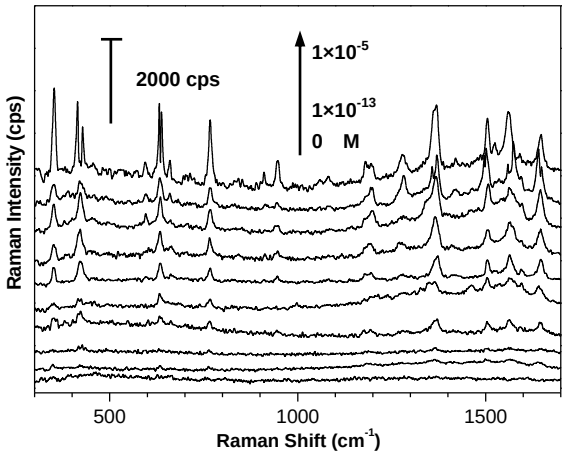

Fig. S11. SERS spectra obtained from the probe-loaded A549 cells after further incubated with $^1O_2$ solution with a concentration increment from 1×10^{-13} to 10^{-5} M by a factor of 10 (the bottom line represents the blank).

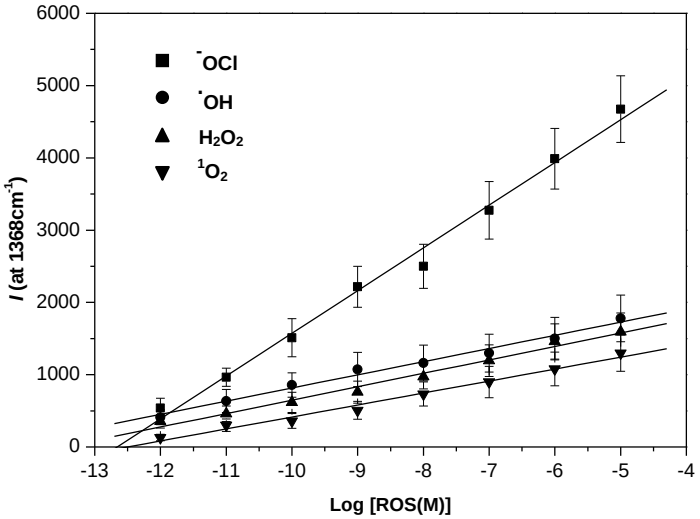

Fig. S12. The linear correlations of Raman intensity (at 1368 cm^{-1}) with the logarithm of four ROS concentrations from 1×10^{-12} to 10^{-5} M in living A549 cells. The probes have the highest sensitivity to ^-OCl because the ^-OCl is more strongly oxidative than the other three ROS.

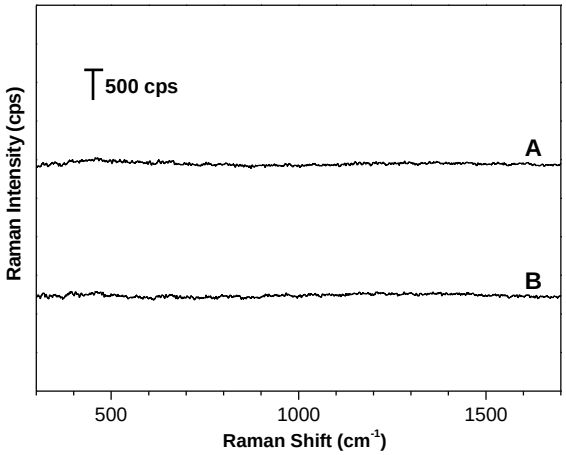

Fig. S13. SERS spectra of the probes after treated with (A) gallic acid (1×10^{-5} M) and (B) curcumin (1×10^{-5} M). It can be seen that no Raman signal was detected, indicating that the probes can not react with the two anti-tumor drugs.

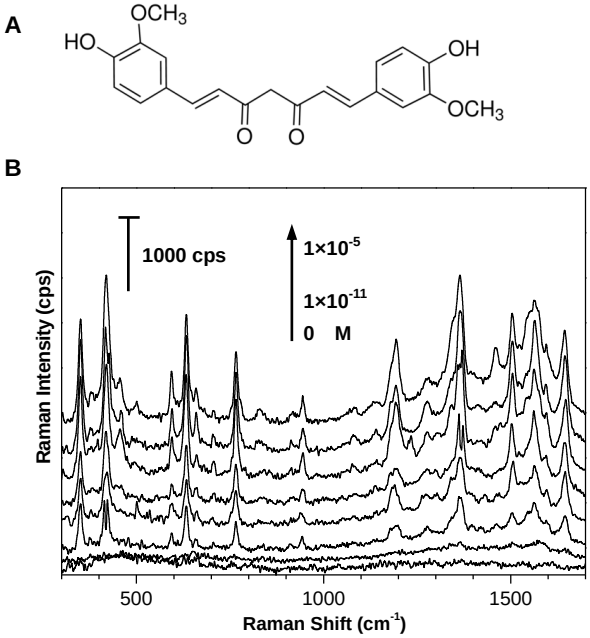

Fig. S14. (A) The structure of curcumin. (B) SERS spectra of the probes in HeLa cells after cultured with curcumin for 30 min. The concentrations are from 1×10^{-11} to 10^{-5} M with an increasing factor of 10 (the bottom line represents the blank).

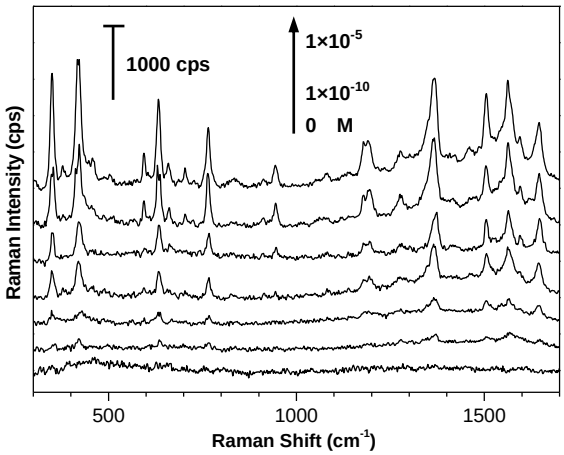

Fig. S15. SERS spectra of the probes in A549 cells after cultured with gallic acid for 30 min. The concentrations are from 1×10^{-10} to 10^{-5} M with an increasing factor of 10 (the bottom line represents the blank).

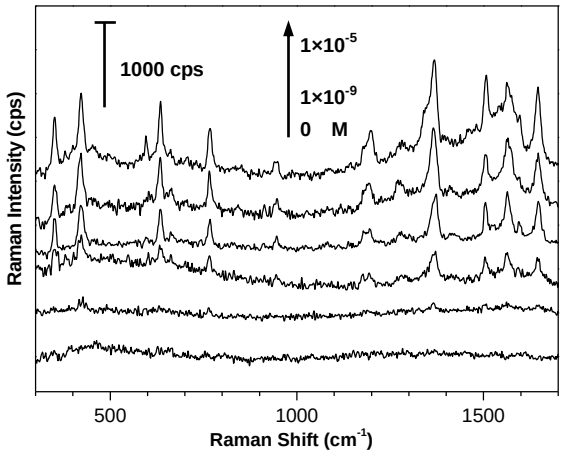

Fig. S16. SERS spectra of the probes in A549 cells after cultured with curcumin for 30 min. The concentrations are from 1×10^{-9} to 10^{-5} M with an increasing factor of 10 (the bottom line represents the blank).