Electronic Supplementary Material (ESI) for Analyst.

Supporting Information

An Au-Se Nanoprobe for Evaluation of the Invasive Potential of Breast Cancer Cells *via* Imaging the Sequential Activation of uPA and MMP-2

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1. Materials and instruments

Trisodium citrate ($C_6H_5Na_3O_7·2H_2O$), hydrogen tetrachloroaurate(III) ($HAuCl_4·4H_2O$, 99.99%), sodium dodecylsulfate (SDS) and dimethyl sulfoxide (DMSO) were purchased from China National Pharmaceutical (Shanghai); N-ethylmaleimide (NEM), lipopolysaccharide (LPS), L-buthionine sulfoximine (BSO), selenocystine, glutathione (GSH), 3-(4,5-Dimethylthiazol-2-yl)-2,5 -diphenyltetrazolium bromide (MTT) and Human caspase-9 recombinant proteins (caspase-9) were purchased from Sigma. The urokinase plasminogen activator (uPA) was purchased from Abcam. uPA and MMP-2 polyclonal antibody were purchased from eBioscience. Recombinant human matrix metalloproteinase-1, 2, 3, 7, 9, 13 were purchased from ProSpec. MMP activator p-aminophenyl mercuric acid was purchased from Genmed Scientifics, U.S.A. UK-371804 was purchased from Topscience Co., Ltd. Marimastat was purchased from MedChemExpress (MCE). All chemicals were analytical grade and used without further purification. Sartorius ultrapure water of 18.2 MΩ·cm was used throughout the experiments. The peptides were synthesized and further purified by Karebay Biochem (Ningbo, China). Detailed peptide sequences were shown in Table S1. MCF-10A (human noncancerous breast cell) and MDA-MB-231 (human breast cancer cell line) were purchased from Procell (Wuhan, China). MCF-7 cells (human breast cancer cell line) were purchased from KeyGEN biotechnology (Nanjing, China). Cell culture products, unless mentioned otherwise, were purchased from GIBCO.

All pH values were measured by a pH-3c digital pH meter (LeiCi, China) with a combined glass-calomel electrode. Transmission electron microscopy (TEM) was carried out on a JEM-2100 electron microscope, and the samples were prepared by carbon-coated copper grids. Fluorescence spectra were acquired with Fluorescence Spectrometer (F-4600, Hitachi, Japan). DLS was measured in Zetasizer Nano ZS. Absorption spectra were collected on a UV–Vis spectrometer (TU-1900, Purkinje General, China). Centrifugation was performed on an Eppendorf 5417R Centrifuge. Absorbance was measured in a microplate reader (Synergy 2, Biotek, U.S.A.) in the MTT assay. Confocal fluorescence images were accomplished with a confocal laser scanning microscopy (Leica TCS SP8, Germany). Imaging flow cytometry was

accomplished on an Amnis Image Stream MarkII (Merck Millipore).

2. Experiments

2.1 Preparation of the gold nanoparticles

The 13 nm Au NPs were synthesized by the classical sodium citrate reduction method reported before.[1] Typically, 70 mL $HAuCl_4$ (0.01% w.p.) aquous solution was heated to boiling with vigorous stirring, and 3.5 mL of trisodium citrate solution (1% w.p.) was rapidly added under stirring. The color of the solution changed from pale yellow to colorless and finally to burgundy. The solution was kept boiling for additional 20 min, and then cooled down to room temperature under stirring. Afterward, the solution was filtered by using a 0.45 μm Millipore membrane filter and stored in refrigerator at 4 °C. The concentration of Au NPs was determined by the intensity of their extinction at 524 nm (ε = 2.7 ×108 $L \cdot mol^{-1} \cdot cm^{-1}$).

2.2 Quantitation of peptide chains loaded on the Au NPs

Each peptide's loading capacity onto the Au NPs was quantified through a previous reported protocol.[2] Briefly, the selenolcysteine (final concentration 10 mM) was added to the nanoprobe (1 nM), which was incubated for 12 h under shaking at room temperature. Afterwards, the released peptides were collected *via* centrifugation, and the fluorescence intensities of the supernatant solution were obtained by fluorescence spectrometer. The fluorescence of RhB-labeled peptide and FITC-labeled peptide were obtained at $\lambda_{ex}/\lambda_{em}$ of 561/583 nm and 490/520 respectively. The fluorescence intensities were converted to molar concentrations of peptides by interpolation from a standard linear calibration curve that was prepared with known concentrations of peptides with identical buffer pH, ionic strength and peptide concentrations. With dividing molar concentrations of each peptide by the original concentration of the nanoprobes, the numbers of peptide on nanoprobe were quantified. All experiments were performed at least three times.

2.3 Response Experiment

For the detection of analytes, the Au–Se nanoprobe was incubated with increasing concentrations of uPA (10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 0.2, 0.3, 0.4 μg/mL) and MMP-2 concentration(10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1} μg/mL), respectively. After

incubation at 37 °C for 1 h, the fluorescent intensities of RhB and FITC were examined with lasers of 561 and 490 nm wavelength as excitation, respectively. In order to study the cross interference of fluorescent signals, the Au–Se nanoprobe (1 nM) with MMP-2 (0, 0.1 or 0.2 μg/mL) was incubated with increasing concentrations of uPA (0, 0.01, 0.05, 0.1, 0.2, 0.4 μg/mL), respectively. The fluorescent intensities of RhB were examined with laser of λ_{ex} = 561 nm as excitation. Similarly, the Au–Se nanoprobe (1 nM) with uPA (0, 0.4 or 0.8 μg/mL) was incubated with increasing concentrations of MMP-2 (0, 0.01, 0.02, 0.05, 0.08, 0.1μg/mL), respectively. The fluorescent intensities of FITC were examined with with laser of λ_{ex} = 490 nm as excitation, respectively. All experiments were performed at least three times.

2.4 Kinetics Study

The Au–Se nanoprobe (1 nM) was incubated with uPA (0.4 μg/mL) and MMP-2 (0.1 μg/mL) at 37 °C, respectively. Afterwards, the fluorescence intensities were determined on a certain time interval (0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60 min or 0, 5, 10, 15, 20, 25, 30, 40, 50, 60 min). The fluorescent intensities of RhB and FITC were examined with lasers of 561 and 490 nm wavelength as excitation, respectively. All experiments were performed at least three times.

2.5 Specificity Experiment

To study the targeting specificity of the Au–Se nanoprobe, the uPA (0.4μg/mL), MMP-2 (0.1 μg/mL) and other targets (caspase-9 of 10 Unit/mL; MMP-7 of 0.1 μg/mL; GSH of 5 mM and Sec of 1.7 μM) were added to the nanoprobe(1 nM) for 1 h at 37 °C. The fluorescent intensities of FITC and RhB were examined with lasers of 561 and 490 nm wavelength as excitation, respectively. All experiments were performed at least three times.

2.6 pH influence study

For the study of pH influence on the Au–Se nanoprobe detection, the experiment was designed with two groups. Groups I: the Au–Se nanoprobe (1 nM) under the pH values ranging from 6.2 to 8.0 serving as the control; Group II: the Au–Se probe (1 nM) incubated with MMP-2 (0.1 μg/mL) and uPA (0.4 μg/mL) under the pH values ranging

from 6.2 to 8.0. After incubation for 1 h at 37 °C, the fluorescence of FITC and RhB were examined with lasers of 561 and 490 nm wavelength as excitation, respectively. All experiments were performed at least three times.

2.7 Inhibitor blocking test

To further confirm the specific cleavage function of uPA and MMP-2 to peptide chains loaded on the Au-Se nanoprobe, three groups with different components were carried out. Group I: the Au-Se nanoprobe (1 nM) was served as control without any treatment; Group II: the Au-Se nanoprobe (1 nM) was incubated with MMP-2 (0.1 μg/mL) or uPA (0.4 μg/mL) at 37 °C for 1 h; Group III: MMP-2 (0.1 μg/mL) or uPA (0.4 μg/mL) was pre-treated with Marimastat (an MMP inhibitor) or UK-371804 (an uPA inhibitor) for 12 h at 37 °C, respectively. Then the Au-Se nanoprobe (1nM) was incubated with the mixed solution for 1 h at 37 °C. The fluorescent intensities of FITC and RhB were examined with lasers of 561 and 490 nm wavelength as excitation, respectively. All experiments were performed at least three times.

2.8 Confocal Fluorescence Image Assay

In a comparative experiment of normal cells and cancer cells, all cells were cultured in confocal dishes for 24 h at 37 ℃. The Au-Se nanoprobe (1 nM) was respectively delivered into MCF-10A, MCF-7, and MDA-MB-231 cells in DMEM culture medium at 37 °C in 5% CO_2 for 4 h at 37 ℃. Then the cells were incubated with Hoechst 33342 for 5 min and imaged by confocal laser scanning microscopy (CLSM) with different laser transmitters.

To study the influence of inhibitors, MDA-MB-231 cells were divided into three groups and cultured in confocal dishes for 24 h at 37°C. Group I was served as control without treatment; Group II was incubated with UK-371804 for 12h at 37°C; Group III was incubated with Marimastat for 12 h at 37°C. The Au-Se nanoprobe (1 nM) was added to the above three groups of MDA-MB-231 cells for 4 h at 37°C. After the above treatment, the cells were incubated with Hoechst 33342 for 5 min and imaged by CLSM with different laser transmitters.

2.9 Intracellular Biothiol Influence on the Two Nanoprobes

To test the influence of biothiols on the Au-S nanoprobe and the Au-Se nanoprobe detection in living cells, MDA-MB-231 cells were divided into two groups and plated in chamber slides for 24 h. One group of MDA-MB-231 cells were incubated with 5 mM BSO for 3 h at 37°C and the other group served as control without treatment. The two nanoprobes (1 nM) were added to the above two groups of MDA-MB-231 cells for 4 h at 37°C, respectively. Then the cells were incubated with Hoechst 33342 for 5 min and imaged by CLSM with different laser transmitters.

To test the influence of biothiols on the nanoprobe by flow cytometer, MDA-MB-231 cells were divided into two groups and plated in confocal dishes for 24 h. One group of MDA-MB-231 cells were incubated with 500 μM nethylmaleimide for 20 min at 37°C and the other group served as control without treatment. The two nanoprobes (1 nM) were added to the above two groups of MDA-MB-231 cells at 37°C for 4 h, respectively. After washed three times with PBS, all groups of cells were trypsinized and collected by centrifugation at 1,000 rpm for 2 min. The cell images were captured and analyzed using the ImageStreamX multispectral imaging flow cytometer (Amnis).

2.10 Cell Culture

All the cells were cultured in Dulbecco's modified Eagle's medium(DMEM) which was supplemented with 10% fetal bovine serum and 100 U/mL 1% antibiotics (penicillin/streptomycin) and kept in a 100% humidified atmosphere containing 5% CO_2 at 37°C.

2.11 MTT Assay

To evaluate the cytotoxicity of the nanoprobe, a tetrazolium-based colorimetric MTT assay was performed. 1). MCF-10A, MCF-7 and MDA-MB-231 cells were seeded into 96-well plates at a density of 1×10^6 cells/well and cultured for 24 h at 37°C. Afterwards, the three types of cells were incubated with the fresh DMEM medium containing 1 nM naked-AuNPs, 1 nM Au-Se nanoprobe and 1nM Au-S nanoprobe for 3, 6, 9, 12 and 24 h, respectively. Following medium removal, cells were washed three times with PBS and cultured with fresh medium containing MTT (0.5 mg/mL) for 4 h.

Next, the remaining MTT solution was discarded and the precipitated formazan crystals were dissolved in 150 μL of DMSO. The absorbance of each well was recorded using a BioTek Synergy2 microplate reader at 490 nm. Cell viability was calculated based on the absorbance normalized to the data from untreated cells. 2). MCF-10A, MCF-7 and MDA-MB-231 cells were seeded into 96-well plates at a density of 1×10^6 cells/well and cultured for 24 h at 37°C. Afterwards, the three types of cells were incubated with the fresh DMEM medium containing a series of Au-Se nanoprobe concentration (10-1000 nM) for 24h, Following medium removal, cells were washed three times with PBS and cultured with fresh medium containing MTT (0.5 mg/mL) for 4 h. Next, the remaining MTT solution was discarded and the precipitated formazan crystals were dissolved in 150 μL of DMSO. The absorbance of each well was recorded using a BioTek Synergy2 microplate reader at 490 nm. Cell viability was calculated based on the absorbance normalized to the data from untreated cells. According to the cell viability data, the IC50 regression curve was established by GraphPad Prism software.

2.12 Western Blotting Assay.

After washed three times with PBS, cells were collected, and lysed using RIPA lysis buffer, containing 1% Sigma protease cocktail, for 30 min at 4 °C. The lysates were centrifuged at 10,000 × g at 4 °C to obtain solubilized cellular proteins. The supernatant protein concentration was measured using a bicinchoninic acid (BCA) protein assay. Equal amounts of protein (50 μg) were separated by 12% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a PVDF membrane, and blocked with Tris buffered saline (TBS) containing 0.05% Tween-20 and 5% nonfat milk powder for 1 h. The membranes were then incubated with primary antibodies against uPA or MMP-2 (1:1000) and β-actin (1:1000) overnight at 4 °C. After washed with 1×TBST 3 times, the membranes were incubated with HRPconjugated secondary antibody (1:50 000) for 1 h at room temperature. Detection was carried out

by incubating the membranes for 5 min with the ECL reagent followed by imaging using a ChemiDoc Touch Imaging system (Bio-Rad, Hercules, CA, USA).

2.13 Cell Invasion Assay

Cell invasion was evaluated using 24-well matregel invasion chambers (8 μm, BD, Biosciences). The filter was coated with 60 μL of diluted Matrigel and then air-dried for 1 h in a 37°C incubator. Three types of cell suspension was plated in the upper chamber of each transwell (2×10^4 cells /well) and further incubated for 24 h. The noninvasive cells on the upper surface of the membrane were removed with a cotton-tipped swab. The invasive cells which adhered to the lower surface of the membrane were then fixed with 4% paraformaldehyde and stained with 0.2% crystal violet before counting the number of invaded cells under microscope.

References

(1) W. Pan, T. Zhang, H. Yang, W. Diao, N. Li, B. Tang, *Anal. Chem*., 2013, **85***,* 10581-10588

(2) B. Hu, F. Kong, X. Gao, L. Jiang, X. Li, W. Gao, K. Xu, B. Tang, *Angew. Chem. Int. Ed.*, 2018**, 57***,* 5306-5309.

Table S1. Peptide sequences employed in this work.

	Sequence
Peptide 1	FITC-Acp-Gly-Pro-Leu-Gly-Val-Arg-Gly-{Se-Cys}
Peptide 2	FITC-Acp-Gly-Pro-Leu-Gly-Val-Arg-Gly-Cys
Peptide 3	RhB-Glu-Ser-Gly-Arg-Ser-Ala-Asn-{Se-Cys}
Peptide 4	RhB-Glu-Ser-Gly-Arg-Ser-Ala-Asn-Cys

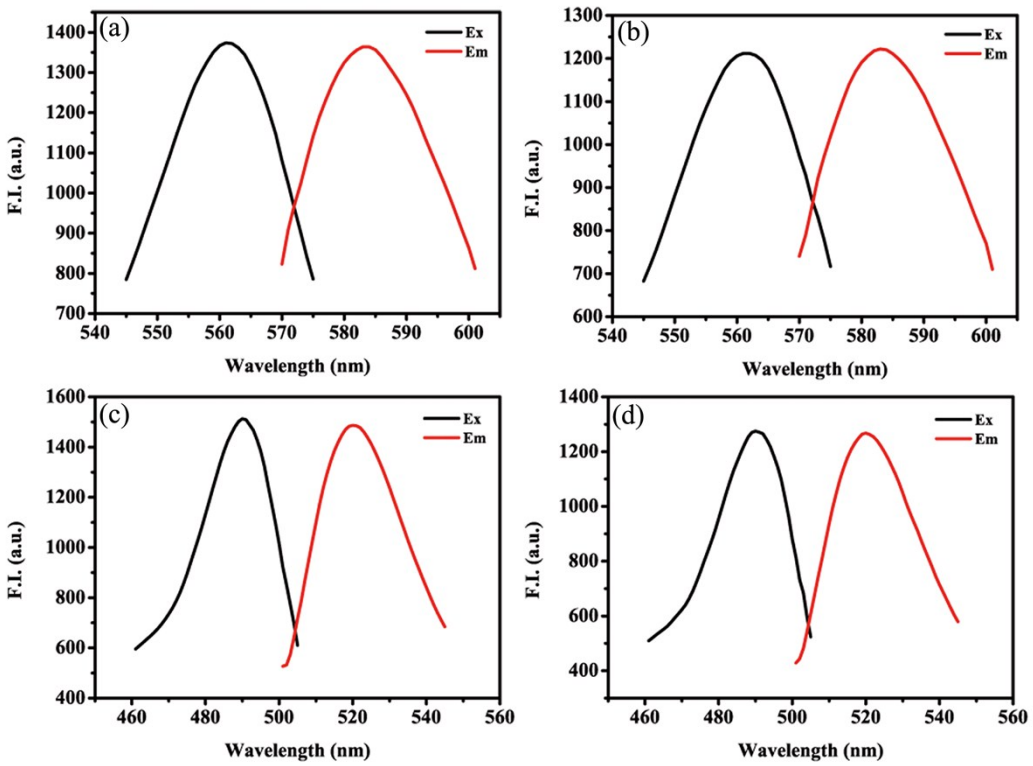

Figure S1. The excitation and emission spectra of RhB for (a) the Au-Se nanoprobe and (b) the Au-S nanoprobe. The excitation and emission spectra of FITC for (c) the Au-Se nanoprobe and (d) the Au-S nanoprobe.

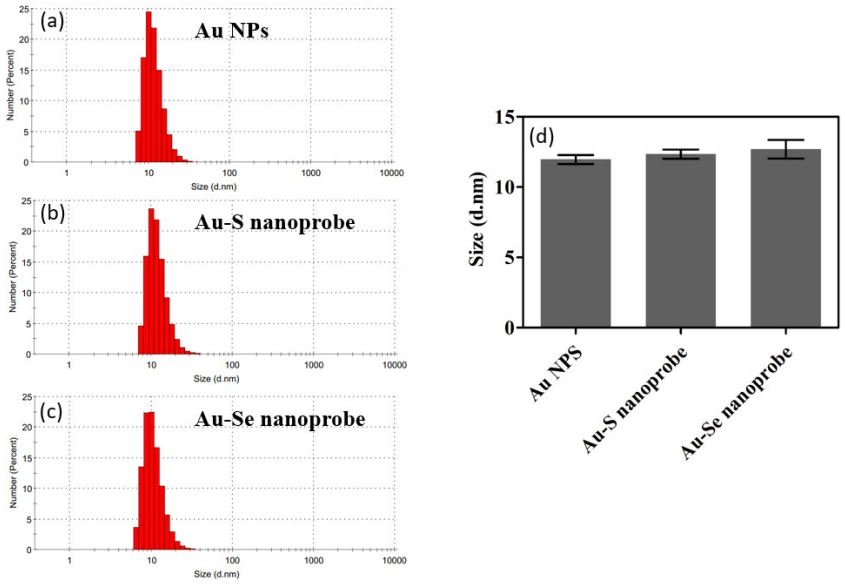

Figure S2. The particle size distribution of (a) the Au NPs, (b) the Au-S nanoprobe and (c) the Au-Se nanoprobe. (d) The average sizes of the three nanoparticles .

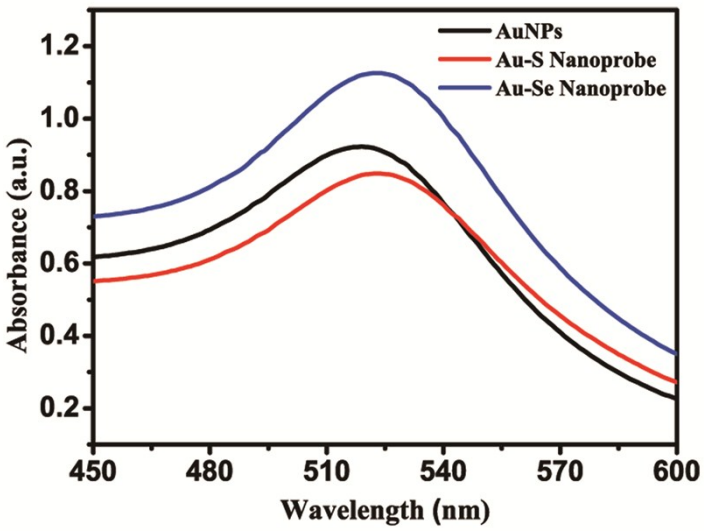

Figure S3. UV-Vis spectra of the Au NPs, the Au-S nanoprobe and the Au-Se nanoprobe

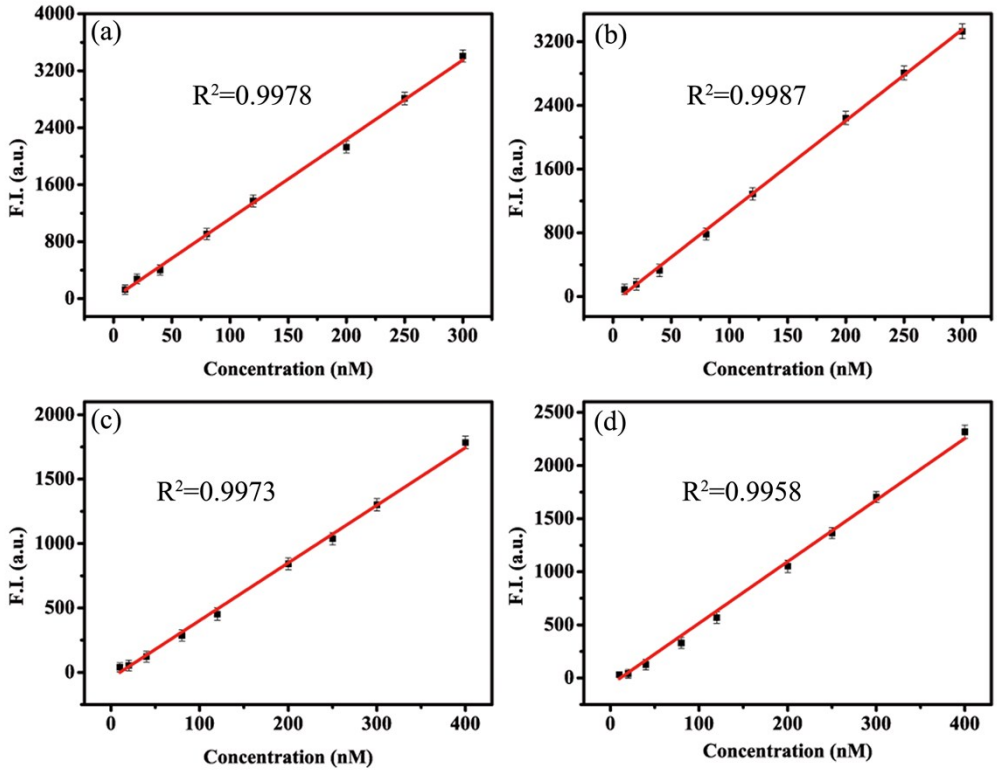

Figure S4. Standard linear calibration curves of the FITC modified peptide chains and the RhB modified peptide chains. (a) the peptide FITC-Acp-Gly-Pro-Leu-Gly-Val -Arg-Gly-{Se-Cys}; (b) the peptide FITC-Acp-Gly-Pro-Leu-Gly-Val-Arg-Gly-Cys; (c) the peptide RhB-Glu-Ser-Gly-Arg-Ser-Ala-Asn-{Se-Cys}; (d) the peptide RhB-Glu-Ser-Gly-Arg-Ser-Ala-Asn-Cys.

Table S2. Quantitative analysis for the loading capacity of the peptides on Au NPs.

Concentration[a]		1:20	1:50	1:100	1:150	1:200	1:300
Counts[b]	Au-Se FITC	23	36	56	74	82	115
	Au-S FITC	15	29	41	53	61	83
	Au-Se RhB	27	34	52	77	86	123
	Au-S RhB	19	25	36	59	67	88

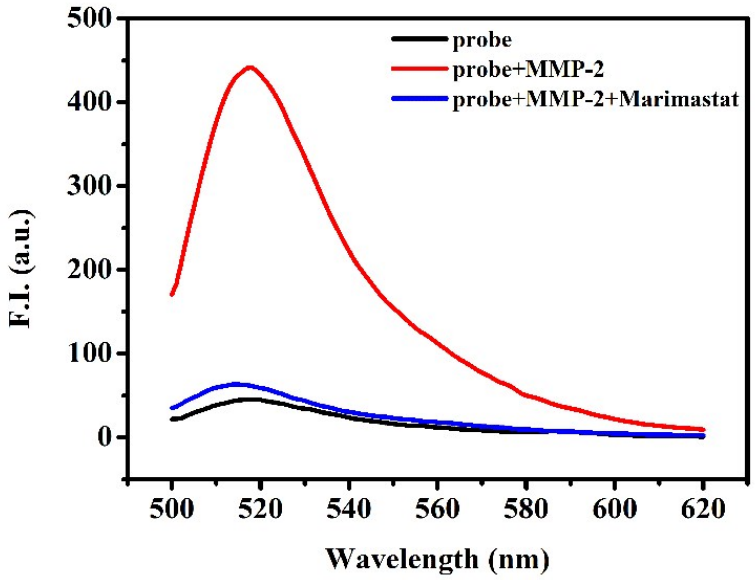

Figure S5. Detection MMP-2 by the fluorescence intensity of FITC in the presence and absence of Marimastat.

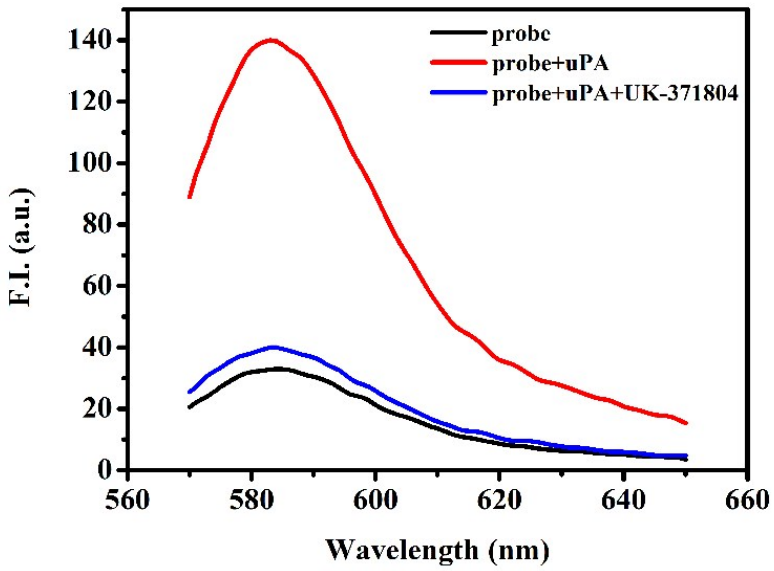

Figure S6. Detection uPA by the fluorescence intensity of RhB in the presence and absence of UK-371804.

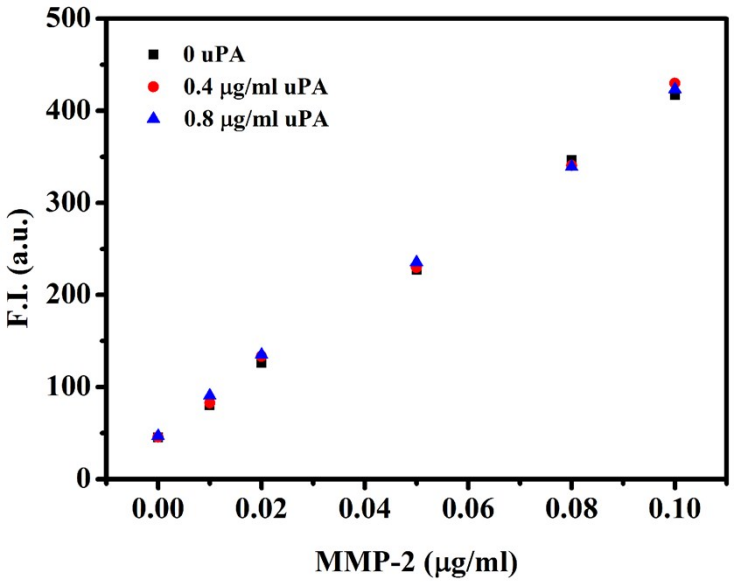

Figure S7. Fluorescence recovery of the Au-Se nanoprobe in the presence MMP-2 and uPA (uPA concentrations denoted by differently shaped data points) measured with 490 nm excitation wavelengths.

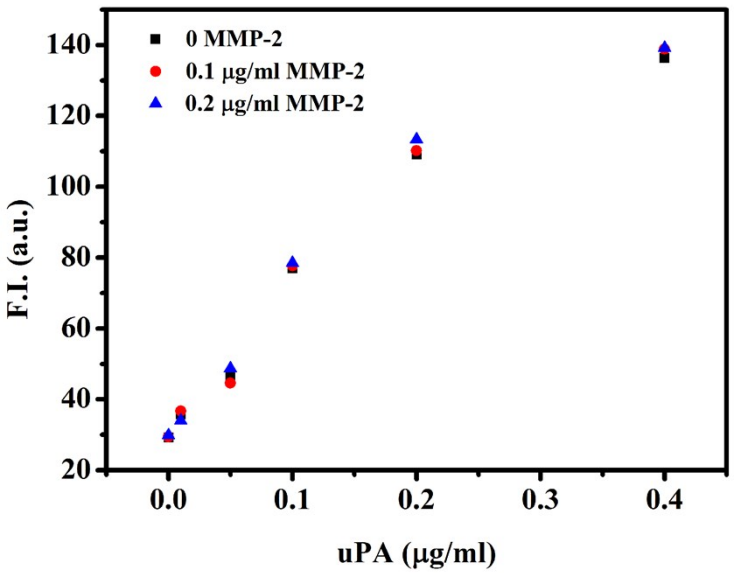

Figure S8. Fluorescence recovery of the Au-Se nanoprobe in the presence of uPA and MMP-2 (MMP-2 concentrations denoted by differently shaped data points) measured with 561 nm excitation wavelengths.

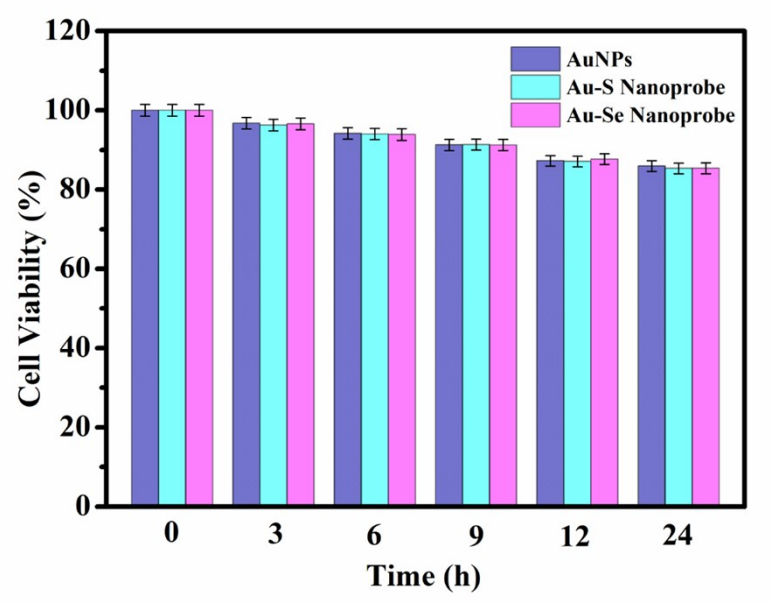

Figure S9. MTT assay of MCF-10A cells. The cells were incubated with naked Au NPs (1 nM), the Au-Se nanoprobe (1 nM) and the Au-S nanoprobe (1 nM) for 0, 3, 6, 9, 12 and 24 h. Dark blue bars represented for the naked Au NPs, light blue bars represented for the Au-Se nanoprobe, and pink bars represented for the Au-S nanoprobe.

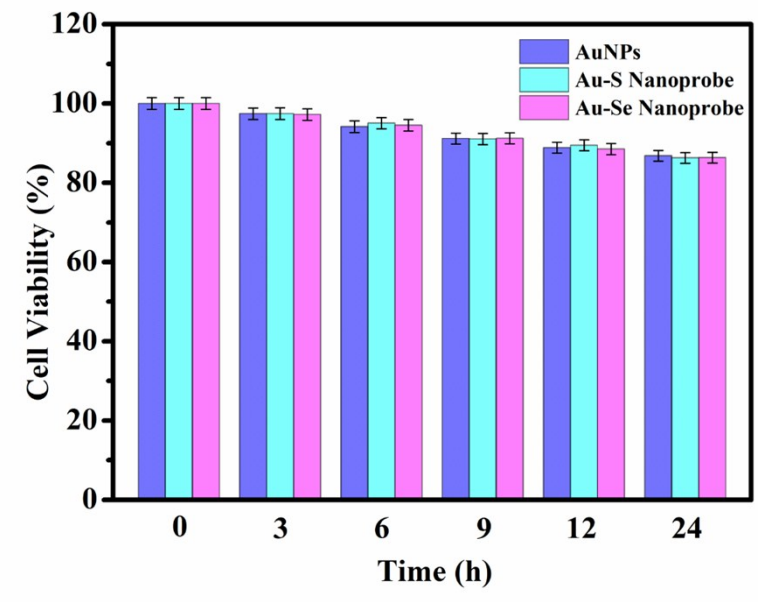

Figure S10. MTT assay of MCF-7 cells. The cells were incubated with naked AuNPs (1 nM), the Au-Se nanoprobe (1 nM) and the Au-S nanoprobe (1 nM) for 0, 3, 6, 9, 12 and 24 h. Dark blue bars represented for the naked Au NPs, light blue bars represented for the Au-Se nanoprobe, and pink bars represented for the Au-S nanoprobe.

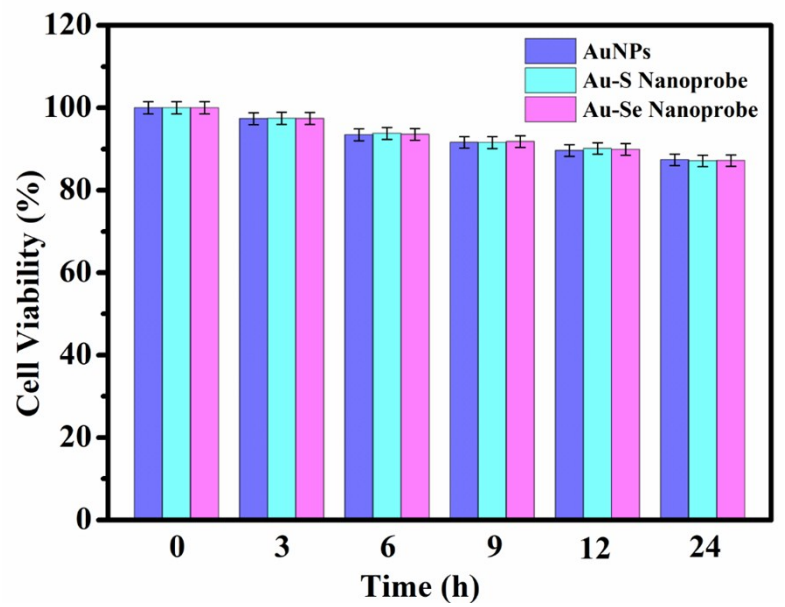

Figure S11. MTT assay of MDA-MB-231 cells. The cells were incubated with naked AuNPs (1 nM), the Au–Se nanoprobe (1 nM) and the Au-S nanoprobe (1 nM) for 0, 3, 6, 9, 12 and 24 h. Dark blue bars represented for the naked Au NPs, light blue bars represented for the Au-Se nanoprobe, and pink bars represented for the Au-S nanoprobe.

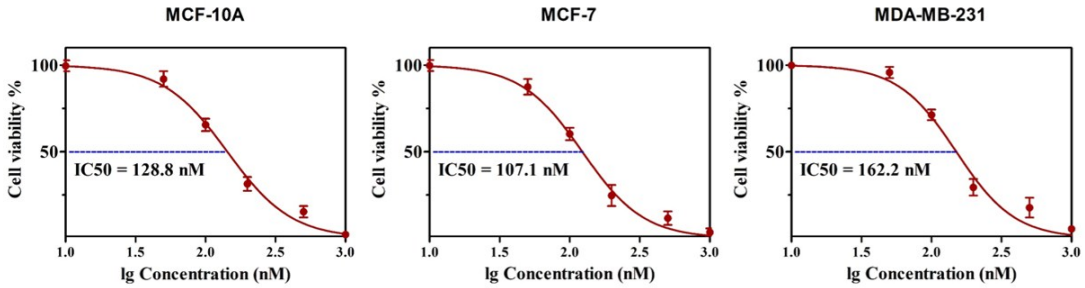

Figure S12. The half maximal inhibitory concentration (IC50) of the Au-Se nanoprobe against MCF-10A, MCF-7 and MDA-MB-231 cells for 24 h were measured by MTT.

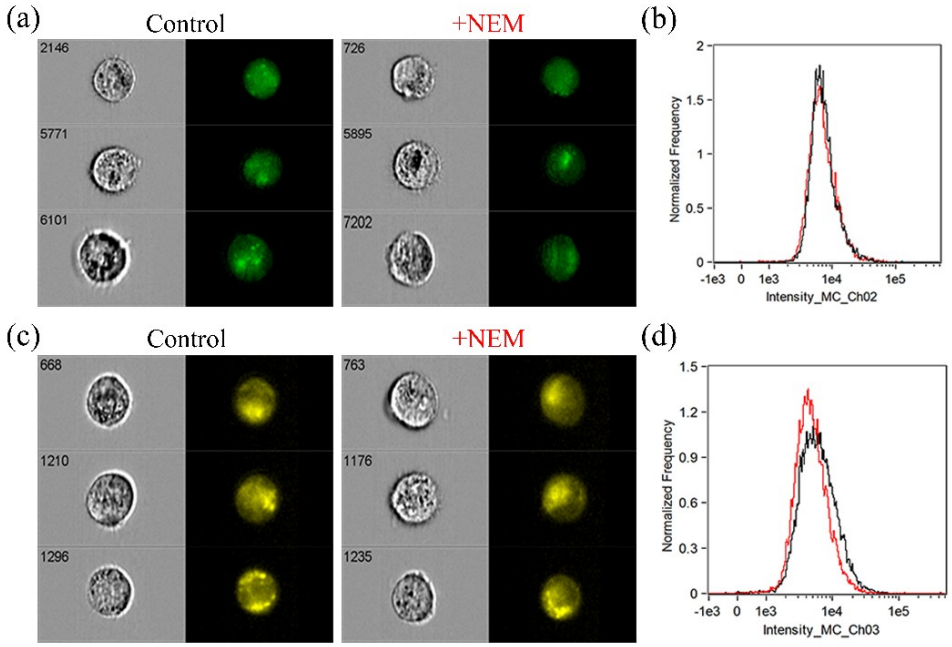

Figure S13. Test of the intracellular interferences of GSH on the Au-Se nanoprobe in MDA-MB-231 cells by imaging flow cytometry. (a) and (b) represent the fluorescence intensities of FITC, (c) and (d) represent the fluorescence intensities of RhB. The flow cytometry data analysis of fluorescence intensity of cells for different treatments of (b) and (d) correspond to the cells in (a) and (c) with the same color black and red, respectively.

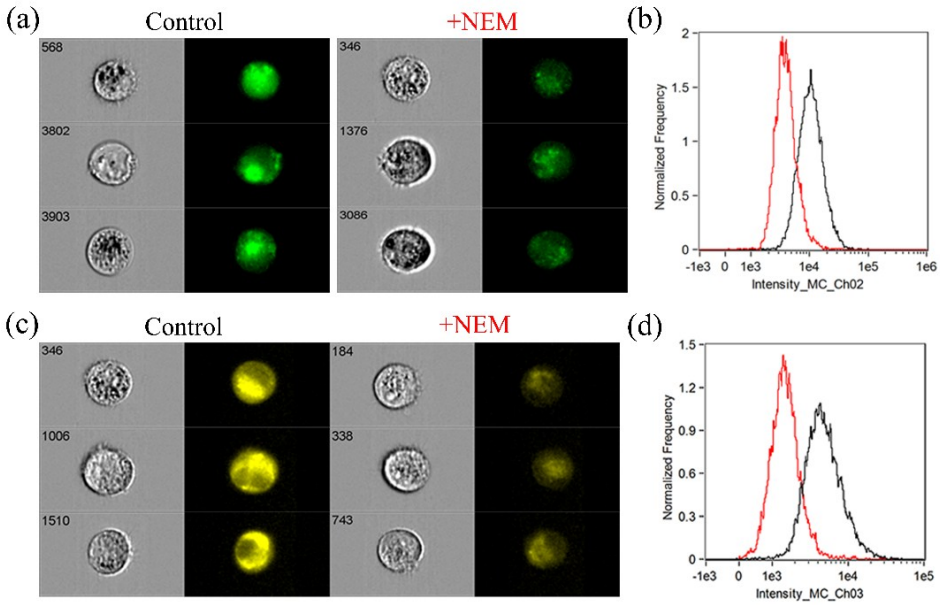

Figure S14. Test the intracellular interferences of GSH on the Au-S nanoprobe in MDA-MB-231 cells by imaging flow cytometry. (a) and (b) represent the fluorescence intensities of RhB, (c) and (d) represent the fluorescence intensities of RhB. The flow cytometry data analysis of fluorescence intensity of cells for different treatments of (b) and (d) correspond to the cells in (a) and (c) with the same color black and red, respectively.

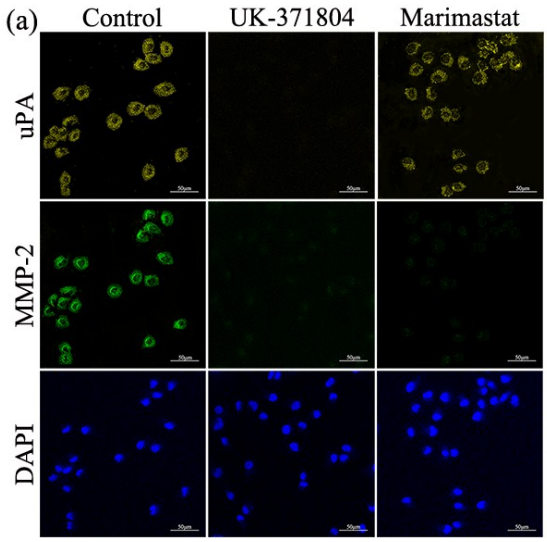

Figure S15. (a) CLSM images of the effects UK-371804 and Marimastat on the Au-Se nanoprobe in MDA-MB-231 cells.

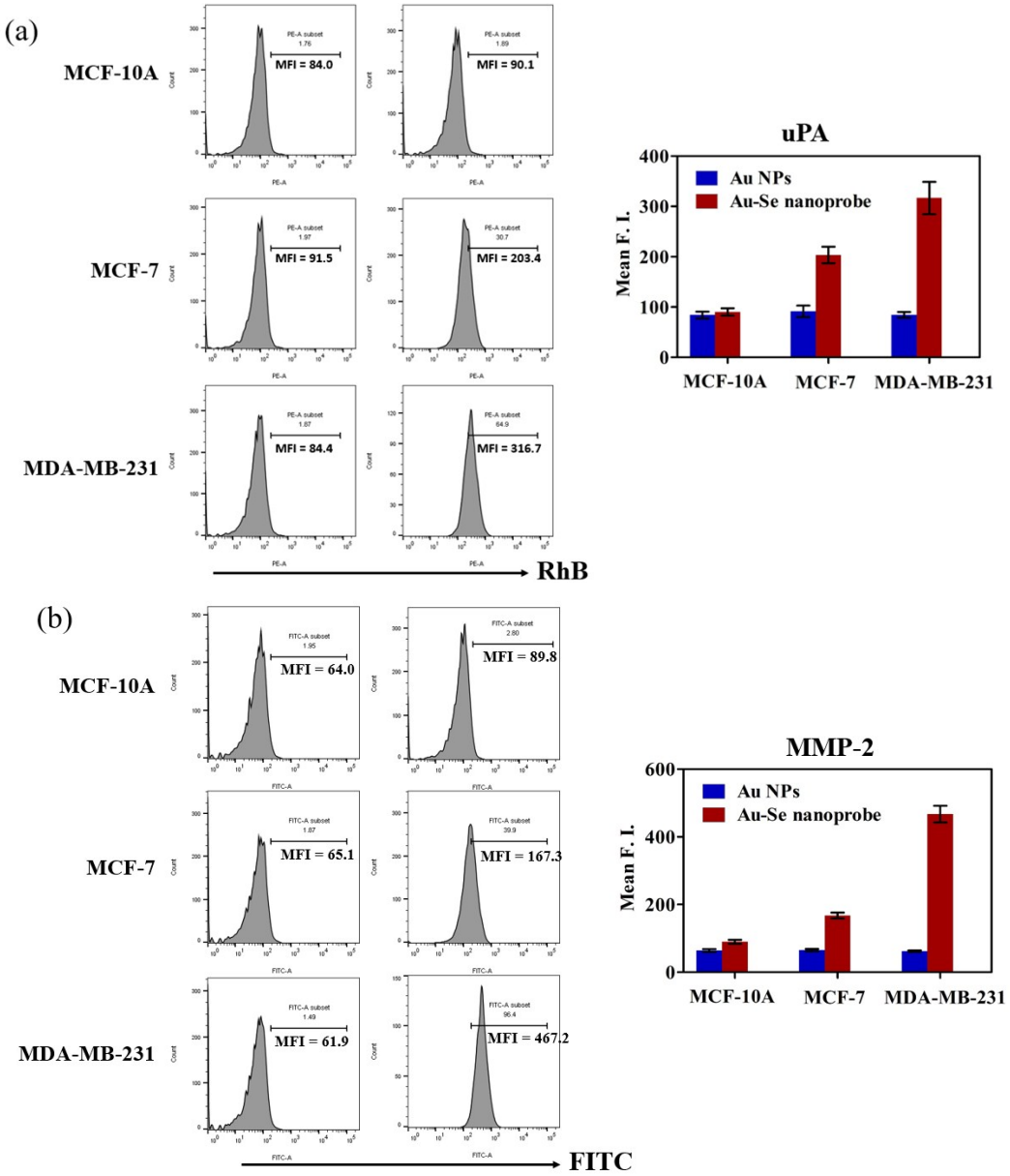

Figure S16. The mean fluorescence intensity of (a) RhB and (b) FITC in MCF-10A, MCF-7 and MDA-MB-231 cells were quantitatively determined by flow cytometry.

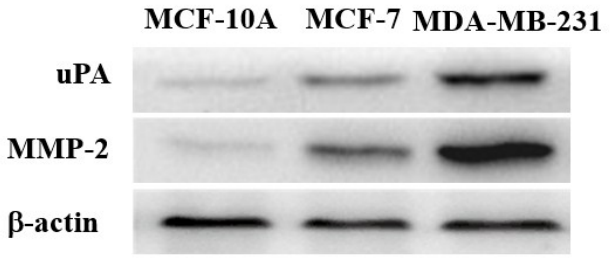

Figure S17. The expression of uPA and MMP-2 in MCF-10A, MCF-7 and MDA-MB-231 cells were measured by western blotting.