Supplementary Information

A gold-selenium-bonded nanoprobe for real-time in situ imaging of the upstream and downstream relationship between uPA and MMP-9 in cancer cells

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**Experimental Section**

**Materials.** N-ethylmaleimide (NEM), glutathione (GSH), lipopolysaccharide (LPS), chlorpromazine and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma. Ethylisopropylamiloride (EIPA) was purchased from J&K Scientific Ltd. UK-371804 and Marimastat was purchased from Topscience Co., Ltd. All the chemical reagents were of analytical grade and used without further purification. All aqueous solutions were prepared using ultrapure water of 18.2 MΩ•cm⁻¹. MMP-9 and uPA were purchased from Abcam Company. The peptides were synthesized and further purified by Kangbei (Ningbo, China). Detailed peptide sequences are shown in Table S1. MCF-7 and PANC-1 cells were obtained from Procell (Wuhan, China). 35 mm glass bottom petri dishes were obtained from Shengyou Biotechnology Co., Ltd (Hangzhou, China). Human uPA/PLAU ELISA Kit and Human MMP-9 ELISA Kit were purchased from Boster Biological Technology Co. Ltd (Wuhan, China).

**Instruments.** Absorption spectra were measured on a UV–Vis spectrometer (TU-1900, Purkinje General, China). High resolution transmission electron microscopy (HRTEM) was acquired on a JEM-2100 electron microscope. Fluorescence spectra were carried out through Fluorescence Spectrometer (FLS-980, Edinburgh, UK). Absorbance was recorded in a microplate reader (Synergy 2, Biotek, USA) in the MTT assay. All pH were measured using a digital pH-meter (pH-3c, LeiCi, China). Confocal fluorescence images were accomplished with a confocal laser scanning microscopy (Leica TCS SP8, Germany). Imaging flow cytometry was accomplished on Amnis ImageStream MarkII (Merck Millipore, Seattle, WA). Centrifugation was performed on an Eppendorf 5417R Centrifuge.

**Preparation of AuNPs.** The 13 nm AuNPs were synthesized by the classical sodium citrate reduction method. All glassware used was first soaked with aqua regia (HNO₃/HCl, 1:3), then washed with ultrapure water before the experiments. Typically, a 100 mL HAuCl₄ aqueous solution (0.01%) was heated to reflux under vigorous stirring, and then (3.6 mL, 1 % w. p.) sodium citrate solution was rapidly added with vigorous stirring. The solution color changed from the initial pale yellow to colorless-
and gradually turned burgundy. The reaction was continued for 10 min with stirring and then the heating was stopped. The vigorous stirring was kept for 15 min until the prepared solution slowly cooled. The prepared AuNPs was filtrated through a 0.45 µm filter and kept at 4 °C for further use.

**Nanoprobe Preparation.** SDS solution (10%) was added to 1 nM AuNPs solution to achieve a final concentration of 0.1% and the mixture was shaken for 4 h. Then the different concentrations (30, 75, 150, 300, 450, 600 nM) of the two selenol modified peptide chains labeled with 5-TAMRA and FITC were added to the mixture. Similarly, the two thiol modified peptide chains were added to another mixture with the same concentration set as well. The mixture was further shaken softly for 48 h in the dark to achieve the preparation of the Au-Se nanoprobe and the Au-S nanoprobe. The prepared nanoprobe was centrifuged (13,000 rpm, 20 min, 4 °C) and resuspended in PBS buffer (pH 7.4) for three times. Finally, the nanoprobe was dispersed in PBS as stock solution kept at 4 °C. The concentration of AuNPs was confirmed by detecting their extinction at 524 nm (ε = 2.7 × 10⁸ L mol⁻¹ cm⁻¹).

**Evaluation of Peptides on the Nanoprobe.** The amount of each peptide loaded on the AuNPs was quantified through a previous report. Generally, the DTT (20 mM final concentration) was added to the 1 nM nanoprobe and shaken overnight to release the peptides. Then, the released peptides were collected via centrifugation (13000 rpm, 30 min) and the fluorescent intensity in the supernatant was obtained. The fluorescence of 5-TAMRA-labeled peptide was measured at λ<sub>ex</sub>/λ<sub>em</sub> = 542 nm/580 nm and the fluorescence of FITC-labeled peptide was acquired at λ<sub>ex</sub>/λ<sub>em</sub> = 480 nm/515 nm, respectively. The fluorescent intensities of the two dyes were converted to molar concentrations of each peptide by interpolation from a standard linear calibration curve that were prepared with known concentration of peptide with the identical pH, buffer, DTT concentration and ionic strength. With dividing molar concentrations of each peptide by the original concentration of the nanoprobe, the numbers of peptides per nanoprobe were determined. All experiments were performed at least three times.

**GSH Influence Test.** To investigate the effect of GSH on the Au-S nanoprobe and the Au-Se nanoprobe, GSH (5 mM) was added to the two nanoprobe (1 nM) for
increasing times (0-12 h) at 37 °C. The fluorescence of 5-TAMRA was obtained at 542 nm excitation and 580 nm emission; the fluorescence of FITC was obtained at 480 nm excitation and 515 nm emission.

**Response Experiment.** For the detection of analysts, the Au-Se nanoprobe was cultured with two protein targets, respectively with increasing uPA concentrations ($10^{-4}$, $10^{-3}$, $10^{-2}$, $10^{-1}$, 0.2, 0.3, 0.4 µg/mL) and MMP-9 concentration ($10^{-7}$, $10^{-6}$, $10^{-5}$, $10^{-4}$, $10^{-3}$, $10^{-2}$, $10^{-1}$ µg/mL) for 1 h at 37 °C. The fluorescent intensities of 5-TAMRA and FITC were examined with 542 nm and 480 nm excitation wavelengths, respectively.

**Kinetics.** The Au-Se nanoprobe solution (1 nM) was incubated with uPA (0.4 µg/mL) and MMP-9 (0.4 µg/mL) at 37 °C, respectively and the fluorescence intensity was measured on a certain time interval (0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60 minutes). The fluorescence of 5-TAMRA and FITC were obtained via the same way described above.

**Specificity Experiment.** To study the specificity of the Au-Se nanoprobe, the uPA (0.4 µg/mL), MMP-9 (0.4 µg/mL) and other peptides were added to 1 nM nanoprobe at 37 °C for 1 h. The FITC fluorescence and 5-TAMRA fluorescence were measured at appropriate excitation and emission wavelengths. All experiments were performed at least three times.

**Cell Culture.** All the cells were incubated in Dulbecco’s modified Eagles medium which was supplemented with 1% antibiotics penicillin/streptomycin and 10% fetal bovine serum (FBS) and kept in a humidified atmosphere of 5% CO$_2$ at 37 °C.

**MTT Assay.** To evaluate the cytotoxicity of the nanoprobe, a tetrazolium-based
colorimetric MTT assay was performed. MCF-7 and PANC-1 cells were seeded in replicate 96-well microtiter plates (1×10^6 cells/well in 200 µL DMEM). The culture was kept in 5% CO₂/95% air incubator at 37 °C. After cell attachment for 24 h, the initial medium was removed and the cells were cultured with 1 nM naked-AuNPs, 1 nM Au-Se nanoprobe and 1 nM Au-S nanoprobe for different time periods, respectively. Then MTT solution (0.5 mg mL⁻¹ in PBS, 100 µL) were added to each well after being washed with PBS and further incubated for 4 h. After discarding the remaining MTT solution, DMSO (150 µL) was added to dissolve the purple formazan. The absorbance at 490 nm was recorded with a Synergy 2 microplate reader.

**Cellular Uptake Mechanism of the Au-Se Nanoprobe.** MCF-7 cells were plated in glass-bottomed dishes for 24 h at 37 °C in a humidified atmosphere with 5% CO₂. All groups of cells were treated with LPS (10 µg/mL) for 12 h to induce uPA and MMP-9. MCF-7 cells were pre-incubated separately with two endocytosis inhibitors: 50 µM EIPA or 20 µM chlorpromazine for 1.5 h. Then, the cells were incubated with the Au-Se nanoprobe for 4 h. Each dish was washed three times with PBS buffer. The fluorescence images of uPA and MMP-9 were examined by CLSM with 542 nm and 488 nm excitation, respectively.

**Intracellular Biothiol Influence on the Two Nanoprobes.** To test the effect of biothiols on the Au-S nanoprobe and the Au-Se nanoprobe in living cells, MCF-7 cells were chosen and separated into two groups. Two groups of cells were pretreated with LPS (10 µg/mL) for 12 h to induce uPA and MMP-9. Then one group was treated with NEM (500 µM, thiol scavenger) at 37 °C for 20 min and the other group
served as control group without NEM treatment. The two nanoprobe solutions (1 nM) were added to the above two groups of MCF-7 cells at 37 °C for 4 h, respectively, and then the fluorescent images of uPA and MMP-9 were examined under CLSM.

**Fluorescence Imaging Assay.** For the dynamical evaluation of the changes in uPA and MMP-9 expression, two cancer cell lines: MCF-7 and PANC-1 cells were chosen and separated into two groups. Two groups of cells were pretreated with LPS (10 µg/mL) for 12 h to promote uPA and MMP-9 expression. Next, the cells were cultured with the Au-Se nanoprobe and Au-S nanoprobe for 4 h at 37 °C, respectively. After washing cells twice with PBS to discard the excess nanoprobe, fresh medium was added to the cells and incubated for 0-10 h. The cells were examined by CLSM to acquire the fluorescence intensity change.

In the experiment of identifying the relationship between uPA and MMP-9, MCF-7 cells and PANC-1 cells were chosen and divided into four groups. Group I was served as control without treatment; Group II was treated with LPS (10 µg/mL) for 12 h; Group III and IV were pretreated with 1 µM UK-371804 (uPA inhibitor) for 12 h and 5 nM Marimastat (MMP-9 inhibitor) for 12 h, respectively, and further treated with LPS for 12 h. Finally, the four groups of cells were incubated with the Au-Se nanoprobe for 4 h and then examined by CLSM to record the fluorescent signal change.

**Flow Cytometry.** The two types of cells, MCF-7 cells and PANC-1 cells were pretreated with LPS (10 µg/mL) for 12 h to promote uPA and MMP-9 expression. Next, the cells were cultured with the Au-Se nanoprobe and Au-S nanoprobe for 4 h
at 37 °C, respectively. After washing cells twice with PBS to discard the excess nanoprobe, the cells were further cultured with the fresh medium for 0-10 h. Finally, all of the cells at different culture time were analyzed on ImageStream MarkII imaging flow cytometry with excitation at 488 and 561 nm laser.

**MMP-9 Protein and uPA Protein Levels.** The two types of cells, MCF-7 cells and PANC-1 cells were treated with different drugs as indicated in the fluorescence imaging assay. MMP-9 protein and uPA protein levels in the cells were quantified using ELISA assay described by Peri et al.1

Reference

**Supplementary Data:**

**Table S1** Peptides employed in this work

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>Peptide 1</td>
<td>5-TAMRA-Ser-Gly-Arg-Ser-Ala-Asn-{Se-Cys}</td>
</tr>
<tr>
<td>Peptide 2</td>
<td>5-FITC-Ahx-Gly-Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln-{Se-Cys}</td>
</tr>
<tr>
<td>Peptide 3</td>
<td>5-TAMRA-Ser-Gly-Arg-Ser-Ala-Asn-Cys</td>
</tr>
<tr>
<td>Peptide 4</td>
<td>5-FITC-Ahx-Gly-Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln-Cys</td>
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**Fig. S1** Au-Se nanoparticle diameter histogram, calculated from the HRTEM image.
**Fig. S2** UV-Vis spectra of AuNPs, Au-S nanoprobe and Au-Se nanoprobe.

**Fig. S3** Hydrodynamic diameters obtained from DLS of AuNPs (a), Au-Se nanoprobe (b) and Au-S nanoprobe (c).
**Fig. S4** Zeta potentials of the AuNPs, Au-Se nanoprobe and Au-S nanoprobe.

**Fig. S5** Standard linear calibration curves of the 5-TAMRA modified peptide chains and the FITC modified peptide chains. (a) the peptide 5-TAMRA-Ser-Gly-Arg-Ser-Ala-Asn-Cys; (b) the peptide 5-TAMRA-Ser-Gly-Arg-Ser-Ala-Asn-{Se-Cys}; (c) the peptide 5-FITC-Ahx- Gly-Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln-Cys; (d) the peptide 5-FITC-Ahx- Gly-Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln-{Se-Cys}.
Table S2 Quantitative analysis of the peptides on AuNPs

<table>
<thead>
<tr>
<th>uPA\MMP:9</th>
<th>30:30</th>
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<td>8</td>
<td>5</td>
<td>12</td>
<td>9</td>
</tr>
<tr>
<td>Au-Se probe</td>
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<td>3</td>
<td>11</td>
<td>7</td>
<td>20</td>
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</tr>
</tbody>
</table>

Fig. S6 Specificity tests of the Au-Se nanoprobe against different protein targets. (a) 1: without target; 2: uPA target; 3: MMP-9; 4: MMP-2; 5: HSA. (b) 1: without target; 2: uPA; 3: MMP-9 target; 4: MMP-2; 5: HSA.

![Graph](image1.png)

![Graph](image2.png)
**Fig. S7** Growth inhibition assay of MCF-7 cells. The cells were incubated with naked AuNPs (1 nM), the Au-S nanoprobe (1nM) and the Au-Se nanoprobe (1nM) for 4 h, 12 h, 24 h. Blank bars represent for the control, red bars represent for the naked AuNPs, blue bars represent for the Au-S nanoprobe, grey bars represent for the Au-Se nanoprobe.

**Fig. S8** Growth inhibition assay of PANC-1 cells. The cells were incubated with naked AuNPs (1 nM), the Au-S nanoprobe (1nM) and the Au-Se nanoprobe (1nM) for 4 h, 12 h, 24 h. Blank bars represent for the control, red bars represent for the naked AuNPs, blue bars represent for the Au-S nanoprobe, grey bars represent for the Au-Se nanoprobe.
**Fig. S9** Cell uptake pathway study. The MCF-7 cells were treated without or with chlorpromazine and EIPA inhibitors before incubated with the Au-Se nanoprobe (1 nM). The fluorescence images of uPA and MMP-9 were recorded with 542 and 488 nm excitation wavelengths, respectively. Scale bars: 100 µm.

**Fig. S10** Fluorescent imaging of intracellular thiol interference on the Au-S nanoprobe (a, b) and the Au-Se nanoprobe (c, d) in MCF-7 cells. The cells were treated with or without NEM. The fluorescence images of uPA and MMP-9 were recorded with 542 and 488 nm excitation wavelengths. Scale bars: 100 µm.
Fig. S11 Quantitative flow cytometry analysis of the MCF-7 cells. The cells pretreated with LPS were incubated with Au-Se nanoprobe (a, b) and Au-S nanoprobe (c, d) for 4 h, respectively. Cellular fluorescence (FL) intensities were detected by flow cytometry from 0 h to 10 h. (a, c) FL intensities of 5-TAMRA for uPA; (b, d) FL intensities of FITC for MMP-9. (e-h) Quantitative data expressing time-dependent changes in mean FL intensity of (a-d).
Fig. S12 The protein levels in MCF-7 cells measured by ELISA assay at different time variations after the treatment of LPS. (a) Quantification of uPA protein levels. (b) Quantification of MMP-9 protein levels.

Fig. S13 Real-time in situ visualization of uPA and MMP-9 in PANC-1 cells. Two groups of cells pretreated with LPS were incubated with 1 nM Au-Se nanoprobe for 4 h, respectively. The fluorescence images of uPA and MMP-9 were recorded with 542 and 488 nm excitation wavelengths from 0 h to 10 h. Scale bars: 100 µm.
Fig. S14 Real-time in situ visualization of uPA and MMP-9 in PANC-1 cells. Two groups of cells pretreated with LPS were incubated with 1 nM Au-S nanoprobe for 4 h, respectively. The fluorescence images of uPA and MMP-9 were recorded with 542 and 488 nm excitation wavelengths from 0 h to 10 h. Scale bars: 100 µm.

![Figure S14](image)

Fig. S15 The protein levels in PANC-1 cells measured by ELISA assay at different time variations after the treatment of LPS. (a) Quantification of uPA protein levels. (b) Quantification of MMP-9 protein levels.

![Figure S15](image)
**Fig. S16** Quantitative flow cytometry analysis of the PANC-1 cells. The cells pretreated with LPS were incubated with Au-Se nanoprobe (a, b) and Au-S nanoprobe (c, d) for 4 h, respectively. Cellular fluorescence (FL) intensities were detected by flow cytometry from 0 h to 10 h. (a, c) FL intensities of 5-TAMRA for uPA; (b, d) FL intensities of FITC for MMP-9. (e-h) Quantitative data expressing time-dependent changes in mean FL intensity of (a-d).
**Fig. S17** Fluorescence images of effect of upstream uPA expression on downstream MMP-9 activity in PANC-1 cells. The cells were incubated with the Au-Se nanoprobe after treated with or without LPS, UK-371804 and Marimastat. Scale bars: 100 µm.

**Fig. S18** The protein levels in MCF-7 cells measured by ELISA assay. (a) Quantification of uPA protein levels. (b) Quantification of MMP-9 protein levels. The cells were treated with or without LPS, UK-371804 and Marimastat. The cells without drug treatment were used as control.
Fig. S19 The protein levels in PANC-1 cells measured by ELISA assay. (a) Quantification of uPA protein levels. (b) Quantification of MMP-9 protein levels. The cells were treated with or without LPS, UK-371804 and Marimastat. The cells without drug treatment were used as control.