Supporting information

A Photothermally Responsive Nanoprobe for Bioimaging Based on the Edman Degradation

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**Materials and instruments.** All reagents and chemicals were procured from commercial sources and used without further purification. Cy5.5 was purchased from Luminprobe Ltd (USA). c(RDGYK) was obtained from CS Bio Co (USA). All others were purchased from Sigma-Aldrich (USA). Waters 600 high-performance liquid chromatography (HPLC) system with a Waters 996 Photodiode Array Detector (PDA) using a semi-preparative C18 HPLC column (XTerra Prep RP18, 10 µm, 7.8 x 300 mm, Waters) was used for the purification of products. A Perkin-Elmer 200 series HPLC pump with a Waters 2487 UV detector using an analytical C18 HPLC column (XTerra 5 µm, 150 x 4.6 mm, Waters) was used for analysis of compounds. HPLC runs a linear gradient starting from 5% A (0.1% TFA in acetonitrile) and 95% B (0.1% TFA in water) for 5 min and increasing to 65% A at 35 min with a flow rate of 5 ml/min for semi-prep HPLC and 1 ml/min for analytical HPLC. The $^1$H NMR spectra were recorded on a Bruker spectrometer at 300 MHz. All chemical shifts are reported in the standard $\delta$ notation of parts per million (ppm). Mass spectra were obtained with Waters LC-MS system (Waters, Milford, MA) that includes an Acquity UPLC system coupled to the Waters Q-Tof Premier high-resolution mass spectrometer. UV-Vis absorption spectra were recorded on a Shimadzu spectrophotometer. Fluorescence emission spectra were measured on an Fluomax-4 spectrofluorometer. Transmission electron microscope (TEM) messages were collected on a JEM 2010 operating at an acceleration voltage of 200 kV. The as-prepared samples were dispersed in water and dripped water onto a copper grid for the TEM tests.
Scheme S1. The Illustration of the Edman degradation mechanism
Scheme S2. Chemical structure and synthetic route of NH₂-Glu(Cy5.5)-c(RGDyK). Reagents and conditions: i) Fmoc-Cl, K₂CO₃, dioxane/H₂O, 25 °C, 4.0 h, 80 %; ii) PyBOP, DIPEA, DCM, 25 °C, 24.0 h, 90 %; iii) TFA, DCM, 25 °C, 1.0 h, 90 %; iv) C₂O₂Cl₂, DCM, 25 °C, 8 h, 99 %; v) RGDyK, DIPEA, DMF/DCM, 25 °C, 4.0 h, 60 %; vi) piperidine, DMF, 25 °C, 4 h, 50 %.

Fmoc-Glu(COOH)-OtBu: To an ice cold stirred suspension of NH₂-Glu(COOH)-OtBu (530 mg, 2.5 mmol) in water was added K₂CO₃ (560 mg, 4 mmol). A solution of Fmoc-Cl (717 mg, 2.75 mmol) in 10 mL dioxane was added. The resulting solution was warmed to room temperature and stirred for 4 h, then poured into 15 mL water, and the dioxane was removed under reduced pressure. The aqueous solution was washed twice with 40 mL ether; the remaining aqueous solution acidified to pH=2 at 0 °C with 6 M HCl and extracted twice with 30
mL ether. The ethereal solutions were combined, dried with MgSO₄, and the solvent was removed under reduced pressure to obtain the required compound as a white crystalline solid in a yield of 80 %. ¹H NMR (300 MHz, CDCl₃) δ 7.76 (d, J = 7.5 Hz, 2H), 7.59 (d, J = 7.3 Hz, 2H), 7.39 (t, J = 7.4 Hz, 2H), 7.31 (td, J = 7.4, 1.3 Hz, 2H), 5.44 (d, J = 7.9 Hz, 1H), 4.41 (dd, J = 15.6, 8.5 Hz, 2H), 4.36 – 4.27 (m, 1H), 4.21 (t, J = 6.9 Hz, 1H), 2.55 – 2.28 (m, 2H), 2.27 – 2.11 (m, 1H), 1.95 (dt, J = 14.3, 7.2 Hz, 1H), 1.47 (s, 9H).

**Fmoc-Glu(Cy5.5)-OtBu:** Fmoc-Glu(COOH)-OtBu (42.0 mg, 0.1 mmol), PyBOP (21.0 mg, 0.04 mmol) and diisopropylethylamine (42 mg, 0.4 mmol) were added to a solution of Cy5.5-NH₂ (15 mg, 0.02 mmol) in dry dichloromethane. The mixture was vigorously stirred at room temperature for 24 h, and the solvent was evaporated under reduced pressure. The residue was washed with 5 mL Et₂O, dried under reduced pressure, then purified by reversed-phase HPLC to afford compound Fmoc-Glu(Cy5.5)-OtBu as a blue solid (21 mg, 90 %). MS (ESI⁺): calcd. For C₇₀H₈₂N₅O₆⁺ 1088.6 [M⁺]; found 1088.6 [M⁺].

**Fmoc-Glu(Cy5.5)-COOH:** Fmoc-Glu(Cy5.5)-OtBu (21 mg, 0.02 mmol ) was added into a solution of 4 mL TFA/DCM (v/v, 1:1). The mixture was stirred at room temperature for 1 h, and then the solvents were removed under reduced pressure, the residue purified by reversed-phase HPLC to obtain the compound Fmoc-Glu(Cy5.5)-COOH as a blue solid (20 mg, 99 %). MS (ESI⁻): calcd. For C₆₆H₇₄N₅O₆⁺ 1032.6 [M⁺]; found 1030.6 [M-2H⁻].

**Fmoc-Glu(Cy5.5)-c(RGDyK):** Oxalyl chloride (2.6mg, 0.02 mmol) was added into a solution of Fmoc-Glu(Cy5.5)-COOH (10 mg, 0.01 mmol) in 2.0 mL dried DMF/DCM (v/v, 1:199) under N₂ atmosphere. The mixture was stirred at room temperature for 8 h, the solvent was removed under reduced pressure and dried under high vacuum to obtain the crude acid chloride.

To crude acid chloride was dissolved in 2.0 mL anhydrous DMF and treated with the diisopropylethylamine (26 mg, 0.02 mmol) and c(RGDyK) (13 mg, 0.02 mmol). The reacted mixture was stirred at room temperature for 4 h under N₂ atmosphere. The solvent was removed, the crude product was purified by the reversed-phase HPLC to obtain Fmoc-Glu(Cy5.5)-c(RGDyK) (10 mg, 60%). MS (ESI⁻): calcd. For C₉₃H₁₁₃N₁₄O₁₃⁺ 1633.9 [M⁺]; found 1631.7 [M-2H⁻].

**NH₂-Glu(Cy5.5)-c(RGDyK):** Piperidine (1.7 mg , 20 µmol) was slowly added into a solution of Fmoc-Glu(Cy5.5)-c(RGDyK) (3.2 mg, 2.0 µmol) in 2 mL anhydrous DMF with stirring, and the mixture was stirred at room temperature for 2h. The DMF was removed under reduced pressure, the residue was purified by the reversed-phase HPLC to give the NH₂-Glu(Cy5.5)-c(RGDyK) (1.4 mg, 50 %). MS (ESI⁺): calcd. For C₇₈H₁₀₃N₁₄O₁₁⁺ 1411.8 [M⁺]; found 706.3 [(M+H)/2⁺].
Scheme S2. Synthetic route for Si-NCS. Reagents and conditions: a) CS₂, THF, 0 °C, 3 h; b) Dicyandiamide (DCDA), TEA, 40 °C, 80%.

Si-NCS: To a solution of (aminopropyl)triethoxysilane (6.6 g, 30 mmol) in 30 mL dry THF was added CS₂ (3.4 g, 45 mmol) and the resulting mixture stirred at 0 °C for 3 h. Then the mixture was warmed to room temperature, DCDA (3.8 g, 45 mmol) and triethylamine (0.1 g, 1 mmol) were added. The mixture was stirred at 40 °C for 4.0 h. The solvent was removed under reduced pressure to obtain the yellow oil. The Si-NCS was purified by reduced pressure distillation (150 °C/10 mm Hg) to give the desired product in the 80% (6.3 g). Si-NCS: ¹H NMR (300 MHz, DMSO) δ 3.77 (q, J = 7.0 Hz, 6H), 3.64 (t, J = 6.5 Hz, 2H), 1.75-1.65 (m, 2H), 1.16 (t, J = 7.0 Hz, 9H), 0.69 – 0.58 (m, 2H).

Scheme S3. Schematic illustration of the synthesis of AuNR@SiO₂@Glu(Cy5.5)-c(RGDyK)

AuNR@CTAB: The AuNR@CTAB was synthesized according to a previous method with some modifications.³ CTAB aqueous solution (7.5 mL, 0.20 M) was mixed with 2.5 mL of 1.0 mM HAuCl₄ aqueous solution. To the stirred solution, 0.60 mL of ice-cold 10.0 mM NaBH₄ aqueous solution was added, which resulted in the formation of a brownish yellow solution. Vigorous stirring of the seed solution was continued for 5 min. After stirred, the seed solution was kept at 30 °C for 2 h.

CTAB (100 mL, 0.20 M) was added to 3.2 mL of 4.0 mM AgNO₃ solution at 30 °C. To this solution, 100 mL of 1.0 mM HAuCl₄ was added to form a brown solution, and after gentle mixing of the solution 1.4 mL of 78.8 mM ascorbic acid was added, which result in a change in the growth solution from brown to colorless. The colorless solution was stirred at 30 °C for 5 min, then 160 µL of the seed solution was added to the growth solution. After 2 min the stirring was stopped and the growth solution was kept at 30 °C for 24 h. The mixture was centrifuged...
(9000 rpm, 10 min), and the collected solid was repeatedly washed with water. The precipitate AuNR@CTAB was redispersed in 30 mL water for subsequent use. The particlesize was 18.0×55.0 nm in accordance to TEM, the absorbance peak was at 760 nm (Fig. S11).

**AuNR@SiO$_2$-NCS**: The above AuNR@CATB solution was adjusted to pH 9 by addition of NaOH (0.1 mM) AuNR@, then 200 µL of 2.5 % TEOS in ethanol solution was added dropwise. The mixture was stirred at 35 °C for 24 h in darkness. The mixture was centrifuged (10000 rpm, 10 min), and the collected solid was washed consecutively with three portions (30 mL) ethanol and water. The precipitated AuNR@SiO$_2$ was redispersed in 20 mL ethanol.

To a solution of AuNR@SiO$_2$ in 20 mL ethanol, 1 mL of 2.5 % Si-NCS ethanol solution was added dropwise. The mixture was heated at 60 °C for 4 h, and then stirred at 40 °C for another 24 h. The mixture was centrifuged (10000 rpm, 10 min), washed twice with 20 mL ethanol. The precipitated AuNR@SiO$_2$-NCS was redispersed in 20 mL ethanol. The silica shell was about 3.8 nm in the TEM, the absorbance peak was at 765 nm (Fig. S11).

**AuNR@SiO$_2$-Glu(Cy5.5)-c(RGDyK)**: To a solution of AuNR@SiO$_2$-NCS in 20 mL ethanol, a solution of 0.25 mg NH$_2$-Glu(Cy5.5)-c(RGDyK) and DIPEA (2 µL) in 2 mL ethanol were added dropwise. The mixture was heated at 50 °C for 1 h, and then stirred at room temperature for another 24 h. The mixture was centrifuged (10000 rpm, 10 min), washed twice with ethanol. The precipitate AuNR@SiO$_2$-NCS was redispersed in 20 mL ethanol. The silica shell was unchanged at about 3.8 nm (TEM), not changed in the TEM, the absorbance peak was at 765 nm, and the shoulder peak at 680 nm attributed to Cy5.5 dye (Fig. S11).

**Procedures for Edman degradation.**

1. As a function of pH. The stock solutions of AuNR@SiO$_2$-Glu(Cy5.5)-c(RGDyK) (containing 1.1 µM) Cy5.5 was prepared in different pH (4, 5, 6, 7) buffer. And then the different pH solutions were heated at 80 °C for different tmin (0, 5, 10, 15, 20, 25, 30 min) and then cooled to room temperature for fluorescence measurements, excitation was fixed at 633 nm, and the emission was collected from 400 to 850 nm.

2. As a function of temperature. The stock solutions of AuNR@SiO$_2$-Glu(Cy5.5)-c(RGDyK) (containing 1.1 µM) Cy5.5 was prepared in pH 6 (50 mM NaH$_2$PO$_4$, adjusted with NaOH) buffer. And then the final solutions were heated at different temperature (60, 80 and 100 °C for various times (0, 5, 10, 15, 20, 25, 30 min) and then cooled to room temperature for fluorescence measurements, excitation was fixed at 633 nm, and the emission was collected from 400 to 850 nm.

3. Photothermally releasing. The stock solution of AuNR@SiO$_2$-Glu(Cy5.5)-c(RGDyK) (containing 1.1 µM) Cy5.5 was prepared in pH 6 buffer. The final solutions were irradiated by the 808 laser (0.2 W/cm$^2$) for different times (0, 2, 4, 6 and 8 min), and then the solution was cooled to room temperature for fluorescence measurements, excitation was fixed at 633 nm, and the emission was collected from 400 to 850 nm. The solutions were centrifuged (12000 rpm, 15 min), the liquid supernatant (released dye Cy5.5-NH$_2$) was collected and lyophilized to obtain powders. The final powders were dissolved in 100 µL MeCN/H$_2$O (v/v, 1:10) for HPLC, respectively.
**Cell culture.** The U87MG and MCF-7 were purchased from the American Type Culture Collection (ATCC) and grown in RPMI-1640 medium (Life Technologies, Grand Island, NY) which was supplemented with 10% fetal bovine serum, penicillin (100 IU/mL), and streptomycin (100 mg/mL) (Invitrogen, Carlsbad, CA) and in a humidified atmosphere containing 5% CO₂ at 37 °C. Cells were passaged three to four times per week.

**MTT.** In vitro cytotoxicity was measured by performing methyl thiazolyl tetrazolium (MTT) assays on the U87-MG cells. Cells were seeded into a 96-well cell culture plate at 5×10⁴ /well, under 100% humidity, and were cultured at 37 °C and 5% CO₂ for 24 h; different concentrations of AuNR@SiO₂-Glu(Cy5.5)-c(RGDyK) (0, 5, 10, 20, 30, 40 and 50 μg/mL, diluted in RPMI 1640) were then added to the wells. The cells were subsequently incubated for 24 h at 37 °C under 5% CO₂. Then, MTT (10 mL; 5 mg/mL) was added to each well and the plate was incubated for an additional 4 h at 37 °C under 5% CO₂. After the addition of 10% sodium dodecyl sulfate (SDS, 100 mL/well), the assay plate was allowed to stand at room-temperature for 12 h. The optical density OD570 value (Abs.) of each well, with background subtraction at 690 nm, was measured by means of a Tecan Infinite M200 monochromator-based multifunction microplate reader. The following formula was used to calculate the inhibition of cell growth: Cell viability (%) = (mean of Abs. value of treatment group/mean Abs. value of control) *100%.

**Fluorescence microscopy imaging.** U87-MG and MCF-7 cells were incubated with 10 μg/mL AuNR@SiO₂-Glu(Cy5.5)-c(RGDyK) in DMEM for 1 h at 37 °C, the cells washed with DMEM for 3 times, and then treated with DAPI for 10 min, cell imaging was then carried out after washing the cells with DMEM. Before and after irradiated with the 808 nm laser (0.1 W/cm²) for 4 min, the cell imaging was then carried out under excitation of 365 and 633 nm, the emission was collected by DAPI and Cy5.5 channels.

**Whole animal imaging.** The mice were treated by intratumoral injection of 50 μL of nanoprobe AuNR@SiO₂-Glu(Cy5.5)-c(RGDyK) (100 μg/mL), and then irradiated with the 808 nm (0.25 W/cm²) for different times (0, 3 and 6 min). After another 5 min at room temperature, whole animal imaging was carried out under excitation of 633 nm, and the emission was collected at 700±30 nm.

**ICP-OES samples:** The nanorod (20 μL) AuNR@SiO₂-Glu(Cy5.5)-c(RGDyK) was dissolved in 1 mL aqua regia for 24 h, and then diluted to volume (10 ml) with 5% HNO₃ solution. This concentration of this diluted solution was assayed for Au³⁺ by ICP-OES.

**Table S1.** Au³⁺ concentration in the sample of AuNR@SiO₂-Glu(Cy5.5)-c(RGDyK).
Fig. S1. The $^1$H NMR spectrum of Fmoc-Glu(COOH)-OtBu.

Fig. S2. The HPLC result of Fmoc-Glu(Cy5.5)-OtBu.
**Fig. S3.** The HPLC result of Fmoc-Glu(Cy5.5)-COOH.

**Fig. S4.** The HPLC result of Fmoc-Glu(Cy5.5)-c(RGDyK).

**Fig. S5.** The HPLC result of NH$_2$-Glu(Cy5.5)-c(RGDyK).

**Fig. S6.** The LCMS spectrum of Fmoc-Glu(Cy5.5)-OtBu.
Fig. S7. The LCMS spectrum of Fmoc-Glu(Cy5.5)-COOH.

Fig. S8. The LCMS spectrum of Fmoc-Glu(Cy5.5)-c(RGDyK).

Fig. S9. The LCMS spectrum of Fmoc-Glu(Cy5.5)-c(RGDyK).
Fig. S10. The \(^1\)H NMR spectrum of Si-NCS.

Fig. S11. FT-IR spectra of \(\text{NH}_2\)-Glu(Cy5.5)-c(RGDyK), AuNR@CTAB, AuNR@SiO\(_2\)-NCS, AuNR@SiO\(_2\)-Glu(Cy5.5)-c(RGDyK): The peaks (2916, 2849 cm\(^{-1}\)) corresponding to C–H stretching of CTAB molecules disappeared after SiO\(_2\)-NCS coating. Bands at 2969 and 2925 cm\(^{-1}\) were assigned to the asymmetric (\(\nu\)\text{as}) and symmetric (\(\nu\)\text{s}) stretching vibrations of the methylene (CH\(_2\)) group in the alkyl chain and bands at 1630 and 1550 cm\(^{-1}\) were attributed to the stretching vibration of the =C–H bond in the phenyl group of the attached \(\text{NH}_2\)-Glu(Cy5.5)-c(RGDyK).
**Fig. S12.** The absorption spectra of Cy5.5 in various concentration, insert: the standard curve between $\text{abs}_{675\text{ nm}}$ vs conc.Cy5.5.

![Absorption spectra](image)

**Fig. S13.** The model of the AuNR@SiO$_2$-Glu(Cy5.5)-c(RGDyK) to calculate the amount of dyes per nanoparticle.

\[
V_{\text{nanorod}} = \pi r^2 h = 1.4\times 10^4 \text{ nm}^3
\]

\[
m_{\text{nanorod}} = \rho V = 2.7\times 10^{-16} \text{ g}
\]

\[
M_{\text{nanorod}} = m 6.0\times 10^{23} = 1.6\times 10^8 \text{ g/mol}
\]

\[
\text{Abs}_{765\text{nm}} = 0.78 \quad c_{\text{Au}} = 31.1 \text{ ppm} = 31.1 \text{ mg/L}
\]

\[
c_{\text{nanorod}} = c_{\text{Au}}/M = 0.2 \text{ nM}
\]

\[
\varepsilon_{\text{nanorod}} = \text{Abs}/c_1 = 3.9\times 10^9 \text{ M}^{-1}\text{cm}^{-1}
\]

\[
c_{\text{dye}} = 1.1 \text{ \mu M}
\]

\[
dye/\text{nanorod} = 5500
\]
**Fig. S14.** The fluorescent emission spectra (top) and lifetime (bottom) of NH$_2$-Glu(Cy5.5)-c(RGDyK), AuNR@SiO$_2$-Glu(Cy5.5)-c(RGDyK) in the same concentration of Cy5.5.

**Fig. S15.** The fluorescent emission spectra NH$_2$-Glu(Cy5.5)-c(RGDyK) (1.1 μM, red line), and the physical mixture of NH$_2$-Glu(Cy5.5)-c(RGDyK) (1.1 μM), and AuNR@SiO$_2$-NCS (OD$_{765 \text{ nm}}$ = 0.78) (blue line).
Fig. S16. The stability of Cy5.5 under different conditions: (a) the emission of Cy5.5 at various pH values; (b) the emission of Cy5.5 under excitation of 808 nm laser for 40 min. (c) the emission of Cy5.5 at 80 °C for 40 min.
**Fig. S17.** The changes of AuNR@SiO$_2$-Glu(Cy5.5)-c(RGDyK) emission in different pH conditions.

**Fig. S18.** The changes of AuNR@SiO$_2$-Glu(Cy5.5)-c(RGDyK) (10 μg/mL) emission in different temperatures.
Fig. S19. (a) Photothermal property of the AuNR@SiO$_2$-Glu(Cy5.5)-c(RGDyK) (OD$_{808\text{ nm}} = 0.5$) in different 808 nm laser powers (0.1, 0.2, 0.3, 0.5, 0.8 W/cm$^2$), (b) photothermal cycling of the 808 nm laser on the AuNR@SiO$_2$@Glu(Cy5.5)-c(RGDyK) (OD$_{808\text{ nm}} = 0.5$) at power of 0.5 W/cm$^2$. 
After Edman degradation:

the released yield is x, the unreleased yield is (1-x)

the emission intensity should include in the emission of released dyes and unreleased dyes.

\[ I_{after} = I_{released} + I_{unreleased} \]

so 6.6 = 7x + 1 * (1-x)

\[ x = \frac{6.6-1}{7-1} = \frac{5.6}{6} = 0.933\% \]

Because the dyes/nanorod = 5749

So the released dyes/nanorod = 5749*0.933% = 5364

**Fig. S20.** The normalized emission of the pure NH\textsubscript{2}-Glu(Cy5.5)-c(RGDyK), AuNR@SiO\textsubscript{2}-Glu(Cy5.5)-c(RGDyK) before and after photothermally responsive Edman degradation (top), and their corresponding calculation of the released amount of dyes per nanoparticles (bottom).
**Fig. S21.** The absorption of the supernatant after Edman degradation of AuNR@SiO$_2$-Glu(Cy5.5)-c(RGDyK), insert: the standard curve between Abs$_{680}$ nm vs conc. Cy5.5.

**Fig. S22.** The HPLC chromatogram of the pure Cy5.5-NH$_2$. 
**Fig. S23.** In vitro cell viability of U87MG cells incubated with AuNR@SiO$_2$-Glu(Cy5.5)-c(RGDyK) at different concentrations (0-50 μg/mL) for 24 h.

**Fig. S24.** Fluorescence images of c(RGDyK) (a1, a2) blocked and unblocked (b1, b2) U87MG cells with nanoprobe AuNR@SiO$_2$-Glu(Cy5.5)-c(RGDyK) (10 μg/mL) without (a1, b1) and with (a2, b2) 808 nm laser irradiation at a power density of 0.1 W/cm$^2$ for 4 min.
In vitro cell viability of U87MG cells incubated with AuNR@SiO₂-Glu(Cy5.5)-c(RGDyK) (10 μg/mL) with irradiation of 808 nm laser (0.1 W/cm²) at different times (0, 2, 4 and 8 min).

**Fig. S25.**

**References**