Supporting Information

Dual-Functional, Receptor-Targeted Fluorogenic Probe for In Vivo Imaging of Extracellular Protease Expressions

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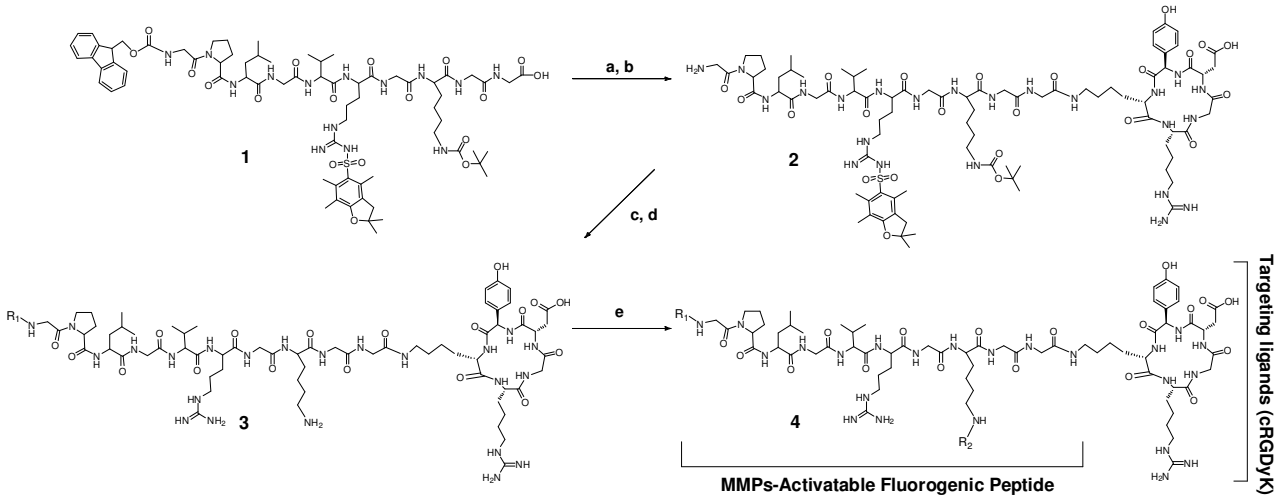
Cell culture. The U87MG human glioblastoma multiforme cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA). U87MG cells were cultured in DMEM medium containing 10% (v/v) fetal bovine serum (GIBCO, Grand Island, NY) supplemented with penicillin (100 μg/mL), streptomycin (100 μg/mL), non-essential amino acids (100 μM) and sodium pyruvate (1 mM) at 37 ˚C with 5% CO_2.

Animal models. Female athymic nude mice were supplied from Harlan (Indianapolis, IN) at 4 to 5 wk of age. U87MG tumor model was generated by subcutaneous injection of 5×10^6 cells in Matrigel into the right front flank of female athymic nude mice at a volume of about 80 μL. The mice were used for optical studies when the tumor volume reached about 300 mm^3. All animal studies were conducted in accordance with the principles and procedures outlined in the

Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Clinical Center, NIH.

Peptide probes. The peptide sequence of 1 was synthesized by standard solid-phase Fmoc peptide chemistry. To synthesize 4 (Scheme S1), 5 mg of 1 was dissolved in 500 µl N,N-dimethylformamide (DMF) solution containing 2% diisopropylethyalamine (DIPEA). 2 mg N,N,N',N'-Bis(tetramethylene)-O-(N-succinimidyl)uronium hexafluorophosphate (HSPyU) was added into the solution to activate the C-terminal carboxylic group and stirred for 30 min at room temperature. Next, 2.5 mg of c(RGDyK) was dissolved in 250 µl DMF solution containing 2% DIPEA and mixed with the activated 1. The reaction mixture was stirred at room temperature and monitored by analytical HPLC. To remove the Fmoc group, 10% piperidine was added into the solution and stirred for 30 min. The crude product was precipitated in cold ether and the pellet was lyophilized and purified by preparative HPLC. The collected fraction 2 was lyophilized and labelled with Cy5.5 NHS ester in DMF solution containing 2% DIPEA. The reaction solution was precipitated against cold ether, lyophilized, and subjected to TFA/H2O/TIS/EDT (85/5/5/5, v/v/v/v) cocktail to remove the protection groups. Crude product was purified by preparative HPLC. The desired fraction containing 3 was collected and lyophilized. In the last step, BHQ-3 NHS ester was conjugated and the final compound 4 was was purified by HPLC on a C18 semi-preparative column using a linear gradient of 10% to 55% acetonitrile/water (0.1% trifluoroacetic acid), for 30 min at a 10 mL/min flow rate. 5 and 6 were synthesized by serial conjugations and deprotections of the peptides as described followed by preparative HPLC purifications. 7 was synthesized by conjugation of CW800 NHS ester and c(RGDyK) in DMF solution containing 2% DIPEA. The reaction solution was precipitated

against cold ether, lyophilized, and purified by HPLC. All purified products were confirmed by analytical HPLC and LC/MS. Figure S1 shows the representative HPLC and LC/MS spectra of 4.



Scheme S1. Synthesis of 4. a) HSPyU in DMF containing 2% DIPEA, c(RGDyK); b) 10% piperidine; c) Cy5.5 NHS ester in DMF containing 2% DIPEA; d) TFA/H_2O/TIS/EDT (85/5/5/5, v/v/v/v); e) BHQ-3 NHS ester in DMF containing 2% DIPEA

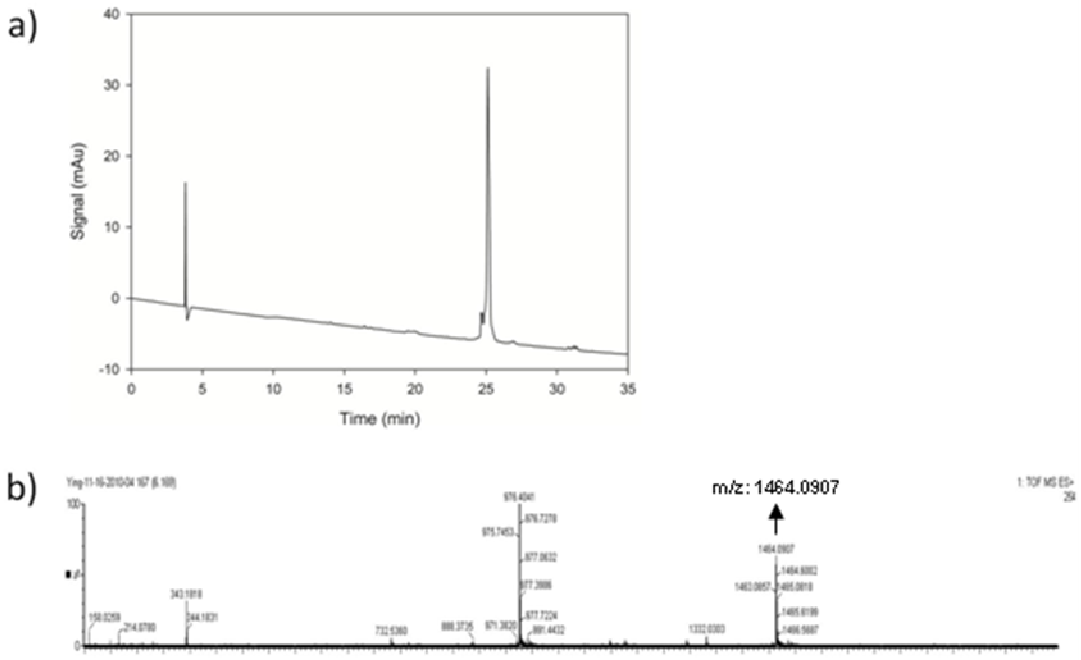


Figure S1. (a) HPLC and (b) LC/MS spectra of 4. MS calculated/found; 2926.20/2926.18

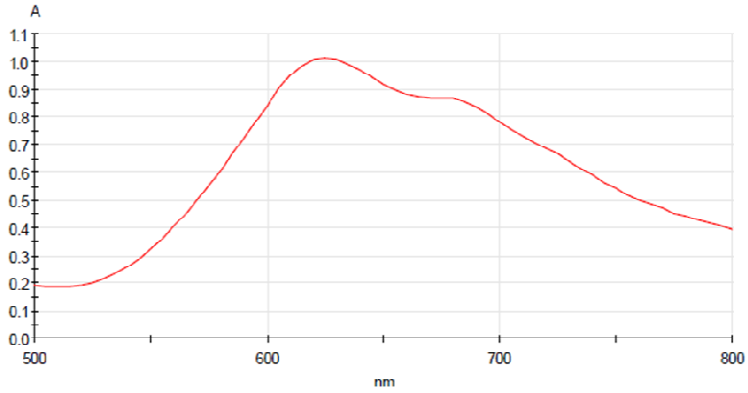


Figure S2. UV-Vis spectrum of 4 in PBS

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In vitro enzyme test. The fluorogenic property of the peptide probes was examined by incubating the probes in the reaction buffer (100 mM Tris, 200 mM NaCl, 5 mM $CaCl_2$, 0.1% Brij, pH 7.5) containing 40 nM of APMA-activated recombinant human MMP-2. Fluorescence intensity was monitored using a spectrofluorometer (F-7000 Fluorescence Spectrophotometer, Hitach, Tokyo, Japan) every 10 min at 37 °C using quartzose cuvette. The excitation wavelength

was set at 675 nm and emission spectra recorded from 680 to 800 nm. The same experimental conditions were applied to various concentrations of MMP-2 in the presence of fixed concentrations of the probes.

Receptor binding assay. Briefly, U87MG cell line was cultured in DMEM medium containing 10% (v/v) fetal bovine serum supplemented with penicillin (100 μg/mL), streptomycin (100 μg/mL), non-essential amino acids (100 mM) and sodium pyruvate (1 mM) at 37˚C with 5% CO_2. After reaching 80% confluence, cells were scraped off and suspended with binding buffer (25 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol, hydrochloride (Tris-HCl), pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 1 mM $MnCl_2$, 0.1% bovine serum albumin (BSA)) at a final concentration of 4×10^6 cells/mL. In a 96-well plate, 2×10^5 U87MG cells/well were incubated with ^{125}I-Echistatin (0.02 mCi/well, PerkinElmer Inc.) in binding buffer in the presence of different concentrations of c(RGDyK) and the peptide probes at room temperature for 2 hrs. After incubation, the plate was washed three times with PBS containing 0.1% BSA, and the radioactivity was measured by γ-counting. The IC_{50} values were calculated by nonlinear regression analysis.

***In vivo* optical imaging.** Optical imaging acquirement and analysis were done using Maestro 2.10 in vivo imaging system (Cambridge Research & Instrumentation, Woburn, MA, USA). U87MG tumor-bearing mice were injected via tail vein with **4**, 5, **6**, **7**, and **4** with MMP inhibitor or unlabeled c(RGDyK), respectively, under isoflurane anaesthesia (n = 3 per group).

The molar concentration of each samples were calculated by the sample weight. 1, 2, 3, 4 hrs post-injection, the mice were subjected to optical imaging using appropriate filters for Cy5.5 and CW800. For the inhibition experiment, MMP inhibitor was intratumorally injected into tumor-bearing mice 30 minutes before the probe injection. After image acquisition, spectral unmixing yielded the pseudocolor images of the pure spectrum of respective dyes. Images were normalized and analyzed using Maestro software. For quantitative comparison, regions of interest (ROI) were drawn over tumors and muscle, and the average signal (10^6 photons×cm^{-2}×s^{-1}) for each area was measured. Values were expressed as mean ± SD for a group of three animals.

***Ex vivo* biodistribution.** *Ex vivo* imaging of excised tumor and organs further confirm the targeting specificity and activation of the probes (Figure S4). At 4 hrs post-injection, the mice were sacrificed and major organ tissues and tumors were harvested, and placed on black paper for *ex vivo* imaging. For quantitative comparison, ROIs were drawn over tumors and the other organs, and the average signal for each area was measured. The results were presented as the average scaled signal from the organs and tumors. Values were expressed as mean ± SD for a group of three animals.

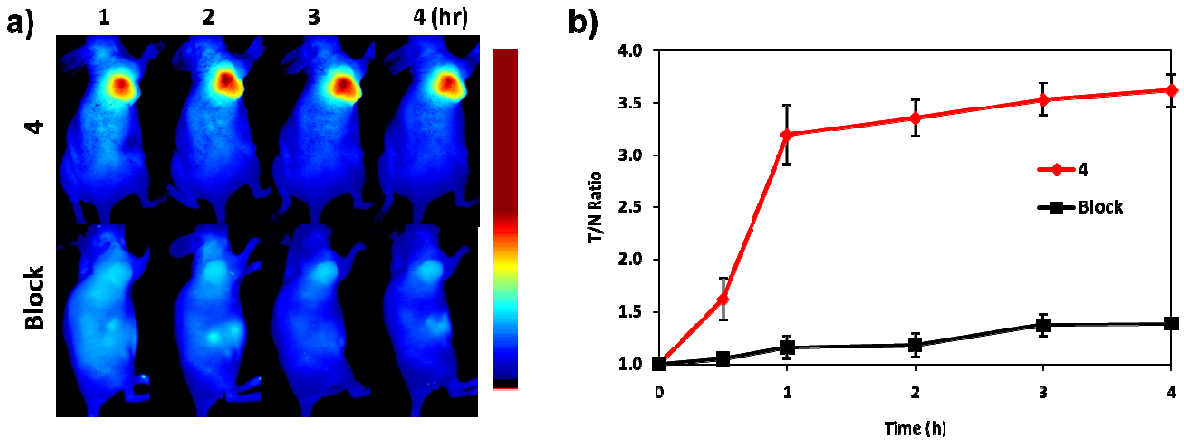
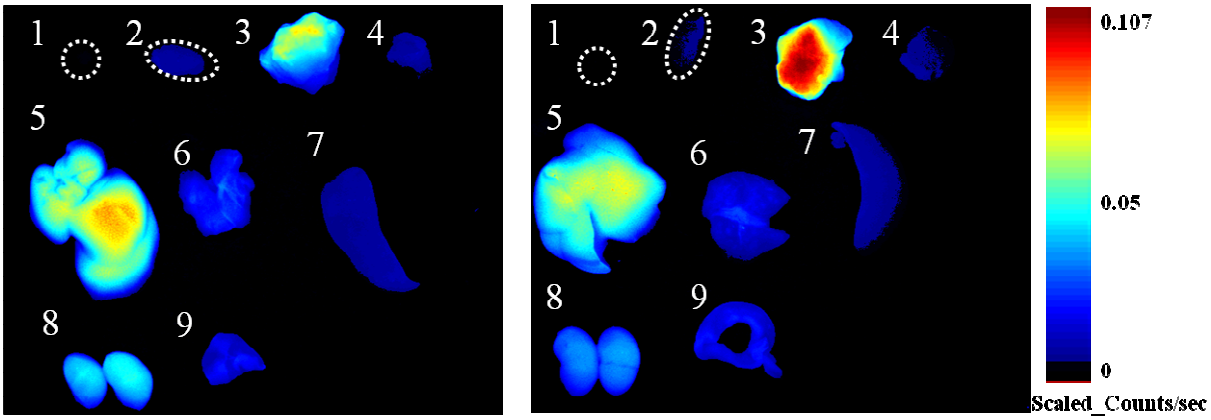


Figure S3. (a) Representative serial in vivo NIR fluorescence images of U87MG tumor-bearing mice injected intravenously with **4** and **4** with unlabeled c(RGDyK) (block). (b) T/N ratio analysis of NIR fluorescence intensity of U87MG tumor *in vivo*.



1. Blood;2. Heart; 3. Tumor; 4. Muscle; 5. Liver; 6. Lung; 7. Spleen; 8. Kidney; 9. Intestines

Figure S4. Biodistribution of **4** (left) and **6** (right).

Immunohitochemistry. Frozen U87MG tumor tissue slices (4 μm) from the tumor-bearing nude mice were fixed with cold acetone for 20 min and dried in air for 30 min at room temperature. After blocking with 10% BSA for 30 min, the sections were incubated with Rabbit anti-integrin αvβ3 and Rabbit anti-MMP-2 and antibodies (10 μg/mL) for 60 min at room temperature in the dark. Samples were then visualized with FITC-conjugated donkey anti-rabbit secondary antibody. Finally, the slices were mounted with DAPI-containing mounting medium under an epifluorescence microscope (Olympus, X81). The tumor slices were also observed directly after mounting with DAPI-containing mounting medium under an epifluorescence microscope and fluorescence images were taken using Cy5.5 filter settings.

Statistical analysis. Results were expressed as mean ± SD. Two-tailed paired and unpaired Student's t tests were used to test differences within groups and between groups, respectively. *P* values < 0.05 were considered statistically significant.