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Supporting Information

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Hybrid Ferritin Nanoparticles as Activatable Probes for Tumor Imaging**

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Experimental Section

Ferritin expression: Ferritin gene was amplified by PCR from cDNA using the forward (5'-ATA TAC CAT GGG CAC CAC CGC GT-3') and reverse (5'-CCA GAC TCG AGT TAG CTC TCA TCA-3') primers to introduce NcoI and XhoI restriction sites flanking the normal start and stop codons, respectively. The double digested PCR product was ligated into NcoI/XhoI double digested plasmid pRSF with T4 DNA ligase. The ligation mixture was then transformed into competent cells of Escherichia coli XL1_Blue by standard protocols. The resulting pRSF/Fn plasmids, which were screened by appropriate restriction digests and verified by DNA sequencing, were transformed into expression strain E. coli BL21(DE3). A 1-L LB-kanamycin (50 μg/mL) culture of E. coli BL21(DE3)/Fn was grown at 37 °C to an OD600 of 0.8, then induced with 1 mM IPTG at 37 °C for 4 h. After sonication, the cell lysate was centrifuged at 10,400 rpm (12,930 g) for 30 min to remove the cell debris. The supernatant was heated at 60 °C for 10 min, and the resultant new supernatant was subjected to HPLC purification using a size exclusion column (Superose 6).

MMP-13 expression: His-tagged MMP-13 protein was expressed in E. coli strain BL21 (DE3) (Novagen, Darmstadt, Germany) induced by 1 mM IPTG at 37°C for 4 hours. The lysates were then centrifuged at 12,000 rpm for 5 min at 4°C, and the inclusion body was dissolved by solubilization buffer (20 mM Tris·Cl at pH 8.0, 8 M Urea, 500 mM NaCl) and purified by Ni-NTA column. The purified denatured protein was refolded by dialysis against refolding buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$ and 0.05% Brij-35) at 4 ˚C. The refolded protein was analyzed with 12% SDS-PAGE.

Bioconjugation: In a typical coupling, N-succinimidyl-4-Maleimidobutyrate (TCI America, 600 nmol in 6 μL DMSO) was added into a ferritin solution in PBS (60 nmol, 500 μl). The pH was monitored to make sure it was around 7.4. The mixture was incubated in a 1.5 ml eppendorf tube at room temperature for 1 h with gentle shaking. The conjugated protein was purified by NAP-5 column (GE Healthcare Life Sciences) pre-equilibrated with PBS buffer, and was concentrated to 0.5 ml by YM-10 centricon. Subsequently, a total of 60 nmol Cy5.5-tagged peptide (Cy5.5-Gly-Pro-Leu-Gly-Val-Arg-Gly-Cys)[1] in PBS was added into the conjugated protein solution, and the reaction proceeded at room temperature for 1 h with gentle shaking. The resulted C-Fn protein was purified by NAP-5 column pre-equilibrated with PBS buffer. For quencher conjugation, BHQ-3 Carboxylic Acid, Succinimidyl Ester (Biosearch Technologies, 300 nmol in 10 μL DMSO) was added into a ferritin solution (60 nmol in 500 μl PBS). The mixture was incubated in an eppendorf tube at room temperature for 1 h with gentle shaking. The conjugated protein was purified by NAP-5 column pre-equilibrated with PBS buffer.

Fluorescent intensity change of hybrid ferritin: The fluorescence activity change of the ferritin particles was tested in 1 ml cuvette. C-Fn and B-Fn (0.43 μM for each) were mixed in Tris buffer (50 mM Tris, 10 mM calcium chloride, 150 mM NaCl, pH=2). After about 10 min, the pH was adjusted to 7.4 with 1 M NaOH. After the protein cage was reassembled, MMP-13 was added to the solution (final concentration of 100 nM). Fluorescence signal intensity was detected with a Hitachi F-7000 spectrofluorometer (Hitachi, Japan) in the "Time Scan" mode. The excitation and emission wavelengths were fixed at 675 nm and 690 nm, respectively.

***In vivo* NIRF imaging:** All animal studies were performed in compliance with the procedures that were approved by the Institutional Animal Care and Use Committee of the Clinical Center, National Institutes of Health. For animal model preparation, about 1 million cells were inoculated subcutaneously into the right flank of mice and the imaging was performed about one week later when the tumor reached a size of 0.5 cm^3. For imaging, a total of 0.02 mg ferritin (C/B=1:1) in 100 μl was injected intratumorally and the imaging was performed at 30, 60, 120, and 240 min post injection on a Maestro all-optical imaging system. For the control group, MMP inhibitor III (EMD Biosciences) was injected into the tumor 30 min prior to the probe injection. After the 240-min imaging, the mice were scarified, and the tumors were collected and imaged with the Maestro imaging system.

Mass spectrum analysis of ferritin, C-Fn and B-Fn: The mass analysis was performed on a Waters LC-MS system (Waters) consisted with an Acquity UPLC system and a Waters Q-Tof Premier high resolution mass spectrometer. Ion detection was achieved in ESI mode using a source capillary voltage of 3.5 kV, source temperature of 110 ˚C, desolvation temperature of 200 ˚C, cone gas flow of 50 L/Hr (N_2), and desolvation gas flow of 700 L/Hr (N_2). The spectra were transformed into a mass scale using MaxEnt 1 software (Waters).

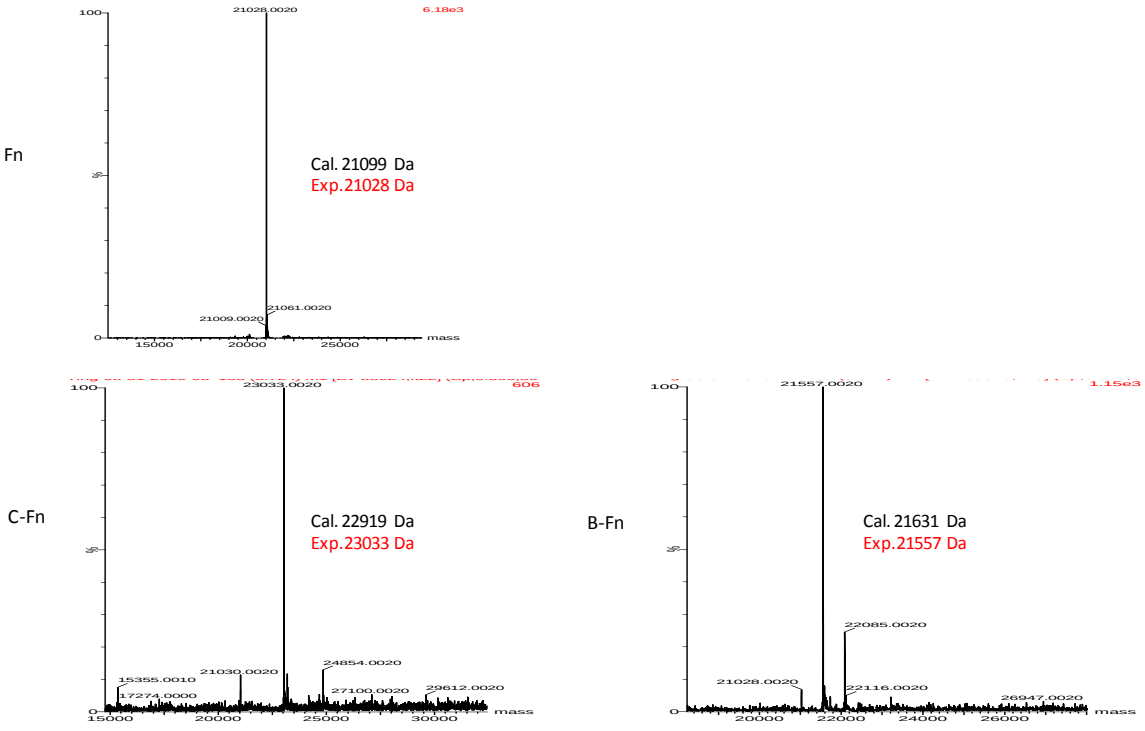

Figure S1. Mass spectrum analysis of ferritin, C-Fn and B-Fn.

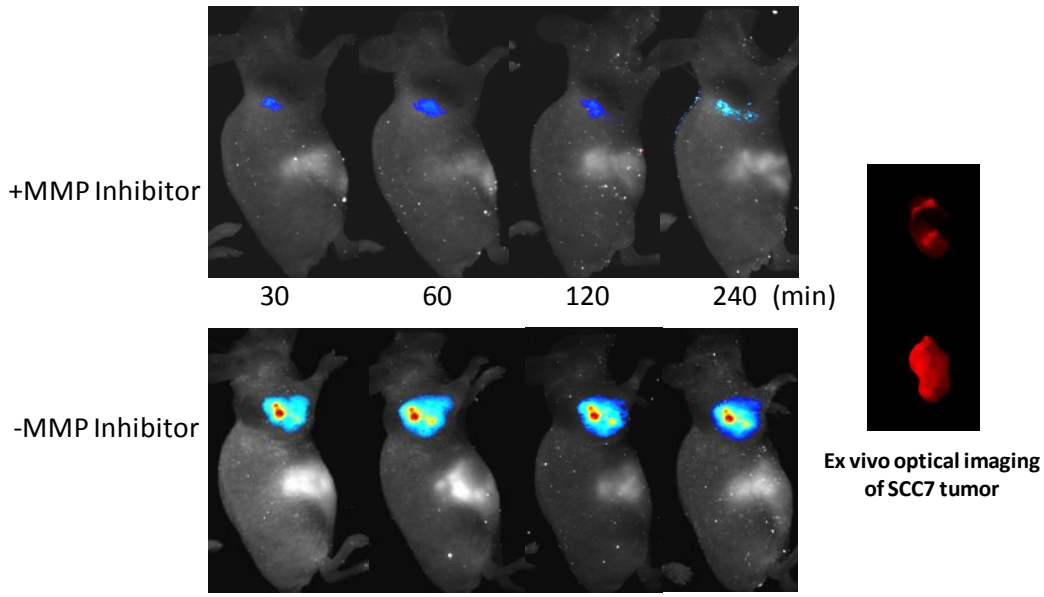

Figure S2. *In vivo* NIRF imaging with a SCC-7 (squamous cell carcinoma) xenograft model.

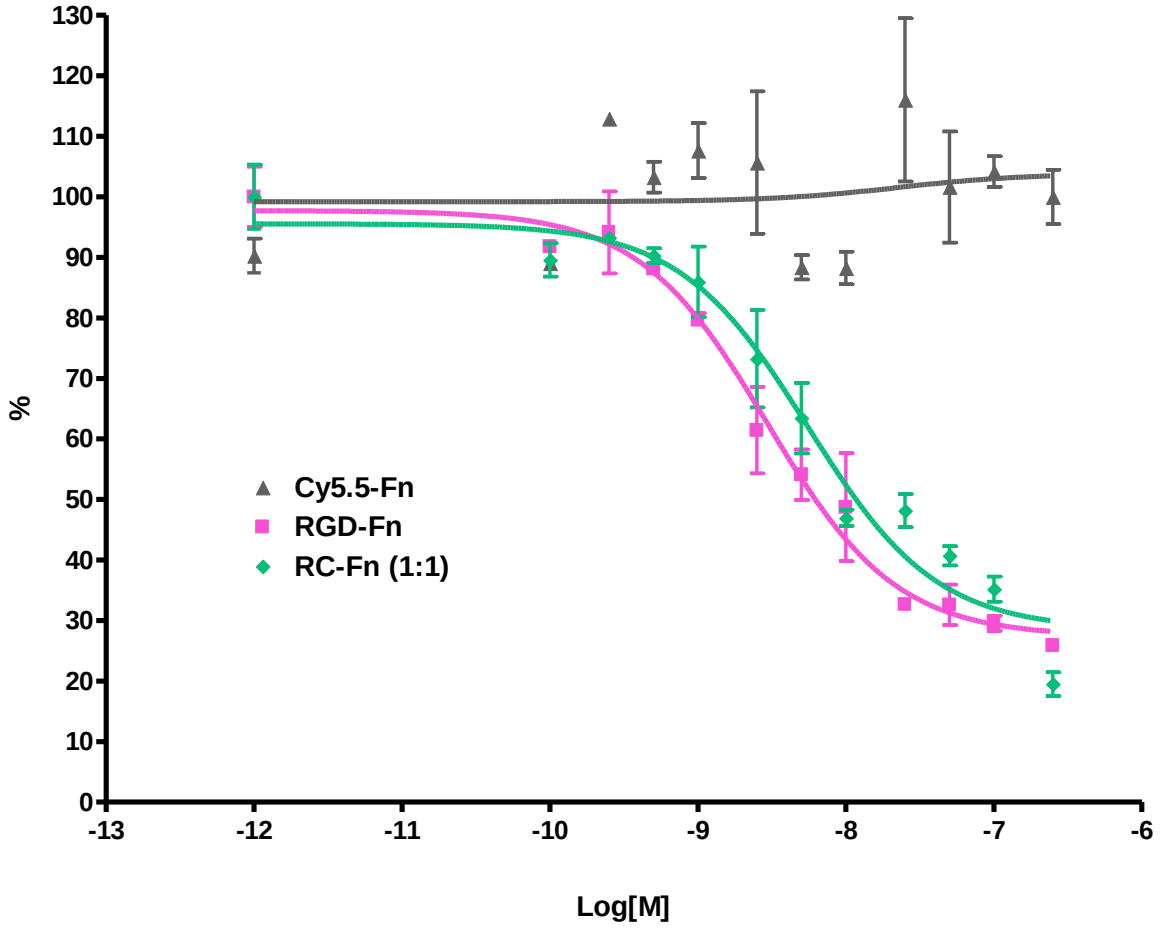

Figure S3. Competitive cell binding assay results. This assay was performed on MDA-MD-435 cells using ^{125}I-echistatin as the competitive ligand. It was performed by following a previously published protocol.[2] RGD-ferritin (R-Fn) was prepared by following a previously published protocol.[3] Cy5.5 conjugated ferritin (Cy5.5-Fn) was prepared by incubating Cy5.5-NHS with ferritin in PBS buffer. The hybrid RGD-Cy5.5-ferritin (RC-Fn) was prepared by mixing the same amount of Cy5.5-Fn and R-Fn and subjecting the mixture to the disassembly/reassembly process. The best-fit 50% inhibitory concentration (IC_{50}) values for MDA-MD-435 were calculated by fitting the data with nonlinear regression using GraphPad Prism (GraphPad Software, Inc.). Cy5.5-Fn showed no binding towards integrin $\alpha_v\beta_3$. The IC_{50}s of R-Fn and RC-Fn were evaluated to be 2.9 nM and 5.5 nM, respectively (based on ferritin monomer concentration). Experiments were performed on triplicate samples.

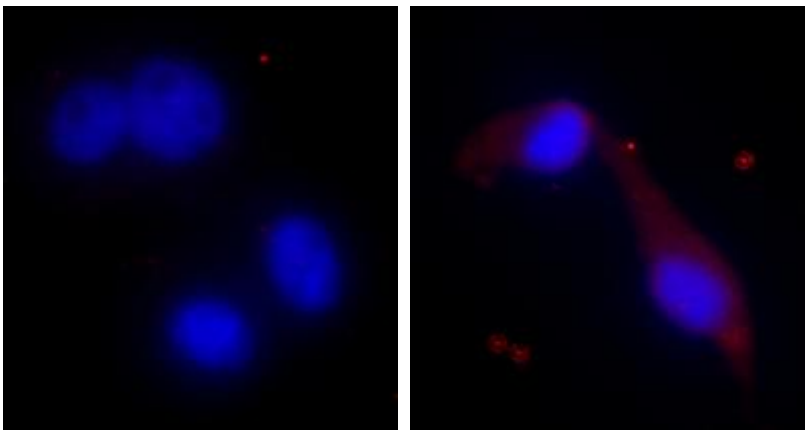

Figure S4. *In vitro* fluorescence staining results. U87MG cells were fixed with cold ethanol at -20 ℃ for 20 min and were blocked with 10% BSA for 60 min at room temperature. The cells were then incubated with Cy5.5-ferritin (Cy5.5-Fn) or RGD-Cy5.5-ferritin (RC-Fn, R/C = 1:1) at the same Cy5.5 concentration (40 nM) for 1 h in the dark. After five washing steps, the cells were mounted with 4', 6-diamidino-2-phenylindole (DAPI)-containing mounting medium and were observed with an epifluorescence microscope (Olympus, X81). In accordance with the binding affinity results, we observed a significant difference in cell staining between the Cy5.5-Fn and RC-Fn groups. Blue, DAPI; Red, Cy5.5.

References:

[1] S. Lee, E. J. Cha, K. Park, S. Y. Lee, J. K. Hong, I. C. Sun, S. Y. Kim, K. Choi, I. C. Kwon, K. Kim, C. H. Ahn, *Angew. Chem. Int. Ed. Engl.* **2008**, *47*, 2804.

[2] Y. Wu, X. Zhang, Z. Xiong, Z. Cheng, D. R. Fisher, S. Liu, S. S. Gambhir, X. Chen, *J. Nucl. Med.* **2005**, *46*, 1707.

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