Supporting Information

Dual-Ratiometric Target-Triggered Fluorescent Probe for Simultaneous Quantitative Visualization of Tumor Microenvironment Protease Activity and pH *in vivo*

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Synthesis of hydrophobic Fe_3O_4 nanoparticles. Fe_3O_4 nanoparticles with core size of 7.2 nm were synthesized according to a previous report.[S1] In brief, 3.6 g (4 mmol) of freshly prepared iron oleate and 3.39 g (4 mmol) of oleic acid were dissolved in 25 mL of 1-octadecene. The resulting solution was quickly heated to 310°C and then maintained at 310°C for 30 min under nitrogen protection. The preparation was terminated by cooling the reaction mixture down to room temperature. The resulting nanoparticles were precipitated by acetone, collected by magnetic separation, washed with acetone for several times, and finally re-dispersed in tetrahydrofuran (THF) or cyclohexane for further experiments.

Ligand exchange. As a typical example, 150 mg of asymmetric PEG2000 ligand bearing a diphosphate group at one end and a maleimide group at the other end (mal-PEG-dp) was dissolved in 10 mL of THF containing 10 mg hydrophobic Fe_3O_4 nanoparticles. Then, the reaction mixture was heated to 60°C and kept at this temperature under stirring for 12 h. After that, the Fe_3O_4 nanoparticles were precipitated and washed using cyclohexane for three times, and then dried under vacuum at room temperature. To remove excess PEG ligand, the resultant aqueous solutions containing the PEGylated Fe_3O_4 nanoparticles were purified through ultrafiltration for 4 cycles using 100 kDa MWCO centrifugal filter (Millipore YM-100) at 2500 g.

Specific binding assays for dual-ratiometric nanoprobes. Fluorescence microscopy studies were carried to qualitatively evaluate the binding specificity of the nanoprobes to LS180 cells. In detail, approximately 1×10^5 LS180 cells were seeded in the wells of two 8-well chamber slides and incubated overnight at 37°C under 5% CO_2 to allow firm adherence. After being rinsed with PBS, the cells were incubated with the nanoprobes at 37°C under 5% CO_2 for 6 h. After that, the cells were rinsed three times with PBS. The fluorescence micrographs were captured with a fluorescence microscope (Olympus X71).

The fluorescence spectroscopy measurements were performed on a microplate reader

(Thermo, Varioskan Flash). In brief, cells were seeded into a 96-well cell culture plate by 1×10^5 cells/well under 100% humidity and incubated overnight at 37°C under 5% CO_2 to allow firm adherence. After being rinsed with PBS, the nanoprobes were added at different time points to different wells, and the cells were incubated with the nanoprobes at 37°C under 5% CO_2. The cells were rinsed for three times with PBS for the following fluorescence spectroscopy measurements after being incubated with the nanoprobes for different periods of time.

Animal tumor model. The tumor models were established upon subcutaneous injections of LS180 cells ($\sim5\times10^6$) into 4-6 weeks old male BALB/c nude mice at the flank region of the right hind legs. The tumor imaging studies were carried out 5-7 d after the inoculation of tumor cells.

Fluorescence imaging of tumors *in vivo*. The fluorescence images of nude mice bearing subcutaneous were acquired with IVIS® Spectrum in vivo imaging system (PerkinElmer Inc., Waltham, Massachusetts). In detail, the nude mice were anesthetized, and then the probes were intravenously injected through tail vein (10 mg of Fe per kilogram body weight). For imaging through ANNA, excitation light of 465-500 nm was adopted, while a 500 nm long-pass filter and was used for collecting the emissions. For imaging through Cy5.5, narrow band filter of 640-675 nm and long-pass filter of 680 nm were used for excitation and emission, respectively, and the exposure time was set as 300 ms. The fluorescence images were analyzed with vendor software to separate autofluorescence from chromophore signals through spectral unmixing algorithms. For pH mapping through emission of ANNA, the fluorescence images based on 500 and 540 nm channels were used for achieving I_{500}/I_{540} ratio mapping. According to the relationship between I_{500}/I_{540} ratio and pH (Figure S5), the tumor pH mapping was finally obtained by taking the absorption of tissue and skin into consideration.[S2] For MMP-9 mapping, the integrated fluorescence signal of ANNA was adopted and normalized with reference to the Cy5.5 signal.

MR imaging of tumor *in vivo*. The MR images were acquired on a 7 T animal MRI instrument (Bruker BioSpec 70/20). Nude mice bearing LS180 tumor xenografts were anesthetized and then the nanoprobe (10 mg Fe per kilogram body weight) was injected through tail vein. T_2-weighted images were acquired at designed time points. The detailed parameters for T_2 imaging were set as follows: FOV= 3 × 3 cm^2, matrix size = 256 × 256, slice thickness = 1 mm, TE = 15, 30, 45, 60, 75, 90, 105, 120 ms, TR= 3000 ms, and NEX = 2. The mice were anesthetized with 1% isoflurane delivered via a nose cone during the imaging sessions.

Histopathological and immunohistochemical assays. Three adjacent slices of the tumor harvested after imaging studies were prepared. The first one was stained with H&E following the standard protocol. The second one was incubated with rabbit anti-MMP-9 antibody followed by horseradish peroxidase (HRP)-goat-anti-rabbit secondary antibody, then 3,3'-diaminobenzidine (DAB) was introduced for eventually staining MMP-9, while the cell nucleus were stained with haematoxylin. The third one was subjected to Prussian blue staining to show the distribution of Fe_3O_4-based probes delivered through tail vein according to standard clinical pathology protocols.

Histopathological and immunofluorescence assays. Three adjacent slices of a tumor harvested after imaging studies were prepared. The first one was stained with H&E following the standard protocol. The second one was incubated with rabbit anti-E-cadherin antibody followed

by Cy3 labeled goat-anti-rabbit secondary antibody for staining E-cadherin, while the cell nucleus were stained with 4',6-diamidino-2-phenylindole (DAPI). The third one was incubated with rabbit anti-MMP-9 antibody, followed by FITC labeled goat-anti-rabbit secondary antibody for eventually showing MMP-9, and the cell nucleus were stained DAPI. The immunofluorescence images of the second and third slices were emerged comparing with H&E staining image as given in Figure 5e in the bodytext.

Determination of number of FA, ANNA, Cy5.5 molecules per Fe_3O_4 particle. UV-Vis absorption spectroscopy was used for determining the number of different functional moieties per particle. The total concentration of Fe_3O_4 particles was determined through 1,10-phenanthroline spectrophotometric method as previously descripted.[S3] The number of folic acid per particle was calculated by subtracting the amount of the un-coupled folic acid molecules in the ultrafiltrate, collected after the purification, from the initial feeding amount of folic acid. The number of ANNA per particle was determined after the final probe was dissolved with HCl to exclude the interference of Fe_3O_4 absorption, while the amount of Cy5.5 was directly measured through UV-Vis spectroscopy.

Determination of MMP-9 expression *in vitro.* The expression level of MMP-9 by tumor tissues was determined through an indirect colorimetric method (MMP-9 assay kit, GMS40057.2 v.a, GENMED SCIENTIFICS INC. USA). The tumor tissues extracted were frozen in liquid nitrogen and stored at -70°C until use. Firstly, the tissues were grinded and homogenized with ice-cold lysis buffer. After centrifugation at 12,000 g for 10 min at 4°C, the soluble fraction obtained was subjected to Bradford assay for determining the total protein concentration. Then, the MMP-9 content was determined as follows. The anti-MMP-9 antibody was immobilized in the 96-well plate upon incubation for 16 h at 4°C and rinsed with PBS buffer. The tissue lysate was then incubated in plate for 60 min at 37°C and rinsed with PBS buffer. The MMP-9 immobilized was activated and incubated with thiopeptide substrate for 60 min at 37°C. The hydrolysis of thiopeptide substrate (Ac-PLG-[2-mercapto-4-methyl-pentanoyl]-LG-OC_2H_5) containing a thioester bond was catalyzed by MMP-9 enzyme present in the above soluble fraction. Then the reaction product was reacted with Ellman's reagent 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB) to form 5-mercapto-2-nitrobenzoic acid (TNB) that exhibits a characteristic absorption at 412nm.[S4] By comparing with a standard curve, the MMP-9 expression level was determined and normalized according to the total protein concentration.

Determination of relative MMP-9 activity and pH *in vivo.* The fluorescence of Cy5.5 and ANNA was recorded from the tumorous region, respectively, with IVIS® Spectrum in vivo imaging system (PerkinElmer Inc., Waltham, Massachusetts). The volume of tumors was measured according to previous reference.[S5] The MMP-9 activity in tumors of different sizes was calculated by normalizing the protease activity (obtained by comparing integrated fluorescence signal of ANNA with that of Cy5.5) according to the tumor volume. The microenvironmental pH of tumors of different sizes was extracted according to the strongest signal of ANNA emission from the tumor site.[S2]

Protease activity against tumor microenvironmental pH. The dual-ratiometric fluorescent nanoprobe was intravenously injected into tumor-bearing mice. Then, Cy5.5 and ANNA emissions

were recorded 50 min postinjection. After that, through intra-tumoral injection of different PBS buffers (6.2 or 7.4) the tumor microenvironmental pH was adjusted. Right after the buffer injection, the tumors was imaged at designed time points to detect time-dependent protease activity *in vivo*. The above experiments were repeated once again for the same tumors with a time interval of 90 min between two PBS shots.

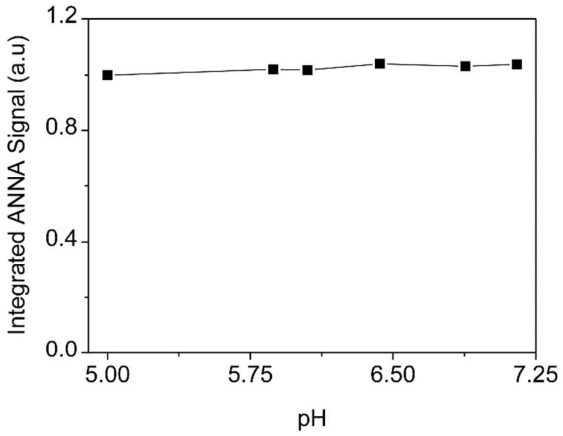

Figure S1. pH-independent ANNA emission integrated from 470 nm to 600 nm. The excitation is 455 nm.

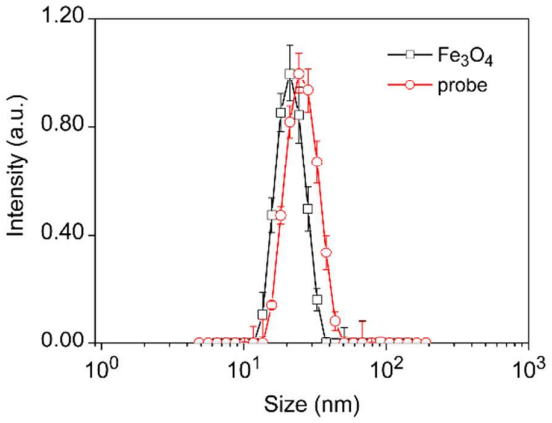

Figure S2. Size distribution profile of the dual-ratiometric probes (red) for comparison with that of the mother PEGylated Fe_3O_4 particles (black) determined by DLS method.

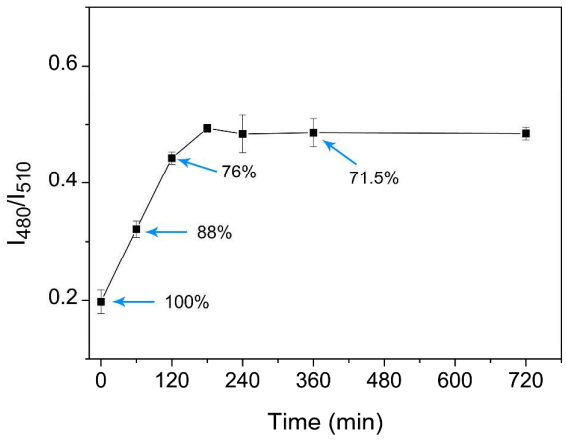

Figure S3. The temporal evolution of I_{480}/I_{510} ratio of ANNA emission recorded during the incubation of the dual-ratiometric fluorescent probe with LS180 cells in 1×PBS, for calculating the percentage of chromophores remaining attached on the cell surface after the overall probe is activated by MMP-9, assuming that lysosomal process following endocytosis of the nanoprobes is involved during the incubation. The pH value of lysosome was taken as 5 according to literature for calculating the percentage of chromophores attached on membrane as inserted.

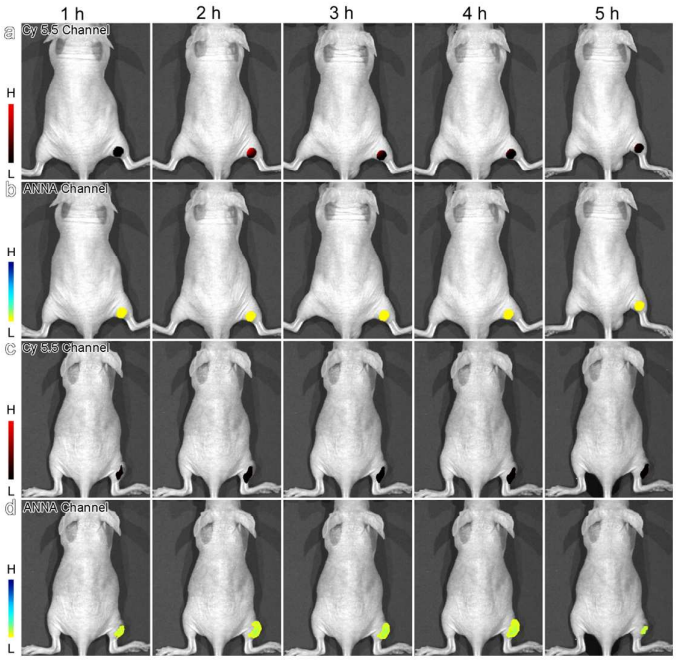

Figure S4. Color-coded fluorescence images of tumor-bearing mice captured after intravenous injection of the control probe (1) constructed using mIgG instead of folic acid (a-b) or control probe (2) bearing no folic acid on the particle surface (c-d) through Cy5.5 and ANNA imaging channels, respectively.

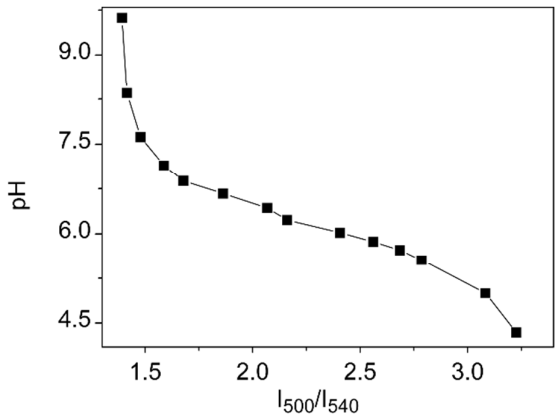

Figure S5. Relationship between the microenvironmental pH and the normalized I_{500}/I_{540} for *in vivo* pH mapping.

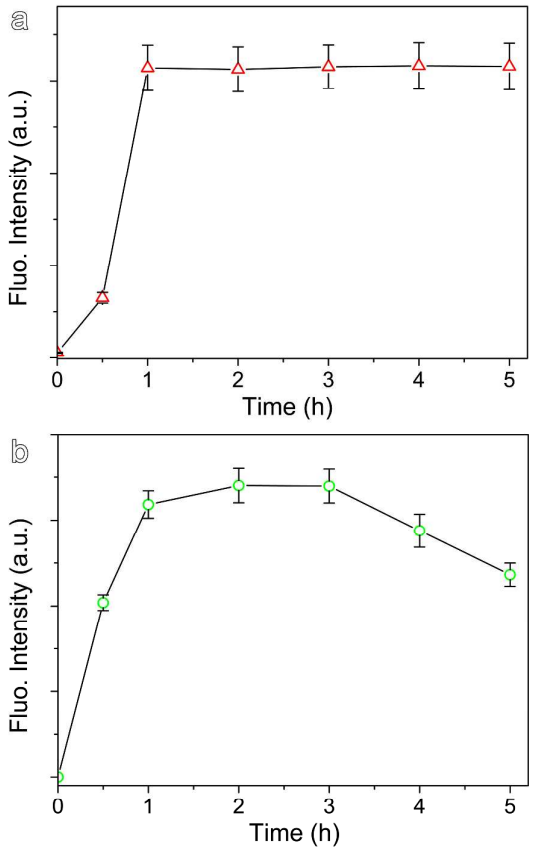

Figure S6. Temporal evolutions of the integrated fluorescence intensity of Cy5.5 (a) and ANNA (b) at the tumorous region.

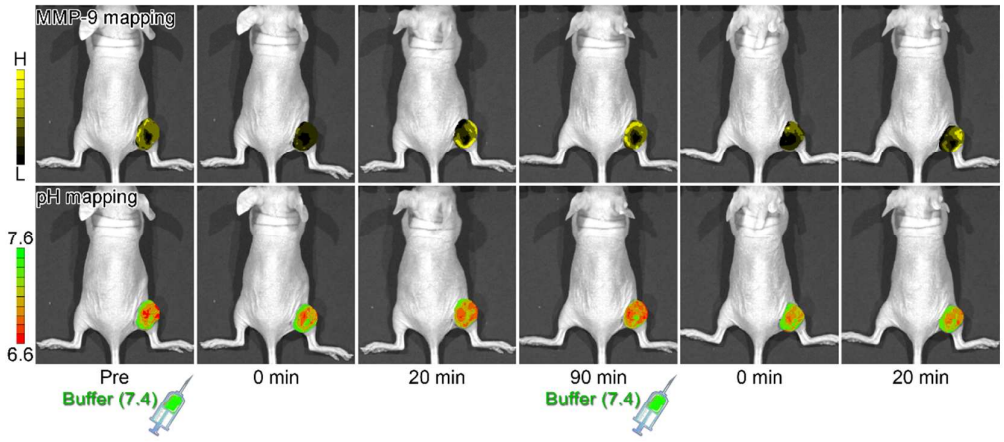

Figure S7. Variation of MMP-9 activities in response to tumor microenvironmental pH adjusted upon intra-tumoral injection of PBS buffer (pH 7.4).

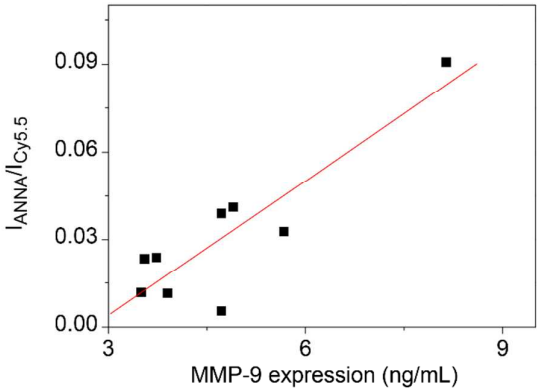

Figure S8. The relationship between the expression level of MMP-9 in tumors of different sizes and $I_{ANNA}/I_{Cy5.5}$ (relative MMP-9 activity) of the corresponding tumors determined through the dual ratiometric fluorescent probe. The red line is a linear fit.

Reference:

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