Supporting Information

A folate receptor-specific activatable probe for near-infrared fluorescence imaging of ovarian cancer

Hawon Lee[a†], Jisu Kim[a†], Hyunjin Kim[a], Youngmi Kim[b] and Yongdoo Choi *[,a]

[a] Molecular Imaging & Therapy Branch, Division of Convergence Technology, National Cancer Center, 323 Ilsan-ro, Ilsandong-gu, Goyang, Gyeonggi-do, Republic of Korea

[b] Department of Chemistry, Institute of Nanosensor and Biotechnology, Dankook University, 126 Jukjeon-dong, Yongin-si, Gyeonggi-do 448-701, Republic of Korea

[*Corresponding author]
Yongdoo Choi, Ph. D.
Tel: +82-31-920-2512
E-mail: ydchoi@ncc.re.kr

† Contributed equally to this work

Materials and Methods

Materials

ATTO655-COOH was purchased from ATTO-TEC GmbH (Siegen, Germany). E64 and E64d (water-soluble and cell-permeable cathepsin B inhibitors) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Folic acid (FA) was obtained from Sigma-Aldrich. Singlet Oxygen Sensor Green (SOSG), which is a singlet oxygen-detecting reagent, was obtained from Life Technologies (Grand Island, NY, USA). Cathepsin B (from human liver), cathepsin S (from human spleen), and cathepsin L (from human liver) were obtained from Calbiochem (La Jolla, CA, USA). Fetal bovine serum (FBS) and the RPMI 1640 cell culture medium were purchased from Life Technologies. The human ovarian carcinoma cell line SKOV3 and the human lung carcinoma cell line A549 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Human serum albumin (HSA) was purchased from GenDEPOT (Barker, TX, USA).

Methods

Synthesis of the FSA probe

Peptides were synthesized by Fmoc SPPS (Solid Phase Peptide Synthesis) using ASP48S (Peptron Inc., Daejeon, Korea): 8 eq. protected amino acid (Fmoc-Arg(Pbf)-OH, Fmoc-Glu(OH)-OtBu) and coupling reagent [8 eq. 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/8 eq. N-Hydroxybenzotriazole (HOBt)/16 eq. 4-Methylmorpholine (NMM)] in dimethylformamide (DMF)] were addedin . The mixture was reacted for 2 h at room temperature and washed with DMF, MeOH, and DMF. For Fmoc deprotection, 20% piperidine in DMF was added as the reactant, and the mixture was incubated for 5 min (repeated two more times), and then washed with DMF, MeOH, and DMF. A peptide backbone attached resin (NH_2-gamma-E(OtBu)-R(Pbf)-R(Pbf)-K(Dde)-2-chloro-Trityl Resin) was prepared by repeating the above procedure, wherein 4 eq. pteroic acid and 4 eq. HBTU/4 eq. HOBt/8 eq. NMM in dimethyl sulfoxide (DMSO) were added to the peptide backbone attached resin. The mixture was reacted for 12 h, suctioned, and the resin was washed with DMF, methanol, and DMF. The protected group Dde of the C-terminal Lys was removed by treatment with 2% hydrazine in DMF. ATTO655-COOH and HBTU/HOBt/NMM in DMF were added above peptide resin. The mixture was reacted for 12 h, suctioned, and the resin was washed with DMF, MeOH, and DMF. The synthesized peptide was cleaved from the resin by

treatment with trifluoroacetic acid (TFA)/EDT/Thioanisole/triisopropylsilane (TIS)/H_2O (90/2.5/2.5/2.5/2.5).The synthesized FSA probes were purified by reverse phase HPLC using a Vydac Everest C18 column (250 mm ×22 mm, 10 μm). Elution was carried out using a water-acetonitrile linear gradient (10~75% (v/v) of acetonitrile) containing 0.1% (v/v) TFA.

Characterization of FSA probe

Molecular weights of the purified FSA probes were confirmed using LC/MSD (Agilent Hewlett Packard 1100 series, California, USA). UV/Vis spectra of free ATTO655 and the FSA probe were measured using an UV/Vis spectrometer (DU730, Beckman Coulter, USA). The highest absorbance peaks of free ATTO655 and the FSA probe were measured at 662 and 665 nm, respectively.

Enzymatic activation of the fluorescence emission of FSA was tested as follows: The FSA (4 nmol) dissolved in 194 μL sodium acetate butter (20 mM sodium acetate, 1 mM EDTA, pH 5.0) was mixed with 97 pmol cathepsin B (6 μL in sodium acetate buffer) for the enzyme-treated sample, or an equal volume of sodium acetate buffer for the buffer-treated sample. The mixture (200 μL) was then incubated for 4 h at 37 ℃. To test for enzymatic activation of FSA by other cathepsins, equivalent molar amounts of cathepsin L or cathepsin S were incubated with the conjugate under the above-described conditions. For samples treated with a specific cathepsin B inhibitor (E64), 97 pmol cathepsin B (6 μL) was incubated with 0.1 mM E64 (20 μL) in 20 mM sodium acetate buffer (pH 5.0), at room temperature and for 30 min. Then, FSA (4 nmol) dissolved in 174 μL sodium acetate butter was applied to E64-pretreated cathepsin B solution and incubated for 4 h at 37 ℃**.** Before measuring the fluorescence spectra of the samples, the samples (50 μL) were diluted with 950 μL phosphate-buffered saline (PBS, 6.7 mM, pH 7.4, 154 mM NaCl). Fluorescence spectra ($\lambda_{ex.}$ 610 nm, $\lambda_{em.}$ 620~850 nm) of the diluted samples were obtained using a multifunctional microplate reader (Safire 2; Tecan, Männedorf, Switzerland). All experiments were performed in triplicate. Data are expressed as the mean (SD).

Stability of FSA in serum conditions

FSA conjugates were dispersed in a PBS solution, a PBS solution containing human serum albumin (20%, v/v), or 100% FBS. The final concentration of FSA probe in the solutions was adjusted to 1 μM. The solutions were maintained at room temperature for 4 h, and their fluorescence spectra ($\lambda_{ex.}$ 610 nm, $\lambda_{em.}$ 620~850 nm) were obtained using a multifunctional

microplate reader.

Cell culture

Folate receptor-positive SKOV3 and folate receptor-negative A549 cancer cells were maintained in RPMI1640 supplemented with 10% FBS and 1% antibiotic/antimycotic (Life Technologies; Invitrogen). Cells were incubated at 37 ℃ under 5% CO_2 in a standard humidified incubator.

Evaluation of fluorescence quenching and recovery inside target cells

In order to demonstrate the turn-on of FSA fluorescence inside the target cells, we applied both FSA and free dye to the folate receptor-positive SKOV3 cells, and then obtained fluorescence images without washing the cells. SKOV3 cells were seeded in a 4-well Lab-Tek chamber (Nalge Nunc International, Penfield, NY, USA) at a density of 1×10^5 cells per well, and incubated for 24 h to allow cell attachment. The FSA conjugate and free dye (ATTO655) were dissolved and diluted with cell culture medium. The existing culture medium was replaced with 300 μL of fresh medium containing FSA or free dye. Then, without washing the cells, NIR fluorescence images ($\lambda_{ex.}$ 633 nm, $\lambda_{em.}$ 646~753 nm) of the cells were acquired at 15 min, 1 h, and 2 h using a confocal laser scanning microscope (CLSM, ZEISS LSM 510 META, Germany). Afterwards, the cells were washed three times to remove the dyes in the extracelluar space, and NIR fluorescence images were obtained again to check if free dyes can be taken up into the cells during the test period.

Confocal images were analyzed using the LSM image 5 browser, and profiling was enabled by Axiovision.

Evaluation of receptor-mediated endocytosis of the FSA probe and subsequent fluorescence activation by cathepsin B within target cells

SKOV3 and A549 cells were seeded in a 4-well Lab-Tek chamber at a density of 1×10^5 cells per well, and incubated for 24 h to allow cell attachment. The FSA probe, FA, and E64d were dissolved and diluted in cell culture medium. The existing culture medium was replaced with 300 μL of fresh medium containing FSA (1 μM), and the SKOV3 and A549 cells were incubated for 2 h. For the free FA competition study, SKOV3 cells were incubated with 1 mM FA for 30 min, and then co-incubated with 1 μM FSA for an additional 2 h. For the enzyme inhibition test, SKOV3 cells were incubated with the cell-permeable cathepsin B inhibitor E64d

(20 μM) for 30 min, and then co-incubated with 1 μM FSA for an additional 2 h. Finally, the cells were washed two times in culture media, and then NIR fluorescence images ($\lambda_{ex.}$ 633 nm, $\lambda_{em.}$ 646~753 nm) were acquired using a CLSM.

In vivo imaging study in a xenograft tumor model

All animal studies were approved by the Institutional Animal Care and Use Committee. Female athymic nude mice (Balb-c/nude, 5 weeks old) were used for the *in vivo* experiments. SKOV3 cells (1×10^7 cells/50 μL of RPMI media) were subcutaneously implanted into the left hind flank of each mouse, and the tumor size was measured periodically. The animals were chosen for *in vivo* studies when the tumor size reached ~60mm^3.

The mice in the ATTO655 and FSA groups received intravenous injections of the free dye or the FSA probe at a dose of 15 nmol/20 g body weight. The mice in the control group received an intravenous injection of PBS (100 μL/mouse). NIR fluorescence images ($\lambda_{ex.}$ 660 nm, $\lambda_{em.}$ 690~730 nm) were obtained using an IVIS Lumina XR (Xenogen Corporation-Caliper, CA, USA) 3 h post-injection.

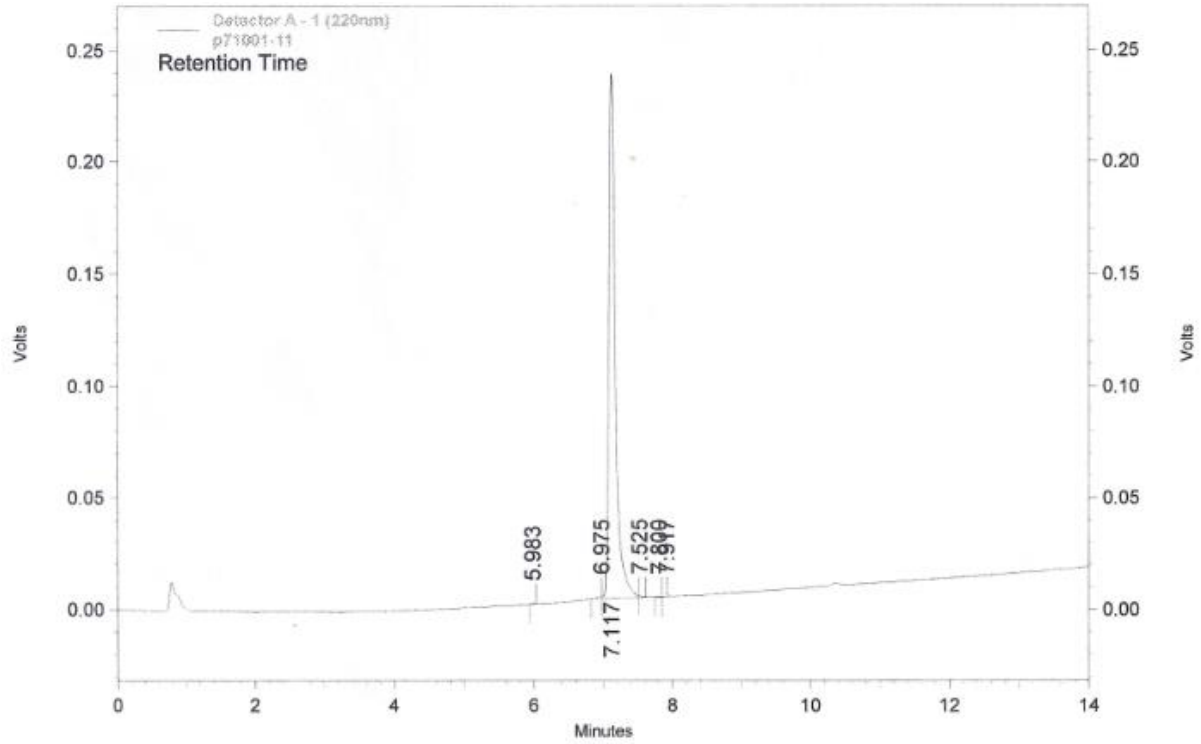

Fig. S1. HPLC chromatogram of synthesized FSA probe. The purified FSA probe was analyzed by reverse-phase HPLC (Shimadzu prominence HPLC, Kyoto, Japan) using a Vydac Everest C18 column (250 mm × 22 mm, 10 μm). Elution was carried out with a water–acetonitrile linear gradient containing 0.1% (v/v) trifluoroacetic acid. The flow rate was 1 mL/min. The composition of the mobile phase was as follows: 100% water and 0% acetonitrile at 0 min, 90% water and 10% acetonitrile at 2 min, 60% water and 40% acetonitrile at 12 min, and 30% water and 70% acetonitrile at 14 min. The FSA probe was detected at 220 nm with a retention time of 7.117 min and a measured purity of 99.415%.

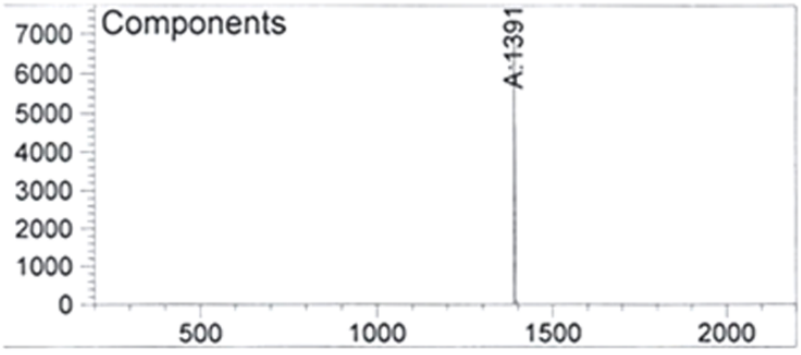

Fig. S2. Mass spectrum of the synthesized FSA probe.

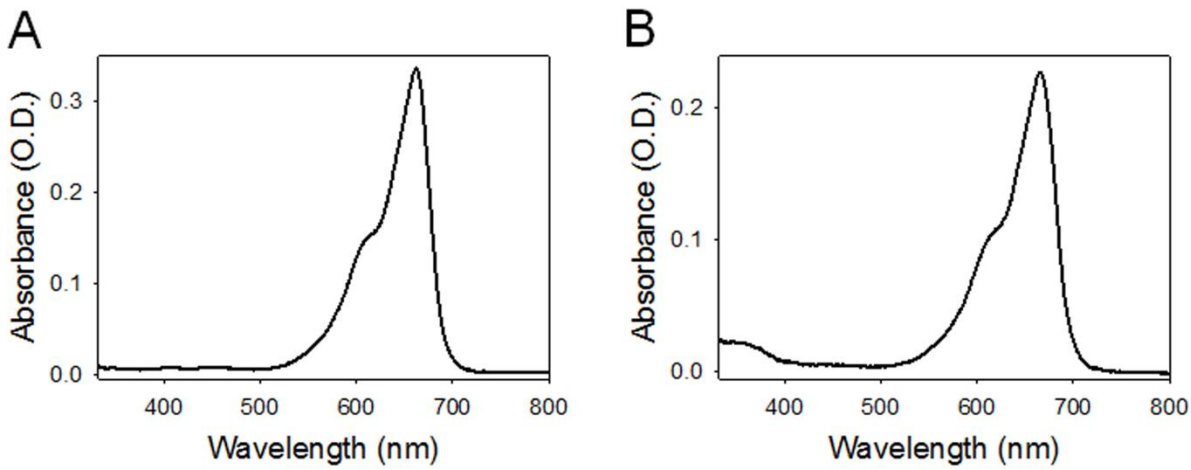

Fig. S3. a) UV/vis spectra of free ATTO655 and b) the FSA probe. Concentrations of ATTO655 and FSA were 2.8 μM and 1.92 μM, respectively. The highest absorbance values for the free ATTO655 and the FSA probe were measured at 662 and 665 nm, respectively.

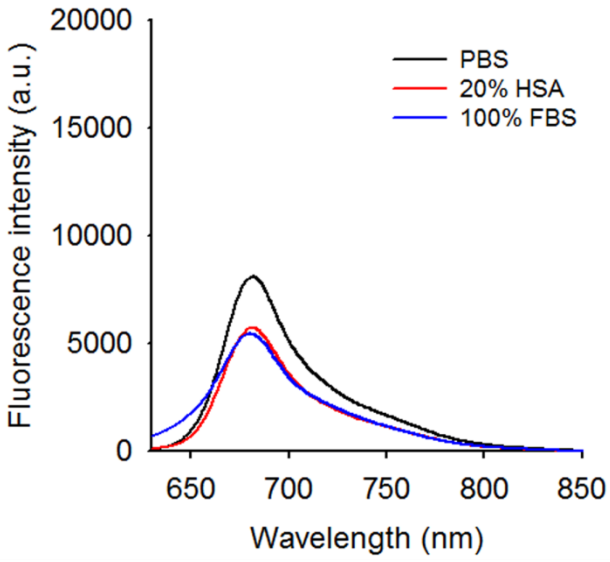

Fig. S4. Fluorescence spectra of FSA probe in the absence and presence of proteins. FSA probes (1 μM) was incubated in PBS, 20% HSA, and 100% FBS solutions for 4 h, and then their fluorescence spectra were measured ($\lambda_{ex.}$ 610 nm, $\lambda_{em.}$ 620~850 nm).

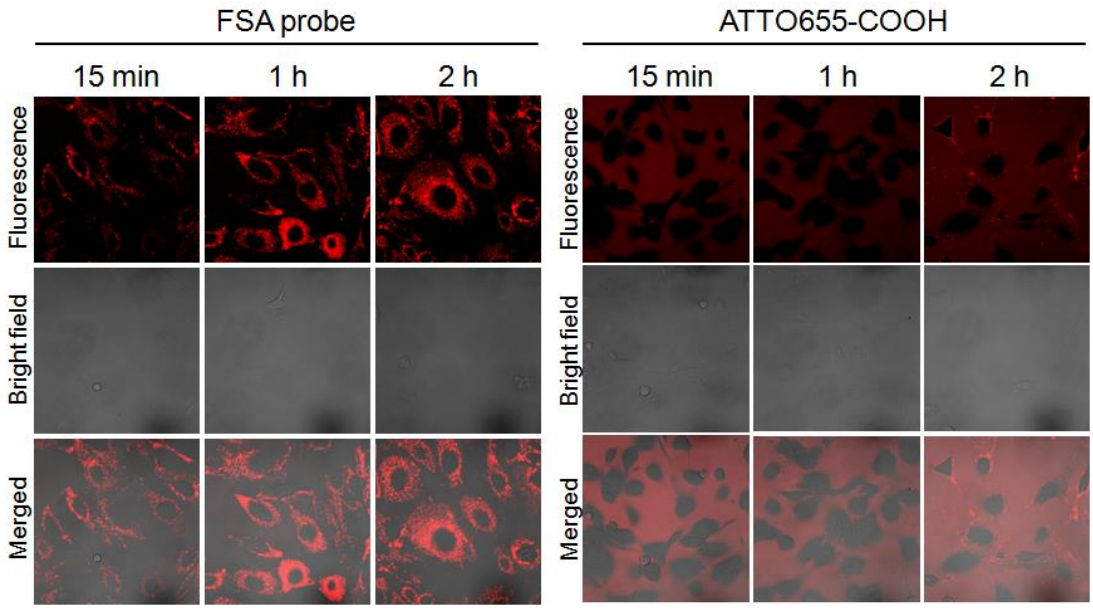

Fig. S5. Fluorescence, bright field, and merged images of FSA- and ATTO655-treated SKOV3 cells (also check Figure 2).