Supplementary Information

Cathepsin B-sensitive nanoprobe for in vivo tumor diagnosis

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Experimental Section.

Synthesis of Cathepsin B-Sensitive Nanoprobe (CB-NP). The CB-NP was synthesized similarly with matrix metalloproteinase probe in our previous paper. The overall synthetic procedure was shown in Scheme S1. First, the cathepsin B substrate peptide probe was prepared by conjugating NIRF dye (Cy5.5, excitation and emission maxima 675nm and 695 nm, respectively) and dark quencher (black hole quencher-3, BHQ-3, absorbance maximum 650 nm) to a cathepsin B-sensitive substrate peptide (Gly-Arg-Arg-Gly-Lys-Gly-Gly, a recognition site is italicized). The peptides were synthesized using standard solid-phase Fmoc peptide chemistry (Peptron, Daejeon, Korea). This peptide probe was characterized by analytical reverse phase-high performance liquid chromatography (RP-HPLC; Purity: >95%), UV-Vis spectra, and emission spectra, and matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS; m/z calculated: 2117, found: 2113) (Figure S2). This CB-peptide probe was chemically conjugated with the self-assembled chitosan nanoparticles (CNPs) to develop CB-NP. Briefly, glycol chitosan (molecular weight 250 kDa; Sigma, St. Louis, MO) was conjugated with 150 ± 5 molecules of hydrophobic 5β-cholanic acid per polymer to make the CNPs (molecular weight 301
kDa). The 5β-cholanic acid conjugated glycol chitosan (22 nmol) were dispersed in phosphate buffered saline (PBS, 7 ml, 100 mM, pH 5.0; Sigma) with a probe-type sonicator (90W, 5 min, Ultrasonic Processor, GEX-600; Sonics & Materials, Newtown, CT). The CB-peptide probe (1.2 μmol) were dissolved in 200 μl of 50% (v/v) dimethyl sulfoxide (DMSO; Sigma) in PBS (pH 6.0) and mixed with 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC, 6.3 μmol; Sigma) and N-hydroxysulfosuccinimide (sulfo-NHS, 6.9 μmol; Pierce, Rockford, IL) dissolved in PBS (100 μl, pH 6.0) for 15 min. This NHS-activated CB-peptide probe was then added to CNP solution, and stirring performed at room temperature in the dark for 6 h. After dialysis against water and lyophilization, CB-NP (molecular weight 345 kDa) was obtained as blue powder.

**Characterization.** The mean diameter and size distribution of CB-NP was measured using dynamic light scattering (DLS) at 633nm and 25°C. TEM was performed using a JEOL 200CX microscope operating at 200 kV. The morphology was observed using transmission electron microscopy (TEM, CM30 electronmicroscope, Philips, CA) operating at 80 kV. UV absorbance of CB-NP (0.8 μM) and CB-peptide probes at various concentrations (2.5, 5.0, 10, 20 μM, respectively) was recorded from 450 to 800nm using UV/VIS spectrophotometer (Optizen 2120, Mecasys, Daejeon, Korea). The absorption intensity of BHQ-3 (690nm) was measured, and the concentration of conjugated CB-peptide probe in CB-NP was calculated from standard curve made by various concentration of CB-peptide probe.

**In vitro Enzyme Specificity.** To test the sensitivity of CB-NP about cathepsin B, CB-NP (20 nM) was incubated for 60 min in 25 mM 2-(N-morpholine)-ethanesulphonic acid (MES) buffer containing various concentrations (1.5, 3.0, 6.0, and 12 nM) of
cathepsin B, respectively. The fluorescence intensity was measured after 60 min using a spectrofluorometer (F-7000 Fluorescence Spectrophotometer; Hitach, Tokyo, Japan) at 37°C. The excitation wavelength was fixed at 675 nm and emission spectra monitored from 676 to 800 nm. To confirm the specificity of the CB-NP, CB-NP (20 nM) was incubated with cathepsin B, cathepsin B plus inhibitor (Z-Phe-Ala-FMK; Calbiochem, San Diego, CA), cathepsin D, and cathepsin L. The similar experimental condition with above sensitivity test was applied to the case of cathepsin B (6.0 nM) with the CB-NP or CB-NP plus inhibitor. In addition, CB-NP (20 nM) was each incubated in assay buffer (0.1 M sodium acetate, 0.2 M NaCl, pH 3.5) containing 23 nM cathepsin D or assay buffer (50 mM MES, 5 mM DTT, pH 6.5) containing 1.0 nM cathepsin L. The fluorescence intensity was measured at every 20 min for 80 min. All cathepsins were purchased from R&D Systems, Inc. (Minneapolis, MN) and activated as individual manuals prior to use.

**Cytotoxicity.** Murine squamous cell carcinoma (SCC7) cells were obtained from the American Type Culture Collection (Manassas, VA). Cathepsin B is expressed in SCC7 cells.4 Cells were cultured in RPMI 1640 (Gibco, Grand Island, NY) containing 10% fetal bovine serum (Invitrogen, Burlington, ON, Canada), 100 U/ml penicillin, and 100 μg/ml streptomycin. The cytotoxicity was evaluated using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. In brief, cell were seeded in a 96-well microplate and incubated with various concentrations of the CB-NP (0.1 to 100 μg/mL) for 24 h before incubation with MTT for 40 min. The formazan product was eluted using DMSO, and absorbance was recorded at 570 nm using a microplate reader. Cell viability was expressed as the percentages of viable cells compared to the survival of a control group, and as mean ± standard deviation (n=3).
In vitro Cellular Uptake. SCC7 cells (1×10⁴ cells/dish) were plated in 35 mm cover-glass bottom dishes and grown to 80% confluence. The cells were washed twice with PBS (pH 7.4), and then incubated with serum-free medium containing CB-NP (200 nM) and cathepsin B substrate peptide probe with same amount of Cy5.5 in the presence or absence of inhibitors for 6 h. The inhibitor was treated 30 min before CB-NP incubation. The cells were washed with PBS (pH 7.4) twice, fixed with fresh 4% (v/v) paraformaldehyde at room temperature for 5 min, and mounted with Fluoromount-G™ (Southern Biotech, Birmingham, AL). Cellular uptake of the probes were observed using IX81-ZDC focus drift compensating microscope (Olympus, Tokyo, Japan) equipped with a 673-nm excitation filter and a 692-nm emission filter.

Immunocytochemistry. SCC-7 cells (1×10⁵ cells/dish) were plated in 35 mm cover-glass bottom dishes and incubated for 36 h. After the incubation, CB-NP (200 nM) was added into each culture dish and additionally incubated for 4 h. Subsequently, the cells were washed with PBS and fixed in 4% (v/v) paraformaldehyde at room temperature for 10 min. The antigens were retrieved in buffer (100 mM Tris, 5% (w/v) urea, pH 9.5) for 10 min at 95°C, and the cells were permeabilized with 0.25% Triton X-100 in PBS for 5 min. Permeabilized cells were incubated with monoclonal mouse anti-cathepsin B IgG (1:200 dilution; Abcam, Cambridge, MA) for 1 h, and then goat anti-mouse IgG-FITC (1:1500 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA) was applied to the cells for 30min at dark condition for visualization. The nuclei of the cells were stained with DAPI. Microscopy was conducted on a DeltaVision system (Applied Precision, Issaquah, WA). The samples were monitored using a microscope (IL-70; Olympus) equipped with a mercury arc lamp, an Uplan Apo 60× oil objective (1.35 numerical
aperture), a CoolSnap HQ digital camera (Roper Scientific, Tucson, AZ) and proper filter sets (Omega Optical, Brattleboro, VT).

**Tumor Models.** All animal care and experimental procedures were performed in compliance with the Institutional guidelines of Korea Institute of Science and Technology (KIST) and the relevant laws, and institutional committees have approved the experiments. SCC7 cells ($1 \times 10^6$ cells/mouse) suspended in 50 µl sterile saline were injected subcutaneously into the flank of athymic nude mice (20 g, n=5 per each group, Institute of Medical Science, Tokyo).

**In Vivo NIRF Imaging.** When tumors reached approximately $5.0 \pm 0.5$ mm in size, CB-NP (1 mg/ml in 100 µl of saline) was injected via tail vein to the tumor-bearing mice to evaluate the cathepsin B activity. The inhibitor was intratumorally administered 30min before the probe injection. *In vivo* NIR fluorescence images were captured using an eXplore Optix system (ART Advanced Research Technologies Inc., Montreal, Canada). Imaging was performed after 1, 3, 6, 9 hour post-injection and images were assessed with an Analysis Workstation (ART Advanced Research Technologies Inc.). The total NIRF intensity in tumor was calculated using the region of interest (ROI) function of Analysis Workstation software (ART Advanced Research Technologies Inc.), and the tumor signal to background ratio (TBR) between the tumor and normal region was also evaluated as a function of time. Values are presented as means ± standard deviations for groups of five animals. A one-way analysis of variance (ANOVA) was used for the comparison of continuous variables between groups.

**Ex Vivo NIRF Imaging.** Tumors and other organs were excised and imaged using a KODAK image station (4000 MM; Kodak, New Haven, CT) after 9 hour post-injection. The image station was equipped with a 150 W halogen lamp and excitation filter sets
proper for Cy 5.5. NIRF images were acquired with a 12-bit CCD camera (Kodak, Japan) equipped with a bandpass emission filter suitable for Cy5.5 (680-720 nm; Omega Optical) and a special C-mount lens. The NIRF intensity was expressed as an average pixel number.

**Histological Analyses.** For the histological evaluation, the each excised tumor was divided into 2 pieces, and one was fixed in 4% (v/v) buffered formalin and embedded in paraffin. The other was embedded in OCT compound for cryo-section preparation. The paraffin-embedded specimens were cut into 5 µm-thick sections, and they were used for immunohistochemistry (IHC) or stained with hematoxylin and eosin (H&E). For IHC, the paraffin-embedded sections were deparaffinized and rehydrated with xylene, graded ethanol and PBS (pH 7.4). Endogenous peroxidase was blocked by incubating the sections in 3% hydrogen peroxide in PBS for 20 min at room temperature. After 3 times of wash, each section was then incubated with primary antibodies to cathepsin B (1:200 dilution; Abcam) for 2 h at room temperature. HISTOSTAIN®-PLUS Kit (Invitrogen, Carlsbad, CA) was applied to detect binding of primary reagents. Sections were counterstained with Harris’s hematoxylin (Sigma) and examined using light microscope (BX51TF, Olympus). The 8 µm-thick frozen sections were prepared for fluorescence microscopic imaging of Cy5.5. The slides were observed under the IX81-ZDC focus drift compensating microscope without histological stains.

**References in Supplementary Information**


Fig. S1 Synthetic scheme of CB-NP.
**Fig. S2** Analytical experiments including (a) HPLC, (b) UV-Vis spectra, (c) emission spectra, and (d) MALDI-TOF mass spectrometry of CB-peptide probe.
Fig. S3 Characterization of the CB-NP including (a) UV absorbance of CB-NP (0.80 µM) and various concentrations of CB-peptide probes (2.5, 5.0, 10, 20 µM), (b) standard curve for calculation of the amounts of CB-peptide probes conjugated to CNPs, (c) size distribution of the CB-NP in PBS; inset: transmission electron microscopy image, and (d) in vitro cytotoxicity of the CB-NP for SCC7 cells.