Dextran Based pH-activated Near-infrared Fluorescence Nanoprobe Imaging the Acidic Compartments in Cancer Cells

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Supporting Information

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Materials and General Experimental Methods

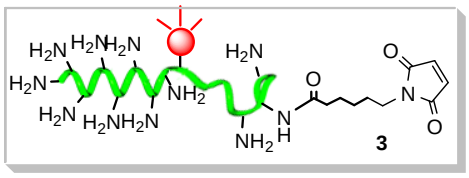
All organic solvents were analytical grade from Fisher Scientific (Pittsburgh, PA, USA) unless otherwise specified. Poly-$_L$-lysine hydrobromide (6.0 kDa), Dextran-NH_2 (70 kDa), IR783, 3-Mercaptopropionic acid, hydrazine anhydrous were purchased from Sigma-Aldrich (St. Louis, MO, USA). *N*-[ε-maleimidocaproyloxy]sulfosuccinimide (Sulfo-EMCS), *N*-succinimidyl S-acetylthiopropionate (SATP), rhodamine succinimidyl ester, FITC labeled dextran (10 kDa), Alexa Fluo-488 labeled transferrin, Alexa Fluo-488 labeled cobra toxin-B subunite, fetal bovine serum, penicillin, streptomycin and cell culture media were form Invitrogen (Carlsbad, CA, USA). Amicon ultra-15 centrifugal filter tubes (5,000 and 10,000 MW cutoff) were from Millipore (Bedford, MA, USA). MTT cell proliferation kit and human glioblastoma-astrocytoma, epithelial-like U87MG cell line were obtained from American Type Culture Collection (Rockville, MD, USA). The molecular weight of conjugate was measured from Waters Ultrahydrogel^{TM} 1000 (7.8 mm × 300 mm) gel permeable column, which was operated by Agilent 1100 series High Performance Liquid Chromatography (HPLC) system. ^{1}H, ^{13}C NMR spectra were recorded at 400 MHz on Varian Mercury400 (Varian Inc. Palo Alto, CA, USA), and chemical shifts were reported in ppm relative to tetramethylsilane. Fast Atom Bombardment (FAB) mass spectra and High Resolution (HR) FAB mass spectra were obtained on a double sector JEOL JMS-A X505HA mass spectrometer (Peabody, MA, USA). The hydrodynamic radius and molecular size distribution of nanoprobe were measured on a NICOMP 380 ZLS Zeta potential/particle seizer (Particle sizing systems, Santa Barbara, CA). pH values of aqueous solutions were measured by a Mettler Toledo DT120 glass electrode operated through a Mettler Toledo MP120 pH meter. Absorbance spectra were recorded on a Shimadzu UV-2401PC UV-vis recording spectrophotometer.

Fluorescence spectra were obtained on a Jobin Yvon-Spex® FluoroMax-3 spectrofluorometer (HORIBA Jobin Yvon Inc., Edison, NJ, USA). *In vitro* fluorescence optical imaging was performed on a Xenogen IVIS® 200 small animal imaging system equipped with an indocyanine green (ICG) band pass filter set. Confocal fluorescence images of live cells were performed with a Zeiss LSM 510 META confocal laser-scanning microscope (Carl Zeiss, Inc., Thornwood, NY) using a Plan-Apochromat 63x/1.4 oil immersion lens (Zeiss). NIR fluorescence microscopic images were obtained with an Olympus IX81 inverted microscope using 60×/1.42 NA oil immersion lenses (Olympus America Inc., Center Valley, PA). The microscope was equipped with a Hamamatsu C9100 EM-CCD digital camera (Hamamatsu Photonics, Bridgewater, NJ).

Synthesis

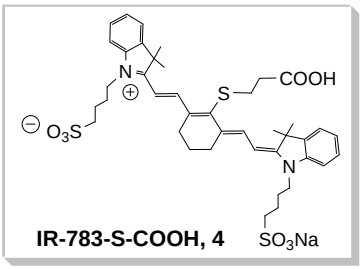
The detail synthetic steps to prepare nanoprobe **NP1** are described below.

Preparation of rhodamine and maleimide group labeled PLL (**3**). Rhodamine-NHS ester (3.8 mg, 7.2 × 10^{-6} mol, 2.0 equiv.) dissolved in 100 μL anhydrous DMSO was added dropwise to PLL (20 mg, 3.6 × 10^{-6} mol) in 1.0 mL 0.1 M HEPES pH 8.5 solution. After stirring at 25 °C for one hour, the mixture was loaded to filtration tube (MW 5000 cut off) and centrifuged with a speed of 4000 rpm to remove the hydrolytic by-products. The purified rhodamine labeled PLL, **2** (3.5 × 10^{-6} mol) was stored in 2.0 mL 0.5 M HEPES pH 8.5 solution and EMCS (2.2 mg, 7.0 × 10^{-6} mol, 2.0 equiv.) in 100 μL DMSO was added slowly in 15 min. After reaction for 1.0 h, the mixture was purified in filtration tube (MW 5000 cut off), and the resulting conjugate **3** as a deep red color clear solution was concentrated in 1.0 mL 0.1 M HEPES. The rhodamine labeling degree was determined by measuring the absorbance of PLL (ε_{213} = 46,800 $M^{-1}cm^{-1}$) and rhodamine (ε_{544} = 60,000 $M^{-1}cm^{-1}$). On average, 1.2 rhodamine per PLL molecule



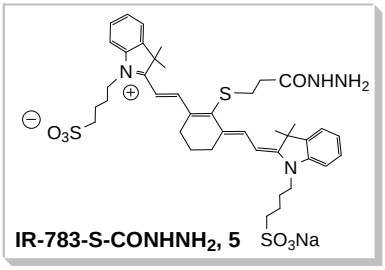
was obtained and the yield of compound **3** is 90%.

Preparation of IR783-S-COOH (**4).** To a solution of IR783 (100 mg, 1.34×10^{-4} mol) in 2 mL anhydrous DMF was added 3-mercaptopropionic acid (14 μL, 17 mg, 1.6×10^{-4} mol, 1.2 equiv.) and triethylamine (22 μL, 16 mg, 1.6×10^{-4} mol). The green color solution was allowed to stir in the dark at r.t. for 15 h. The crude product was precipitated by addition of excess diethyl ether and further purified through gravity chromatography (silica gel, CH_3CN:H_2O = 100:20 (V:V). The purified product dissolved in water was lyophilized as a deep green powder (yield: 82%, 1.1×10^{-4} mol, 90 mg). ^{1}H NMR (400 MHz, CD_3OD): δ 8.70 (d, 2H, *J* = 14.0 Hz), 7.33 (d, 2H, *J* = 7.6 Hz), 7.27-7.23 (t, 2H, *J* = 7.2 Hz), 7.19 (d, 2H, *J* = 8.0 Hz), 7.11-7.07 (t, 2H, *J* = 7.2 Hz), 6.17 (d, 2H, *J* = 13.6 Hz), 4.05-4.02 (t, 4H, *J* = 6.4 Hz), 2.92-2.88 (t, 2H, *J* = 6.8 Hz), 2.77-2.73 (t, 4H, *J* = 6.8 Hz), 2.55-2.52 (t, 4H, *J* = 6.4 Hz), 2.44-2.41 (t, 2H, *J* = 7.2 Hz), 1.83-1.74 (m, 10H), 1.58 (s, 12H); ^{13}C NMR (100 MHz, CD_3OD): 175.68 (C), 173.88 (2 × C), 157.91 (C), 147.23 (2 × C), 143.81 (2 × CH), 142.52 (2 × CH), 135.14 (2 × CH), 129.99 (2 × C), 126.32 (2 × C), 123.57 (2 × CH), 112.24 (2 × CH), 102.46 (2 × CH), 51.95 (2 × CH_2), 50.57 (2 × CH_2), 47.87 (2 × C), 36.19 (CH_2), 34.20 (CH_2), 28.54 (4 × CH_3), 27.40 (2 × CH_2), 27.34 (2 × CH_2), 23.72 (2 × CH_2), 22.30 (CH_2); HRFAB-MS: $C_{41}H_{51}O_8N_2S_3Na$ $[M+H]^+$, found 819.2794 (44.7%), calculated 819.2784.



IR-783-S-COOH, 4

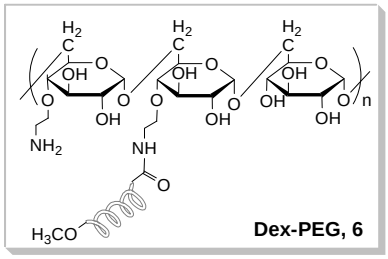
Preparation of IR783-S-COONHNH$_2$ (**5**). Hydrazine monohydrate (2.2 μL, 2.2 mg, 4.4×10^{-5} mol, 1.2 equiv.), IR783-S-COOH (30 mg, 3.7×10^{-5} mol) and DCC (8.2 mg, 4.0 $\times 10^{-5}$ mol, 1.1 equiv.) were dissolved in 2.0 mL DMF. The mixture was stirred for 8.0 h in the ice bath. At the end of the reaction, diethyl ether



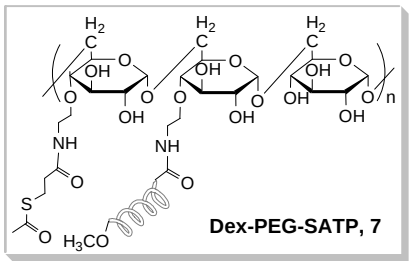
IR-783-S-CONHNH$_2$, 5

was added and the crude product was precipitated as a green solid. The crude product was re-dissolved in 3 mL CH_3CN, and purified on a gravity chromatography (silica gel, $CH_3CN:H_2O$ = 100:25 (V:V)). The purified product **5** was lyophilized as a deep green powder (yield: 74%, 2.7×10^{-5} mol, 23 mg). ^{1}H NMR (400 MHz, CD_3OD): δ 8.80 (d, 2H, *J* = 13.6 Hz), 7.37 (d, 2H, *J* = 7.2 Hz), 7.28-7.26 (t, 2H, *J* = 7.8 Hz), 7.20 (d, 2H, *J* = 8.0 Hz), 7.14-7.10 (t, 2H, *J* = 7.6 Hz), 6.18 (d, 2H, *J* = 14.4 Hz), 4.08-4.04 (t, 4H, *J* = 6.4 Hz), 2.94-2.90 (t, 2H, *J* = 8.0 Hz), 2.79-2.76 (t, 4H, *J* = 6.6 Hz), 2.59-2.56 (t, 4H, *J* = 6.0 Hz), 2.37-2.33 (t, 2H, *J* = 8.0 Hz), 1.85-1.81 (m, 10H), 1.64 (s, 12H); ^{13}C NMR (100 MHz, CD_3OD): 174.02 (2 × C), 159.12 (C), 157.91 (C), 147.73 (2 × C), 143.92 (2 × CH), 142.64 (2 × CH), 135.21 (2 × CH), 129.96 (2 × C), 126.25 (2 × C), 123.59 (2 × CH), 112.18 (2 × CH), 102.31 (2 × CH), 51.97 (2 × CH_2), 50.65 (2 × CH_2), 45.02 (2 × C), 35.76 (CH_2), 34.20 (CH_2), 28.57 (4 × CH_3), 27.40 (2 × CH_2), 22.30 (2 × CH_2), 23.79 (2 × CH_2), 22.40 (CH_2); HRFAB-MS: $C_{41}H_{53}O_7N_4S_3Na$ $[M+H]^+$, found 833.3017 (32.7%), calculated 833.3047.

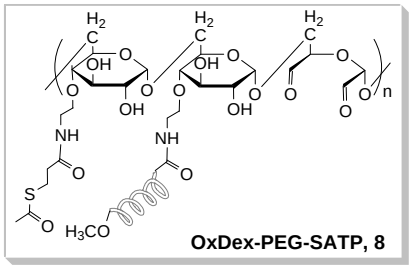
Preparation of Dex-PEG (**6**). Dextran-NH_2 (70 kDa, 70 mg, 1.0×10^{-6} mol) was dissolved in 1.5 mL 0.1 M HEPES pH 8.3 and PEG^{5k}-NHS (6.0×10^{-6} mol, 30 mg, 6 equiv.) in 200 μL DMSO was added. After reaction for 1.0 h, the product was purified in 50 kDa filter tube by 2 × 0.1 M HEPES buffer and 2 × pure water. The product was lyophilized as a white cotton-like solid (82 mg, 0.86×10^{-6} mol, yield: 86%). The molar ratio between PEG and Dextran in Dex-PEG conjugate was determined by quantifying the integrated proton number of CH_2 in the PEG δ (3.67 ppm) and the integrated proton number of dextran at C1 position δ (4.95 ppm) in the ^{1}H NMR spectrum. .



Preparation of Dex-PEG-SATP (**7**). Conjugate **6** (100 mg, 1.1 × 10^{-6} mol, MW is considered as 95 kDa) was dissolved in 1.0 mL 0.1 M HEPES pH 8.3. SATP (6.6 × 10^{-6} mol, 6.0 equiv., 1.6 mg) in 50 μL DMSO was added. After stirring for 1.0 h at r.t., the product was purified in 10 kDa centrifugal filtration tube by 2 × 0.1 M HEPES buffer and 2× pure water. The product was lyophilized as a white cotton-like solid (88 mg, 9.3 × 10^{-7} mol, yield: 85%). The determination of sulfhydrylation labeling degree on the dextran moiety was further measured by using Ellman's reagent as the protocol described in our previous work[1]. Averagely, 2.5 sulfhydryl groups were functionalized on each dextran moiety.

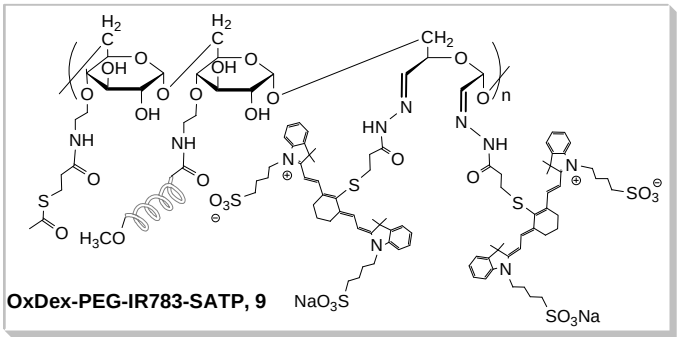


Preparation of OxDex-PEG-SATP (**8**). Conjugate **7** (50 mg, 5.3 × 10^{-7} mol) dissolved in 2 mL pure water and $NaIO_4$ (46 mg, 2.1 × 10^{-4} mol, 400 equiv.) was added as solid form. The mixture was stirred at r.t. for 24 h, and diethyene glycol (22 mg, 2.1 × 10^{-4} mol, 400 equiv.) was added at the end of reaction. The product was purified in 10 kDa filter tube by washing with pure water for 4 times. The purified product was lyophilized as a white solid (33 mg, 3.7 × 10^{-7} mol, MW is considered as 90 kDa, yield: 69%). The oxidation degree of the compound **8** was measured by a modified method described by Maia et al[2]. Briefly, 1.0 mL compound **8** (1 mg/mL) in tricholoracetic acid (0.2% w/v) was mixed with 0.5 mL of tert-butylcarbazate (5 mM in 0.2% tricholoracetic acid) and incubated at r.t. for 24 h. At the end of reaction, 0.2 mL above mixture was transferred to a vial containing 1.0 mL of 2,4,6-trinitrobenzene sulfonic acid (TNBS) working solution (0.01% in 2X PBS, pH 8.0) solution, and the reaction was conducted at r.t. for 1.5 h. 0.2 mL above mixture was added to 0.8 mM 1.0 M HCl, and its absorbance at 335 nm was measured. To plot the

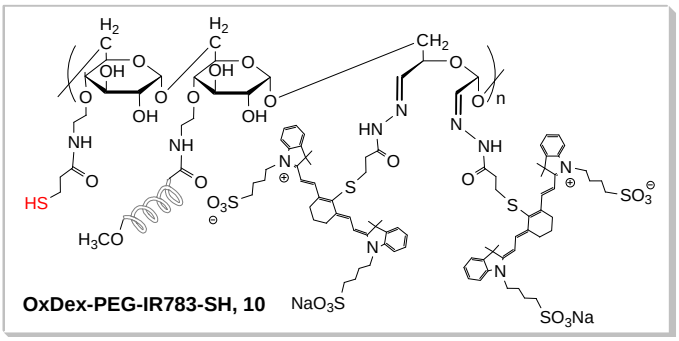


standard calibration curve, aqueous solutions of tert-butylcarbazate with concentrations of 2, 1, 0.5, 0.2, 0.05 and 0.01 mM were prepared. 0.2 mL tert-butylcarbazate with various standard concentrations was added in 1.0 mL TNBS working solution. After 1.5 h reaction, 0.2 mL above mixture was added to 0.8 mM 1.0 M HCl, and the absorbance at 335 nm was measured. Plotting the absorbance at 335 nm against the carbazate concentrations obtained the standard calibration curve. The oxidation degree of the compound **8** was measured as 15%, which 15% of glucose monomers in this dextran-derivative were oxidized.

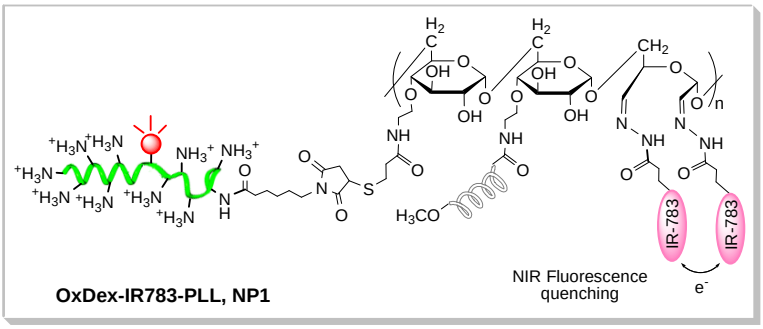
Preparation of OxDex-PEG-IR783-SATP (**9**). Compound **8** (30 mg, 3.3×10^{-7} mol) dissolved in 1.5 mL 1.0 M MES buffer pH 4.7 was added IR783-S-$CONHNH_2$ (35 mg, 4.3×10^{-5} mol, 1.0 equiv. of the aldehyde number in the compound **8**) in 0.5 mL DMSO. After stirring at r.t. for 16 h, the product was purified in 10 kDa centrifugal filtration tube and lyophilized as a deep green color powder (24 mg, 2.6×10^{-7} mol, MW is considered as 90 kDa, yield: 81%). The IR783 fluorophore labeling efficacy in compound **9** was calculated as 35% (35% of aldehyde moieties were labeled with NIR dye) by measuring the absorbance of IR783 (ε_{783} = 180,000 $M^{-1}cm^{-1}$).



Preparation of OxDex-PEG-IR783-SH (**10**). The purified compound **9** (20 mg, 2.2 × 10^{-7} mol) was concentrated to 1.0 mL PBS buffer pH 8.0, and combined with 100 uL deacetylation solution (0.5 M hydroxylamine, 25 mM EDTA in PBS, pH 8.0). After incubation for 2 hours at r.t., the mixture was purified into 10 mM EDTA in PBS pH 8.0 by centrifugal filtration (MW 10 kDa cut off) to minimize the formation of disulfide bond. The presence of sulfhydruls can be qualitatively monitored by the thionitrobenzoate (TNB) assay with the absorbance variation at 412 nm. The yield of compound **10** was calculated as 94% (18.5 mg, 2.1 × 10^{-7} mol) by weighting the product after lyophilization.



Preparation of OxDex-IR783-PLL (**NP1**). Rhodamine labeled PLL **3** (3.3 × 10^{-7} mol, 1.1 equiv.) in 0.5 mL 0.1 M HEPES pH 8.3 was mixed with compound **10** (27 mg, 3.0 × 10^{-7} mol) in 1.0 mL 0.1 M HEPES pH 8.3 solution. After stirring at 25 °C for one hour, the mixture was purified in 50 kDa centrifugal filtration tube. The resulting conjugate **NP1** was lyophilized to give a rose-color powder with a yield of 90% (2.7 × 10^{-7} mol, 27 mg, MW is determined as 102 kDa). The conjugation ratio between PLL and dextran was determined as 1.0 by measuring the absorbance of IR783 (ε_{783} = 180,000 $M^{-1}cm^{-1}$) labeled in dextran and the rhodamine (ε_{544}



= 60,000 $M^{-1}cm^{-1}$) labeled in PLL moiety. FT-IR: 3440 cm^{-1} v(O-H, N-H), 2947 cm^{-1} v(C-H), 2832 cm^{-1} v(C-H), 1649 cm^{-1} v(C=O, amide), 1602 cm^{-1} v(C=N, hydrazone), 1183 cm^{-1} v(C-N), 1046 cm^{-1} v(C-O).

Characterization

Determination of Molecular Weight of Nanoprobe NP1 by Gel Permeation Chromatography (GPC)

All the size exclusion chromatographic (SEC) studies were carried out as the below typical method: mobile phase: 1X PBS, pH 7.4; isocratic flow rate: 0.7 mL/min; operating temperature: 25 °C; injected volume: 20 μL. The gel-permeable column was firstly calibrated by the Dextran GPC Molecular Weight Standards with MW of 80, 150, 270, 410 and 670 kDa (Aldrich, St. Louis, MO). The eluting times of the dextran MW standards with the injection concentration 2.0 mg/mL were measured with the same HPLC method. The molecular weight calibration curve was obtained by plotting molecular weight *vs*. elution times of each dextran standard (Figure S1B). The effluent peaks of dextran standards were monitored at 280 nm, while the peaks of **NP1** were monitored at 780 nm (IR783 absorbance) and 540 nm (rhodamine absorbance) (Figure S1A). The molecular weight of **NP1** was determined as 102 kD by applying its eluting time against the calibration curve (Figure S1B). The overlapping of the eluting peaks of **NP1** monitored at 540 and 780 nm verifies the entity of **NP1** (Figure S1A).

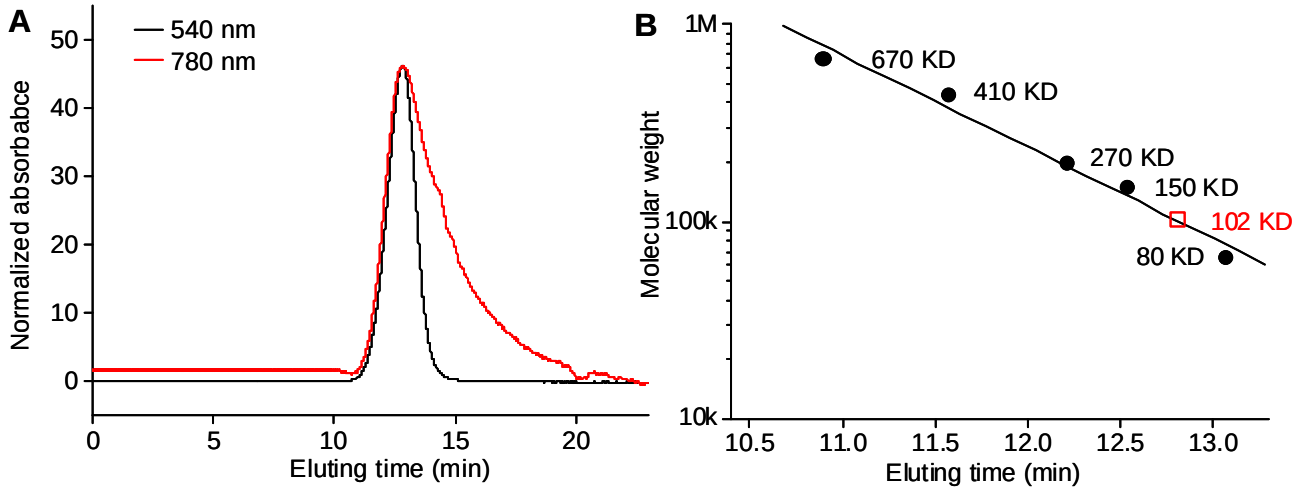


Figure S1. Determination of the molecular weight of nanoprobe **NP1**. (**A**) Gel permeable chromatography of **NP1** monitored at 540 nm (black line) and 780 nm (red line); (**B**) Determination of the molecular weight of **NP1** with the reference of the calibration line, which was fitted by measuring dextran standard (molecular weight ranged from 80–670 kDa) with the same HPLC method.

Determination of Hydrodynamic Radius and Size Distribution of NP1 by Dynamic Light Scattering (DLS)

Hydrodynamic radius of **NP1** and unmodified Dextran-NH_2 was determined by dynamic light scattering (DLS). The measurements were performed at room temperature using a 20 sec acquisition time. The standard solution of bovine serum albumin (BSA) in distilled water with concentration 2.0 mg/ml was used for the calibration of the instrument. The samples were filtered through 0.45 μm filter and diluted to 1.0 mg/mL by PBS pH 7.4. The hydrodynamic radius and the size distribution were calculated with the regularization algorithm provided by ZPW 388 software.

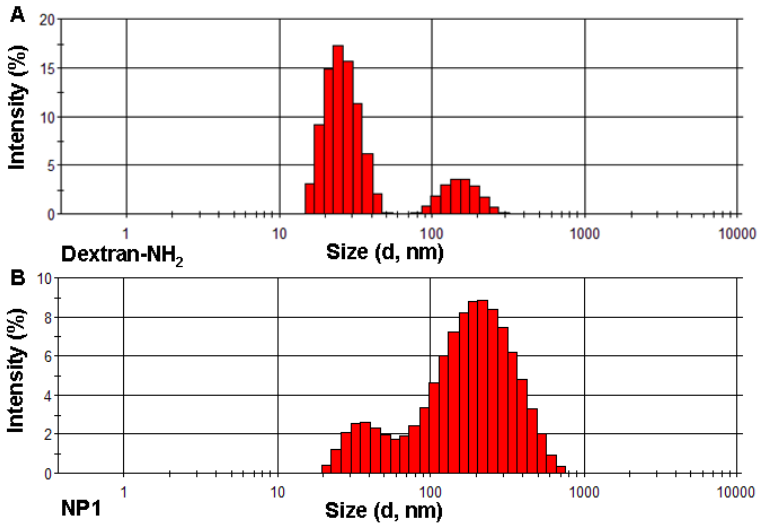


Figure S2. Hydrodynamic diameters and size distributions of Dextran-NH_2 (A) and **NP1** (B) measured by dynamic light scattering in PBS pH 7.4. The average diameter of Dextran-NH_2 is 33 nm (peak 1: 26 nm, peak 2: 157 nm). The average diameter of **NP1** is 124 nm (peak 1: 39 nm, peak 2: 228 nm).

Photospectroscopic and Optical Imaging Studies

(a) Absorbance and Fluorescence Spectroscopy Studies.

All spectra were recorded in a quartz cuvette (10 × 10 mm) at 25 °C. Stock solutions of free NIR dye **5** (2 mM) in DMSO and **NP1** (2 mg/mL) in 10 mM HEPES pH 8.3 were prepared. Before the measurement, stock solution of **5** was diluted to 2 μM in PBS pH 7.4 and **NP1** was diluted to 20 μg/mL in PBS pH 7.4, MES pH 6.5, MES pH 5.5 and citric acid/sodium citrate buffer pH 4.5 respectively. Photospectroscopic spectra of the working solutions of **5** and **NP1** at pH 7.4 in the wavelength range of 400–950 nm were recorded in Fig. S3.

Fluorescence emission spectra of all working solutions were collected in a quartz cuvette (10 × 10 mm) at a 90-degree angle relative to the excitation light path. The fluorescence spectra of **5** and **NP1** in PBS pH 7.4 were demonstrated in Fig S3. Compound **5** was excited at 784 nm, and **NP1** was excited at 708 and 776 nm respectively. For the time-dependent fluorescence intensity studies, **NP1** working

solutions with pH 7.4, 6.5 and 5.5 were excited at 776 nm and the fluorescence intensities at 824 nm were monitored at selected time-points (Fig. 3B). Excitation and emission slits were set to 4.0 nm. Average fluorescence emission intensity values were obtained from three independent measurements.

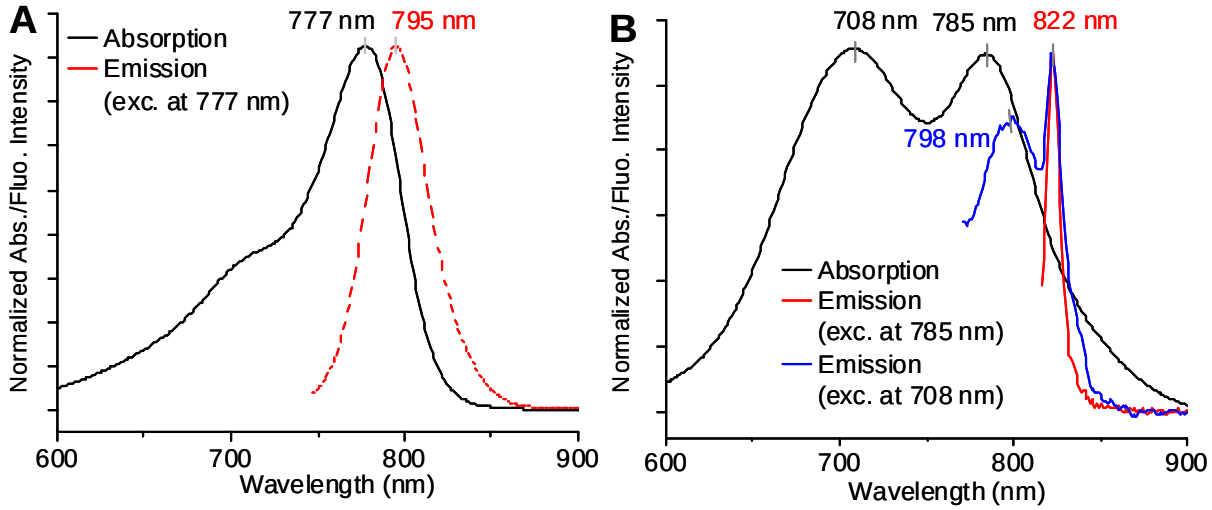


Figure S3. Normalized absorption and fluorescence spectra of free NIR dye **5** and nanoprobe **NP1** in PBS buffer (pH 7.4). (A) Absorption (solid black line) and emission (dashed red line, excited at 777 nm) spectra of free NIR fluorescence **5**. The stokes shift is 18 nm and the emission peak width is 42 nm. (B) Absorption (black line) and emission (red line, excited at 785 nm; blue line, excited at 708 nm) spectra of **NP1**. The stokes shifts are 37 and 114 nm respectively when the probe **NP1** was excited at 785 and 708 nm. The peak width of emission spectrum excited at 785 nm is 9 nm.

(b) NIR Fluorescence Imaging Studies. Stock solution of **NP1** with a concentration of 2 mg/mL was prepared in 10 mM HEPES pH 8.3. The stock solution was diluted 100 times to PBS pH 7.4, 10 mM MES pH 6.5, 10 mM MES pH 5.5 and 10 mM citric acid/sodium citrate pH 4.5 respectively with a final concentration of 20 μg/mL and a total volume of 100 μL in a 96-well plate. The plate was incubated in 37 °C for 1.0 h and imaged under a Xenogen IVIS® 200 small animal imaging system equipped with an indocyanine green (ICG) band pass filter set (Fig. 3A, excitation passband: 710–760 nm; emission passband: 810–875 nm). In Fig. 3B, **NP1** with a final concentration of 20 μg/mL in 10 mM MES pH 5.5 was incubated for 10 min, 1, 4 and 24 h in 37 °C before the imaging. All fluorescence images were

acquired with 1.0 s exposure time (FOV = 12.8 cm, f/stop = 4, Bin = high resolution, FOV = 12.8 cm). The fluorescence intensities were scaled as units of $ps^{-1}cm^{-2}sr^{-1}$ (Fig. 3). The pH and time-dependent fluorescence intensities of **NP1** in Fig. 3 were quantified (Fig. S4) by using ImageJ software (National Institutes of Health, Bethesda, MD). In Figure S4A–B, the values were normalized to the fluorescence intensity of the sample incubated in pH 7.4 for 1.0 h.

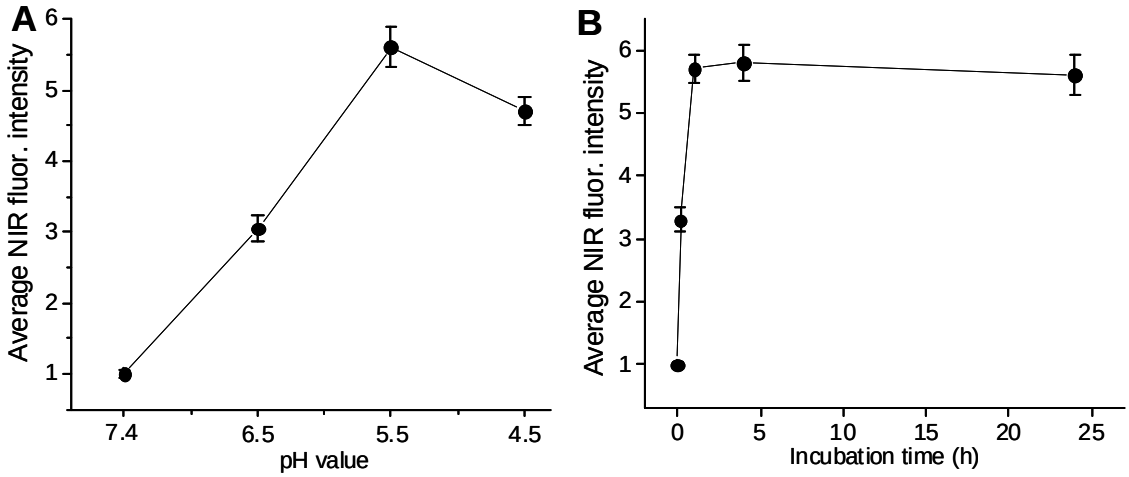


Figure S4. Quantification of the pH and time-dependent NIR fluorescent images of **NP1** in aqueous solution at 37 ℃. (A) pH-dependent average NIR fluorescence intensity of the images that were presented in Fig. 3A. (B) time-dependent average NIR fluorescence intensity of the images that were presented in Fig. 3B.

In vitro Cytotoxicity Studies

(a) Cell Culture. Human glioblastoma U87MG cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were grown as mono-layers in 75-cm^2 flasks containing Minimum Essential Medium, Alpha 1X (MEM, Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% penicillin and streptomycin (Invitrogen, Carlsbad, CA) in a fully-humidified incubator containing 5% CO_2 at 37 °C. Cells were harvested when they reached 80% confluence to maintain exponential growth.

(b) *In Vitro* Cytotoxicity Studies. The MTT cell proliferation assay was applied to determine the viabilities of the cells treated with **NP1** or Dextran-NH_2 used as a control. A cell monolayer in exponential growth was harvested using 0.25% trypsin, and a single-cell suspension was obtained. Cells were counted using a hemocytometer and a light microscope (OLYMPUS BH-2). Cell numbers were optimized to remain in exponential growth phase throughout the entire duration of an MTT assay experiment. To this purpose, cell suspensions containing cell density of 2×10^3 cells/well in 100 μL cell culture medium were added to 96-well plates by serial dilutions. Eight replicates were prepared under the same condition. 24 h after the cell attachment, the cells were treated with **NP1** or Dextran-NH_2 that was sterile filtered through MILLEX®-HV 0.45 μm syringe filter with concentrations in a range of 1–1000 μg/mL. After incubation for 4 days at 37 °C in 5% CO_2, cells were washed with PBS, and the cell viabilities were measured by MTT assay as we reported previously[3]. The cell viabilities after the treatment of **NP1** or Dextran-NH_2 were normalized to the value without any treatment (Fig. S5).

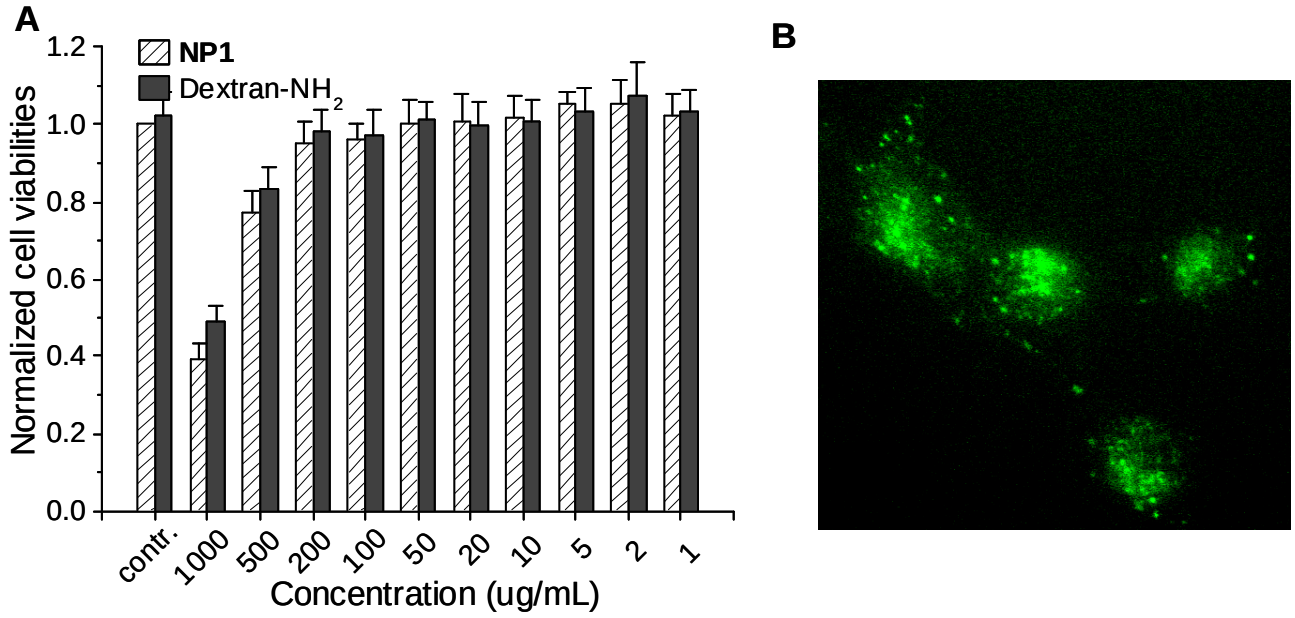


Figure S5. NP1 demonstrated low cytotoxicity and rapid intracellular uptake in the human brain U87MG cancer cells. (A) **NP1** demonstrated comparable cytotoxicity compared with Dextran-NH_2 in U87GM cancer cell cultures. Data are expressed as mean ± SD (n = 8 for each concentration tested). (B) Merged NIR and rhodamine fluorescence microscopic image of live U87MG cells treated with **NP1** (200 ug/mL) for 30 min. Predominated rhodamine fluorescence (displayed in green color) was observed at this time-point.

In vitro Fluorescence Microscopic Imaging

To avoid artifacts that occur during fixation, all the fluorescence microscopic experiments were conducted in live cells. Generally, U87MG cells (2×10^4) cultured on 35 mm glass bottom culture dishes (14 mm microwell, MatTek, Ashland, MA) to approximately 70% confluence were treated with selected probe. At the end of treatment, the cells were washed with Hanks balance salt solution (HBSS) 3× prior to addition of 1.0 mL of phenol red free media, and the cells were immediately imaged.

(a) *In Vitro* Confocal Fluorescence Microscopic Imaging. All confocal fluorescence microscopic images (Figure 4A–C and S6) were performed with a Zeiss LSM 510 META confocal laser-scanning microscope (Carl Zeiss, Inc., Thornwood, NY) using a Plan-Apochromat 63×/1.4 oil immersion lens (Zeiss). Rhodamine labeled in **NP1** was excited with a HeNe 543 nm laser, and fluorescence emission was detected by a photomultiplier tube using a 560 nm band-pass filter. FITC or Alexa Fluor-488 labeled in the markers were excited with an Ar laser (488 nm), and fluorescence was detected by a secondary photomultiplier by applying a 505–550 nm band pass filter.

(a1) Fluorescence colocalization between NP1 and lysosomal marker: Dextran-FITC. As demonstrated in Figure 4A and S6A, U87MG cells were treated with 200 μg/mL FITC labeled dextran (10 kDa), a well-known lysosomal marker in live cells for 3 h at 37 °C. After washing with Hanks 3×, the cells were continually cultured in dextran-FITC free medium for 16 h to facilitate the lysosomal chasing with this probe. To minimize recycling labeling events, media were changed and cells were treated with 200 μg/mL conjugate **NP1** for 1.0 h at 37 °C prior to the microscopy studies.

(a2) Fluorescence colocalization between NP1 and clathrin-mediated endocytosis marker: Transferrin. U87MG cells were simultaneously incubated with 100 ng/mL Alexa Fluor-488 labeled transferring (Tf): a classic clathrin-mediated endocytosis marker[4] and 200 μg/mL **NP1** in 2 mL serum free

medium for 30 min at 37 °C. The cells were washed 3× with HBSS, and 1.0 mL phenol red free medium supplemented with 10% FBS was added prior to microscopic analysis (Fig. S6B).

(a3) Fluorescence colocalization between NP1 and caveolae-mediated endocytosis marker: Cobra Toxin B Subuint. U87MG cells were pre-incubated with 5 μg/mL Alexa Fluor-488 labeled cobra toxin B subunit (CTB): a caveolae-mediated endocytosis marker[5] in serum-free media for 30 min following the addition of a mixture of **NP1** (200 μg/mL) and Alexa Fluor-488 labeled CtxB (5 μg/mL) for 1.0 h. After washing with Hanks 2×, 1.0 mL phenol red free medium supplemented with 10% FBS was added prior to microscopic analysis (Fig. S6C).

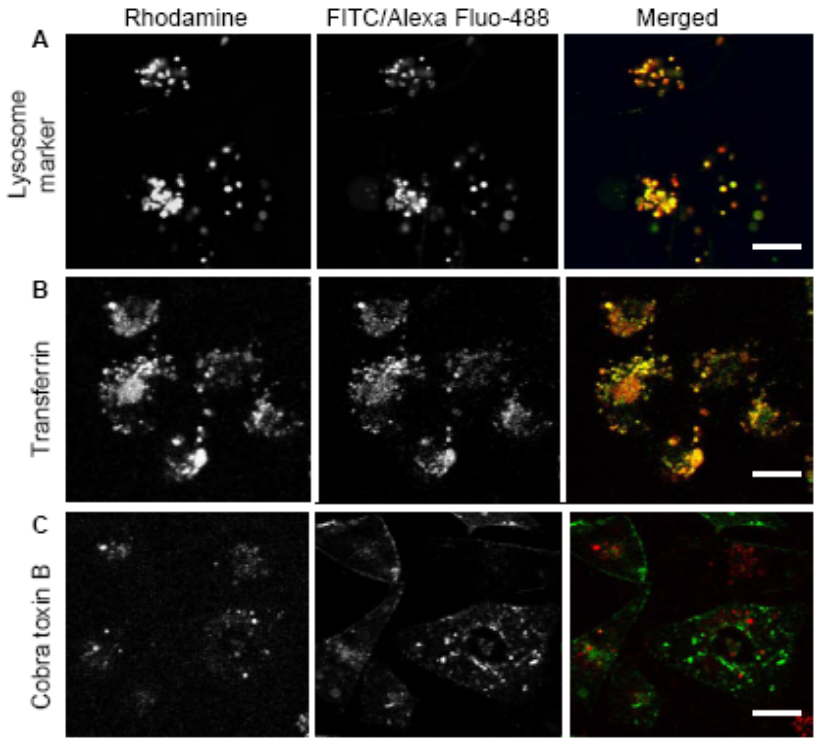


Figure S6. Confocal fluorescence microscopic studies of live U87MG cells showed that **NP1** was internalized into the lysosomes through a clathrin-mediated endocytosis pathway. (A) Cells pre-stained with lysosomal marker: Dextran-FITC were treated with **NP1**. The colocalization between the fluorescence of rhodamine labeled in **NP1** and the FITC labeled in the lysosomal marker verifies the intracellular lysosomal delivery of **NP1**. (B) Cells were treated with the claithrin-mediated endocytosis marker Alexa Fluo-488 labeled transferrin (Tf) and **NP1**. The colocalization between rhodamine and Alexa Fluo-488 showed **NP1** was mainly internalized through the claithrin-mediated endocytosis. (C) Cells were treated with caveolae-mediated endocytosis marker Alexa Fluo-488 labeled cobra toxin-B and **NP1**. The obvious single stained-vesicles indicated that **NP1** was not internalized via caveolae-mediated endocytosis. The scale bars present 10 μm.

(b) *In Vitro* NIR Fluorescence Microscopic Imaging.

All NIR fluorescence microscopic images (Figure 4D–F and S7) were obtained with an Olympus IX81 inverted microscope by using 60×/1.42 NA oil immersion lenses (Olympus America Inc., Center Valley, PA). The microscope was equipped with a Hamamatsu C9100 EM-CCD digital camera (Hamamatsu Photonics, Bridgewater, NJ) and IPLab 4.0 software (Scanalytics BD Biosciences, Rockville, MD). Near-infrared fluorescence was measured using an indocyanine green (ICG) filter cube (Chroma Set 41030; Chroma Technology Corp., Rockingham, VT; excitation: 775 ± 50 nm and emission: 845 ± 55 nm). Rhodamine fluorescence was detected using a Texas Red filter cube (Olympus America Inc.).

U87MG cells cultured in 35 mm glass bottom culture dishes were treated with 200 μg/mL **NP1** for 1, 2 and 24 h at 37 °C. At the end of incubation, the cells were washed with ice-cold Hanks for 3×, and 1.5 mL media without the phenol red was added prior to the microscopic imaging (Fig. 4D–F, Fig. S7).

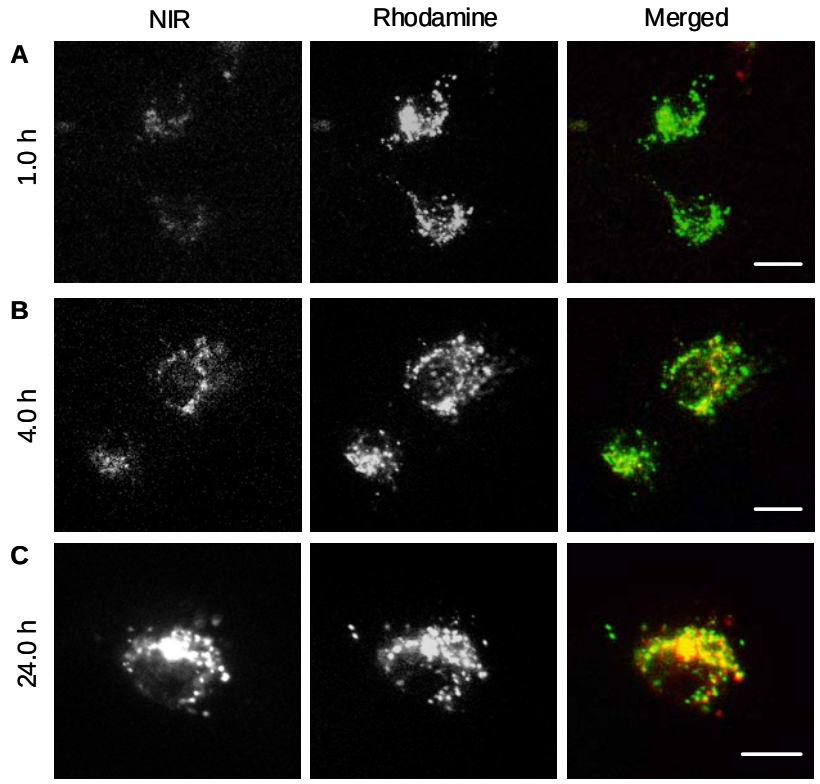


Figure S7. Fluorescence microscopic studies showed the activation of NIR fluorescence of **NP1** in the acidic compartments of live U87MG cancer cells. While the obvious rhodamine fluorescence as the vesicular structures in the cytoplasiam were observed in all time-points, the NIR fluorescence was low at 1 h post-incubation and increased significantly with the incubation time. 24 h afer incubation, the NIR fluorescence attained the high value and colocalized well with the rhodamine with the evidence of yellow color. The exposure times of rhodamine fluorescence and NIR fluorescence were 15 and 300 ms respectively in all experiments.

The incubation time dependent NIR fluorescence intensities in U87MG cells were further investigated in cell lysates. Briefly, cells were treated with 200 μg/mL **NP1** for 1 h and washed with PBS for 3×. The cells were re-cultured and incubated for another 0, 3 and 23 h. At the end of incubation, cells were washed, harvested, centrifuged and resuspended in home-made RIPA lysis buffer supplemented with protease inhibitor cocktail (1:200 dilution; Sigma- Aldrich). After the incubation and gently shaking at 4 °C for 30 min, the cell lysates were centrifuged at 20,000 g for 30 min at 4 °C. The clear supernatant was collected, and the total protein concentration was determined by using the DC protein assay (Bio-Rad,

Hercules, CA). The supernatants were diluted and the NIR fluorescence was measured by the fluorospectrometer. The integrated fluorescence intensities were normalized to the corresponding protein concentrations and the resulting data were further normalized to the sample in which the cells were lysated without the treatment of **NP1** (Figure S8).

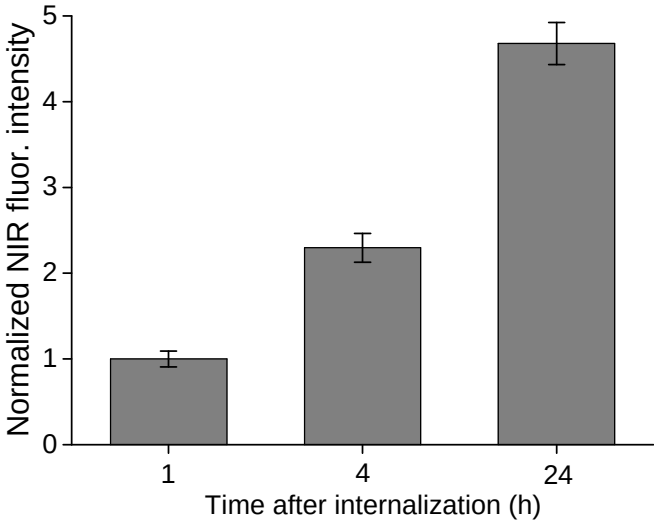


Figure S8. Internalized **NP1** demonstrated time-dependent NIR fluorescence intensity enhancements in the U87MG cell lysates. Values represent Mean ± S.D.

References

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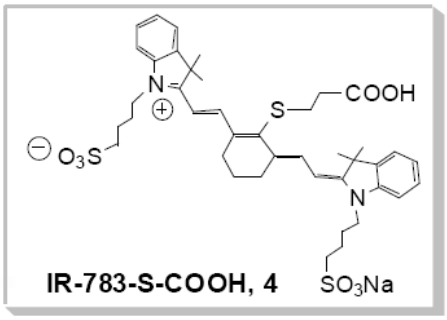
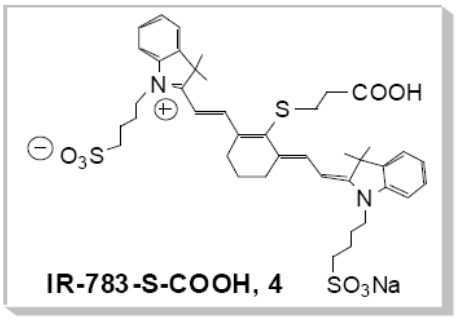


IR-783-S-COOH, 4

^{1}H NMR spectrum



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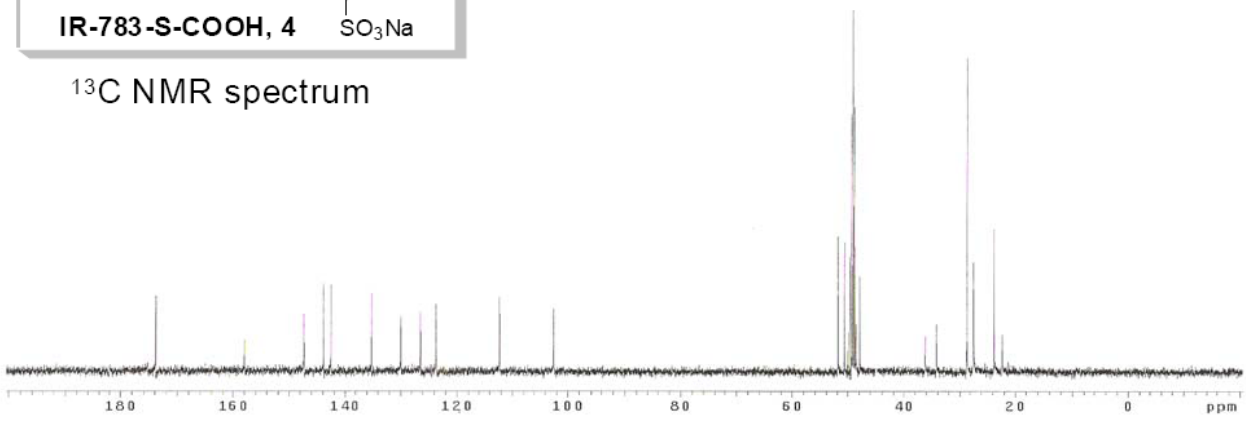
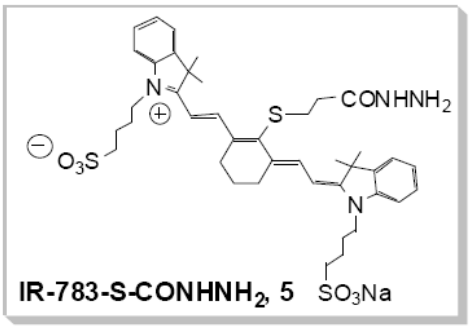
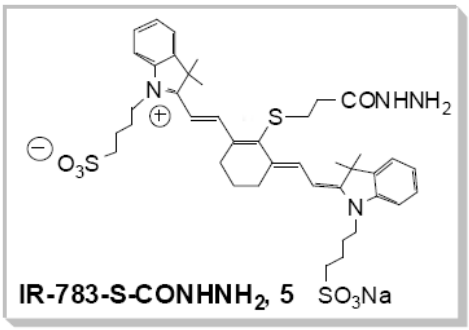
^{13}C NMR spectrum





IR-783-S-CONHNH$_2$, 5

^{1}H NMR spectrum



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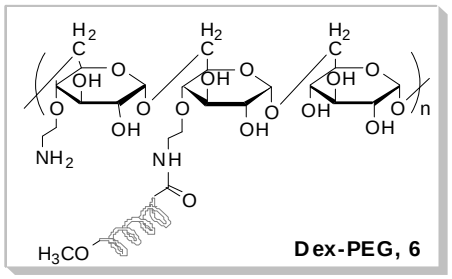

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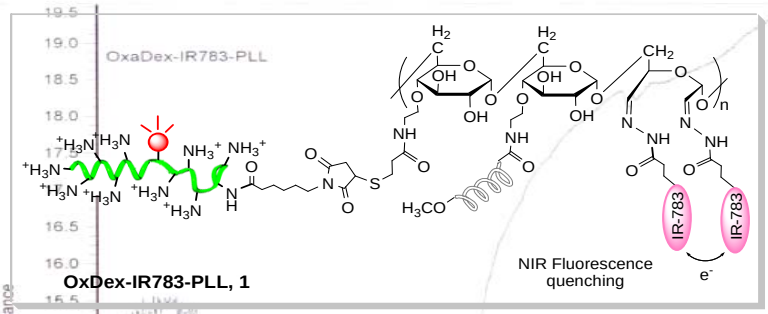

OxDex-IR783-PLL, 1

FT-IR spectrum