

Imaging Brain Tumor by Dendrimer-based Optical/Paramagnetic Nanoprobe across the Blood-Brain Barrier

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Materials and General Experimental Methods

All organic solvents were analytical grade from Aladdin Reagent (Shanghai, China) unless otherwise specified. PAMAM G5 dendrimer (77.35 mg/mL in methyl alcohol, containing 128 primary amino groups, MW: 28,826 Da) was purchased from Dendritech Inc (Midland, MI, USA). Rhodamine succinimidyl ester, Cy5.5 succinimidyl ester, *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP), fetal bovine serum (FBS), penicillin and streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). Activated polyethylene glycol derivatives PEG^{2k}-NHS ester and NH₂-PEG^{2k}-Maleimide were purchased from JenKem Technology Co. Ltd (Beijing, China). DOTA-NHS ester was prepared according to previous report.¹ Peptide TFFYGGSRGKRNNFKTEEYC in which a cysteine residue modified at the C terminal of angiopep-2 peptide TFFYGGSRGKRNNFKTEEY was prepared by the solid-phase peptide synthetic method and the purification was performed by a preparative reverse-phase Waters SymmetryPrepTM C18 7 μm 19 × 300 mm column equipped on a Waters 2487 dual absorbance detector and a Waters 600 controller with a gradient solvent system of acetonitrile in water (0.1% trifluoroacetic acid). The molecular weights of the nanoprobe were measured from Waters UltrahydrogelTM 500 (7.8 mm × 300 mm) gel permeable column, which was operated by High Performance Liquid Chromatography (HPLC) system equipped with a Waters 1525 binary pump and Waters 2487 dual wavelength absorbance detector (Waters Inc. Milford, MA). Human glioblastoma-astrocytoma, epithelial-like U87MG cell line and MTT cell proliferation kit were obtained from American Type Culture Collection (Rockville, MD, USA). Cell culture media (Minimum Essential Medium, Alpha 1X) was purchased from Mediatech (Manassas, VA). Amicon ultra-15 centrifugal filter tubes (10,000 MW cutoff) were from Millipore (Bedford, MA, USA). ¹H, ¹³C NMR spectra were recorded at Varian Mercury400 spectrometer. The Electron Spray Ionization (ESI) mass spectra were obtained on a SHIMAZDU LCMS-2020 (Kyoto, Japan) LCQ quadrupole ion trap mass spectrometer with methanol as the carrier solvent. MALDI-TOF mass spectra were collected on an AB SCIEX TOF/TOFTM 5800 mass spectrometer (Applied Biosystems, CA, USA). The hydrodynamic radius,

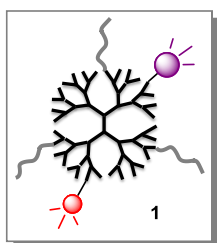
particle size distribution and Zeta potentials of the nanoprobe were measured on a Malvern Zetasizer (Malvern Instruments Inc., Southborough, MA) dynamic light scattering instrument. Gadolinium ion concentrations were determined with a Hitachi P-4010 (Tokyo, Japan) ICP-AES system in the research centre for analysis and measurement, Fudan University. The 35-mm glass bottom culture dishes for live cell microscopic imaging were purchased from MatTek, Ashland, MA. *In vitro* NIR fluorescence optical imaging was performed on a Kodak In Vivo Multispectral Imaging System equipped with a 630 nm excitation filter and a 700 nm emission band pass filter set. *In vivo* MR imaging was carried out on a Bruker Biospec 47/30 MRI scanner in Wuhan Institute of Physics & Mathematics, The Chinese Academy of Sciences. Confocal fluorescence images were performed with a Leica TCS SPE confocal laser-scanning microscope (Leica Inc., Wetzlar, Germany) using a HCXPL APO CS 40 × 1.25 oil immersion lens and a HCPL APO CS 10 × 0.40 immersion lens. Bio-distribution of the radiolabeled nanoprobe in selective organs of tumor-bearing mice was measured on a gamma-counter

Synthesis

Synthesis of cysteine modified Angiopep-2 peptide. To conjugate the angiopep-2 peptide into the G5 dendrimer without the compromising of the receptor binding specificity, TFFYGGSRGKRNNFKTEEYC (MW = 2402 Da) with a cysteine residue labeled at the C' terminal of the angiopep-2 was synthesized *via* Boc-protected solid-phase peptide synthesis strategy. The obtained fully protected linear peptide H-Thr(Bzl)-Phe-Phe-Tyr(Br-Z)-Gly-Gly-Ser(Bzl)-Arg(Tos)-Gly-Lys(Cl-Z)-Arg(Tos)-Asn(Xan)-Asn(Xan)-Phe-Lys(Cl-Z)-Thr(Bzl)-Glu(OcHex)-Glu(OcHex)-Tyr(Br-Z)-Cys(PMeBzl)-OH was deprotected in hydrofluoric acid (HF) and purified by preparative HPLC followed lyophilization. The purity was verified by analytic HPLC. ESI-MS: found 802.5 [M³⁺], calculated 2404.6 [M + H⁺].

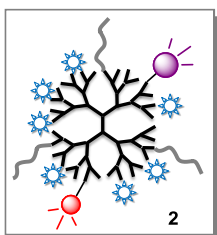
Synthesis of control nanoprobe **Den-PEG**. Treatment of *N*-hydroxysuccinimidyl (NHS) esters of rhodamine, Cy5.5 and PEG^{2k} with G5 dendrimer (29 kDa) in 0.1 M HEPES pH 8.3 respectively

gave compound **1**, which was further treated with DOTA-NHS ester to give **2**. The complexation of **2** with $\text{Gd}_2(\text{CO}_3)_3$ offered the control nanoprobe **Den-PEG**.



Preparation of compound **1**. Rhodamine-NHS ester (0.4 mg, 8×10^{-7} mol, 2.0 equiv.) dissolved in 50 μL anhydrous DMF was added dropwise to G5 dendrimer (11.6 mg, 4×10^{-7} mol) in 1.0 mL 0.1 M HEPES pH 8.5 solution. After stirring at 25 $^\circ\text{C}$ for one hour, the mixture was loaded to the centrifugal filter (MW 10,000 cut off) and centrifuged with a speed of 4000 rpm to remove the hydrolytic by-products.

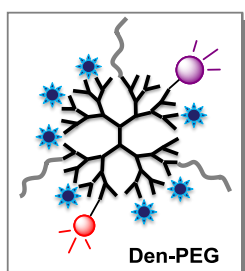
Cy5.5-NHS ester (1.2 mg, 8.0×10^{-7} mol, 2.0 equiv.) in 50 μL anhydrous DMF was added to the rhodamine-labeled dendrimer in 1.0 mL HEPES (0.1 mmol, pH 8.3). After stirring for one hour and the dual-fluorophore conjugated dendrimer was purified by centrifuge. To a solution of dual fluorophore labeled dendrimer in 2.0 mL 1X PBS (pH 7.4) was added PEG^{2K}-NHS ester (10.4 mg, 5.2×10^{-6} mol, 13 equiv.) in 2.0 ml PBS. The mixture was allowed to stir at the room temperature for 1 h. The product was purified in a centrifugal filter (MW 10,000 cut off) and the resulting compound **1** was stored in PBS (pH 7.4) as a purple color solution (yield: 82%, 3.28×10^{-7} mol, 18.9 mg). The rhodamine and Cy5.5 labeling degree was determined by measuring the absorbance of rhodamine ($\epsilon_{552} = 60,000 \text{ M}^{-1}\text{cm}^{-1}$) and Cy5.5 ($\epsilon_{675} = 250,000 \text{ M}^{-1}\text{cm}^{-1}$). The molar ratio between dendrimer and PEG was quantified by measuring the proton integration of dendrimer (3.3–2.2 ppm) and PEG (3.7 ppm, O- CH_2) in the ^1H NMR spectrum. On average, 1.5 rhodamine, 1.1 Cy5.5 and 6 PEG were labeled on each dendrimer.



Preparation of DOTA conjugated G5 dendrimer (**2**). To the solution of compound **1** (3.28×10^{-7} mol, based on dendrimer) in 2 mL 0.5 M HEPES buffer pH 8.3, DOTA-NHS ester (41.6 mg, 4.2×10^{-5} mol, 128 equiv.) as a white solid was added. The pH of the solution was monitored by pH meter and adjusted around 8.5 by

adding 5.0 M NaOH solution. The mixture was stirred at r.t. for further 3 h and the resulting product was purified into 0.1 M HEPES pH 8.3 by the centrifugal filtration (MW 10,000 cut off) with a yield of 92% (based on dendrimer). The number of DOTA chelators labeled on dendrimer was quantified as 75 by measuring the proton integration of dendrimer and DOTA in the ^1H NMR spectrum of compound **2**.

Preparation of control nanoprobe **Den-PEG**. The complexation of compound **2** with gadolinium ion

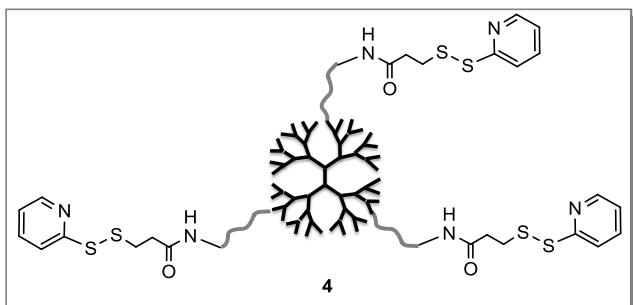


was conducted as our previous work.² $\text{Gd}_2(\text{CO}_3)_3$ (9.5mg, 1.93×10^{-5} mol, 64 equiv.) was added to **2** (3.0×10^{-7} mol, based on dendrimer) in 4 mL 0.1 M HEPES pH 8.3. The suspension was allowed to stir at 60 °C for 12 h. The excess $\text{Gd}_2(\text{CO}_3)_3$ was spin down by the centrifugation (2000 rpm) and the clear supernatant was purified in the centrifugal filter (MW 50,000 cut off) as

described above with a yield of 95% (based on dendrimer).

Synthesis of aiming nanoprobe **Den-Angio**. Bis-activated PEG moiety $\text{NH}_2\text{-PEG}^{2k}\text{-Maleimide}$ reacted with *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) to give **3**, which further reacted with G5 dendrimer to obtain **4**. Treatments of NHS esters of rhodamine, Cy5.5 and DOTA with **4** respectively gave **5**. The condensation between **5** and home-made TFFYGGSRGKRNNFKTEEYC peptide followed the complexation with $\text{Gd}_2(\text{CO}_3)_3$ offered the aiming nanoprobe **Den-Angio**.

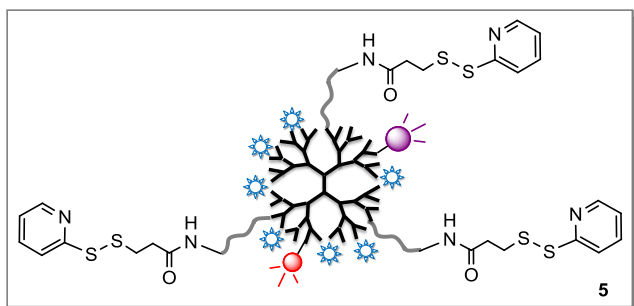
Preparation of SPDP labeled dendrimer derivative (**4**). *N*-succinimidyl 3-(2-pyridyldithio) propionate



(SPDP) (2.1 mg, 6.8×10^{-6} mol, 1.3 equiv.) in 300 μL DMF was added to the solution of $\text{NH}_2\text{-PEG}^{2k}\text{-Maleimide}$ (10.4 mg, 5.2×10^{-6} mol, MW is considered as 2 kDa) in 1.0 mL 1X PBS (pH

7.4). After reaction at r.t. for 45min, the resulting PEG derivative **3** with maleimide and SPDP [3-(2-pyridyldithio) propionate] modified at its two terminals was added to G5 dendrimer (11.6 mg, 4×10^{-7} mol) in 1.0 mL 1X PBS (pH 7.4) without further purification. After stirring at r.t. for overnight, compound **4** in which the dendrimer and SPDP connected through a PEG linker was purified by centrifugation. The molar ratio between dendrimer and PEG was quantified by measuring the proton integration of dendrimer and PEG in the ^1H NMR spectrum of compound **4**. The labeling degree of SPDP in **4** was quantified by DL-Dithiothreitol (DTT) assay. Briefly, excess DTT was added to compound **5** in PBS solution and vortexed for 15 min. The absorbance of above solution at 343 nm was measured. The molar ratio between SPDP to dendrimer was calculated with the equation: $R = \Delta A_{343}/8080 \times C_{\text{dendrimer}}$, where R represents the molar ratio between SPDP and dendrimer; ΔA_{343} represents the change of the absorbance at 343 nm before and after DTT addition; $C_{\text{dendrimer}}$ represents the molar concentration of G5 dendrimer; the value of 8080 represents the extinction coefficient of pyridine-2-thione at 343 nm. On average, 5.0 SPDP moieties were labeled in each dendrimer.

Preparation of rhodamine, Cy5.5, DOTA and SPDP labeled dendrimer derivative (**5**). As

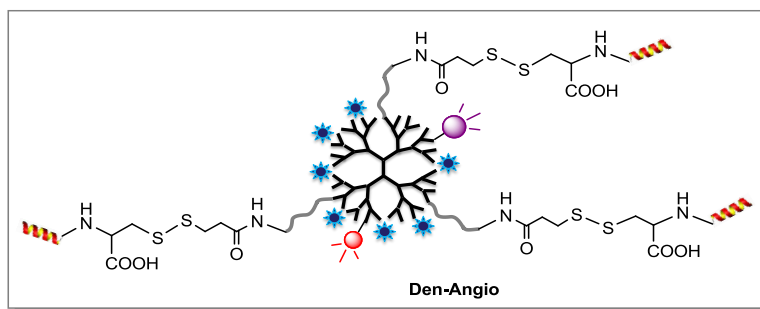


demonstrated before, the NHS esters of rhodamine (0.4 mg, 8×10^{-7} mol, 2.0 equiv..) and Cy5.5 (1.2 mg, 8.0×10^{-7} mol, 2.0 equiv) dissolved in 50 μL anhydrous DMF was added to **5** in 1.0 mL 0.1 M HEPES pH 8.3 solution

consequently. After reaction for 1.0 h, the fluorophore labeled dendrimer was purified via centrifugation and then concentrated into 2.0 mL 0.5 M HEPES pH 8.5. DOTA-NHS ester (50.7 mg, 5.12×10^{-5} mol, 128 equiv.) was added to above solution with the pH maintained at 8.5. After stirring at 25 $^\circ\text{C}$ for 3 h, the mixture was centrifuged (MW 10,000 cut off) to remove unconjugated DOTA

chelators. The rhodamine, Cy5.5 and DOTA modified dendrimer **6** was stored in PBS (pH 7.4) as a purple color solution. Averagely, there are 1.4 rhodamine, 1.1 Cy5.5 and 56 DOTA chelators labeled in compound **5**.

Preparation of the aiming nanoprobe **Den-Angio**. To a solution of compound **5** in 2 mL PBS buffer



solution, cysteine labelled angiopep-2 peptide (12 mg, 5.2×10^{-6} mol, 13 equiv.) in 200 μ L DMF was added. The mixture was stirred at r.t. for overnight, and the angiopep-2 conjugated dendrimer was

purified in the centrifugal filter (MW 50,000 cut off) and concentrated into 2.0 mL PBS. The labeling degree of Angiopep-2 peptides was measured by quantifying the proton integration of dendrimer and the aromatic proton of phenylalanine (7.4–6.6 ppm) according to the ^1H NMR spectrum of **7**. In the next step, $\text{Gd}_2(\text{CO}_3)_3$ (12.7 mg, 2.56×10^{-5} mol, 64 equiv.) was added to the **7** in 2 mL PBS pH 7.4. The suspension was allowed to stir at 60 $^\circ\text{C}$ for 12 h. The excess $\text{Gd}_2(\text{CO}_3)_3$ was spin down by the centrifugation (2000 rpm) and the clear supernatant was purified in the centrifugal filter (MW 10,000 cut off) to obtain **Den-Angio** with a yield of 92% (based on dendrimer).

Characterization

Determination the Purity of Nanoprobes by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Nanoprobe (50 μ g) in 20 μ L treatment buffer [100 mM Tris, pH 6.8, 2% (w/v) sodium dodecyl sulfate (SDS), 12% (v/v) glycerol, 0.01% (w/v) bromphenol blue] was loaded on a 10% SDS-PAGE gel. After electrophoresis under a vantage of 100 V for 2 h, fluorescent of the resulting SDS-PAGE gels were acquired by Kodark In Vivo Multispectral Imaging System equipped with a 540 nm

excitation filter and a 590 nm emission band pass filter set (FOV = 12.8 cm, $f/\text{stop} = 4$, bin = high resolution, exposure time = 2 s).

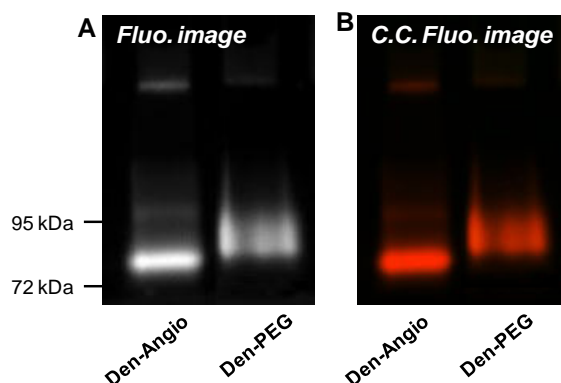


Figure S1. Representative fluorescent image of a SDS-PAGE gel loaded with the indicated nanoprobe (50 μg nanoprobe/well). The fluorescence (A) and color coded (C. C.) fluorescence (B) images of the resolved SDS-PAGE gel were collected by an optical imaging system with exposure time of 2 second.

Determination of Molecular Weight of Nanoprobes by Gel Permeation Chromatography

All gel permeation chromatographic (GPC) studies were carried out as the below typical method if there is not any specific mention. Mobile phase: 1 X PBS, pH 7.4; isocratic flow rate: 0.7 mL/min; operating temperature: 25 °C; injected volume: 20 μL . The gel-permeable column was calibrated by the Gel Filtration Molecular Weight Kit (Aldrich, St. Louis, MO), which comprises a set of protein markers in the range of 29 kDa to 669 kDa. The void volume (V_o) of the column is determined by applying the blue dextran at a concentration of 2 mg/mL. The leading peak monitored at 280 nm indicates the void volume. The elution volumes (V_e) of the different protein 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. V_e/V_o of each protein standard. The elution volumes (V_e) of nanoprobe were measured with the same method. The effluent peak of nanoprobe was monitored at 211 (absorbance of dendrimer) and 577 nm (absorbance of rhodamine) respectively (Figure S2A–B). The overlapping peaks monitored at 211 and 577 nm confirm the conjugation of rhodamine into dendrimer. The molecular weights of **Den-PEG** and **Den-Angio** were obtained as 92 kDa and 83 kDa respectively by applying their V_e/V_o values against the calibration curve (Figure S2C).

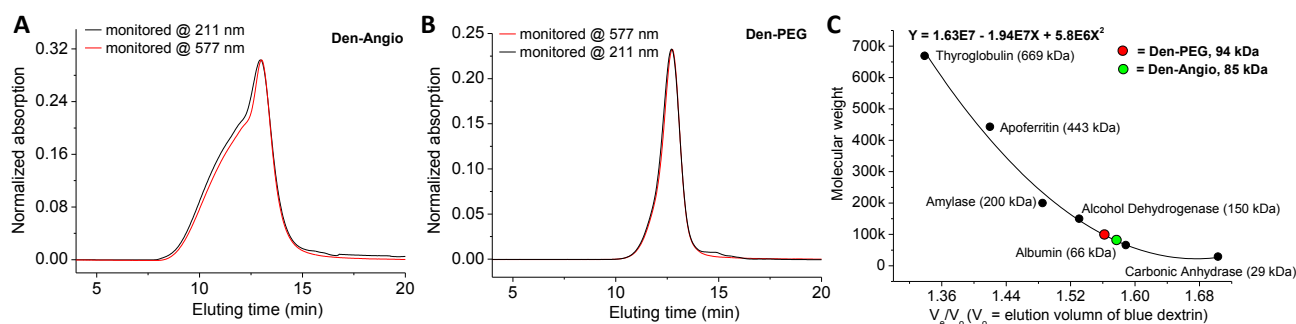


Figure S2. Determination of the molecular weight of nanoprobes by GPC. Gel permeable chromatography of **Den-Angio** (A) and **Den-PEG** (B) monitored at 211 nm (black line, dendrimer) and 577 nm (red line, rhodamine). (C) The molecular weight of nanoprobes was determined by fitting with the calibration curve of protein markers (molecular weight ranged from 29–669 kDa) that was measured under the identical HPLC method.

Determination of Molecular Weight of Nanoprobes by MALDI-TOF MS

The MALDI-TOF MS were obtained using an AB SCIEX TOF/TOFTM 5800 mass spectrometer that was calibrated by BSA (Sigma A-0281) and HSA (Sigma A-3782). The specimens were prepared by mixing 1.0 μ L nanoprobe solution (10 μ M in methanol) with 1 μ L matrix (10 mg/mL CHCA in a solution of 50% ACN, 50% H₂O and 0.1% TFA) on the measuring plate and air-drying.

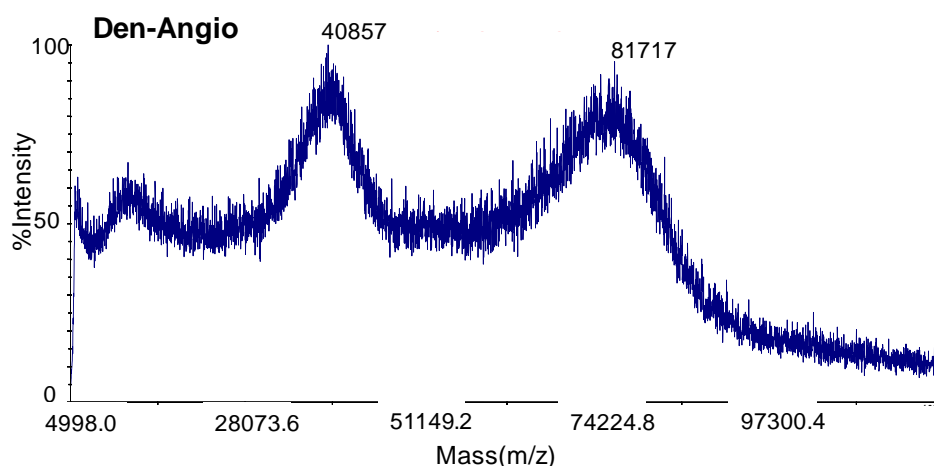


Figure S3. Determination of the molecular weight of **Den-Angio** by MALDI-TOF MS. Representative MALDI-TOF MS of nanoprobe **Den-Angio**. Two main peaks with molecular weight of 41 kDa and 82 kDa were monitored. The peak at 82 kDa can be attributed to the molecular ion peak and the peak at 41 kDa can be attributed to the double charged molecular ion peak.

Determination of Hydrodynamic Size Distribution and Zeta Potentials of Nanoprobes

Hydrodynamic radius of nanoprobes and unmodified G5 dendrimer was determined by dynamic light scattering (DLS) (Figure S1) at room temperature. The standard solution of bovine serum albumin (BSA) in distilled water with a concentration 2.0 mg/ml was used for the instrument calibration. The samples were filtered through 0.45 μm filter and diluted to 100 $\mu\text{g}/\text{mL}$ by PBS pH 7.4. The hydrodynamic radius and the size distribution were calculated with the regularization algorithm. To measure the surface charges of nanoprobe, the instrument was calibrated with the standard solution with a Zeta potential of -50 mV. The nanoprobes were satirized by filtrating through a 0.45 μm filter and diluted to 200 $\mu\text{g}/\text{mL}$ in 10 mM NaCl solution.

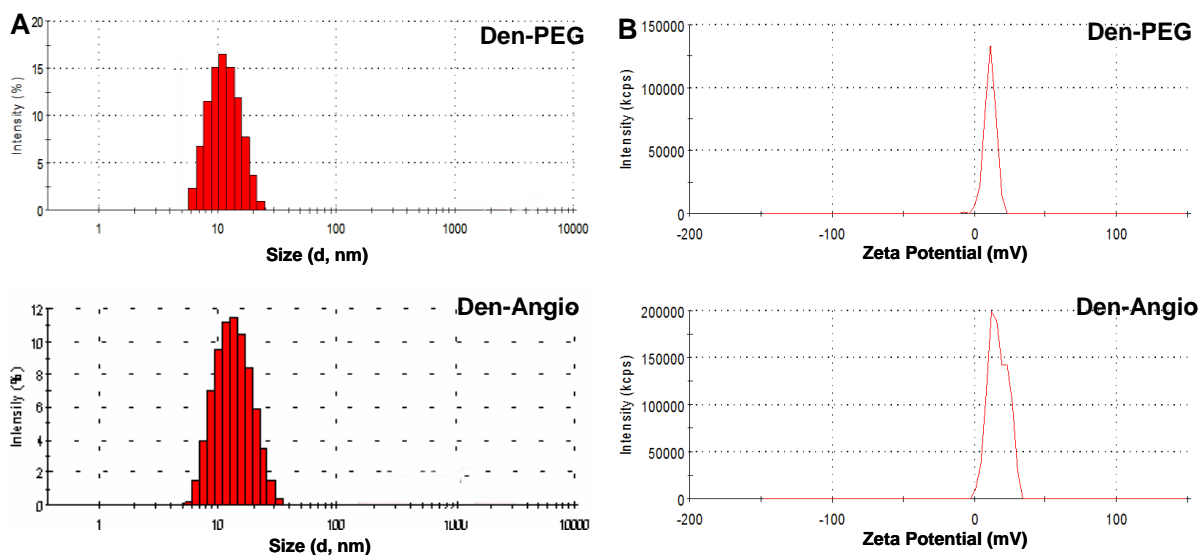


Figure S4. Hydrodynamic size distributions (A) and the Zeta potentials (B) of nanoprobes measured by dynamic light scattering (DSL). The average diameters of **Den-PEG** and **Den-Angio** were measured as 11.3 and 13.3 nm. The average Zeta potentials of **Den-PEG** and **Den-Angio** were measured as +16.7 and +11.6 mV.

Determination of the Gd^{3+} Concentration in Nanoprobe by ICP-AES

Gd^{3+} concentration of nanoprobe was determined by Hitachi P-4010 model ICP-AES (Inductively Coupled Plasma Atomic Emission Spectroscopy) with RF power at 1100 W and nebulizer gas flow at 0.9 L/min. The standard solutions with the Gd^{3+} concentration of 1, 5, 10, 20, 50, 100, 200 ppm in 3% nitric acid were prepared, and a calibration curve was made by plotting the corresponding chromatographic peaks vs. the Gd^{3+} concentrations. Stock solution of nanoprobe (0.1 mM) was

diluted 100 times by 3% nitric acid. The Gd^{3+} concentration of the sample was obtained by applying the detected Gd^{3+} peak against the calibration curve.

Determination of the Relaxivity of Nanoprobes

The longitudinal relaxivities of the nanoprobes and the commercial available MR contrast agent Gd^{3+} -DOTA were determined according to the equation of $r_{1p} = (1/T_{\text{sample}} - 1/T_{\text{PBS}})/[\text{Gd}]$. The T_1 values of PBS and selected compounds with four different concentrations in PBS pH 7.4 were measured with a 4.7 T MR spectrometer at 25 °C. Plotting the $(1/T_{\text{sample}} - 1/T_{\text{PBS}})$ values of nanoprobes with corresponding Gd^{3+} concentrations measured by ICP-AES provided the relaxivity of the nanoprobes. The longitudinal relaxivities r_{1p} of Gd^{3+} -DTPA, **Den-Angio** and **Den-PEG** were measured as 4.7, 6.9 and 7.4 $\text{mM}^{-1}\text{s}^{-1}$ respectively.

In Vitro Studies

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.

Live Cell Confocal Fluorescence Microscopic Imaging

To avoid the artifacts that occur during fixation procedures, all the experiments were conducted in live U87MG cells. Cells (2×10^4) cultured on 35 mm glass bottom culture dishes (14 mm microwell, MatTek, Ashland, MA) to approximately 50% confluence were added with 2 μM

nanoprobe in 2 mL media supplemented with 10% FBS for selected period at 4 or 37 °C. At the end of incubation, the cells were washed with Hanks Balance Salt Solution (HBSS) 3× prior to addition of 1 mL phenol red free media, and the cells were immediately imaged by confocal fluorescence microscopy.

Competitive Studies of Cellular Uptake of Nanoprobe *In Vitro*.

Live U87MG cells cultured in 35-mm glass bottom culture dishes were pre-incubated with regular medium supplemented with 2 μM low-density lipoprotein receptor-associated protein (RAP) that was utilized as the universal competitor for LRP receptors for 30 min at 4 °C. At the end of incubation, the cells were washed and the media supplemented with 2 μM RAP and 2 μM **Den-Angio** was added and continue to incubate for 15 min at 4 °C. At the end of incubation, cells were washed 3× with HBSS and subjected to microscopy imaging.

Cytotoxicity Studies

The MTT cell proliferation assay was applied to determine the viabilities of the cells treated with nanoprobe and the unmodified G5 dendrimer 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 nanoprobe or unmodified G5 dendrimer that was sterile filtered through MILLEX[®]-HV 0.22 μm syringe filter with final concentrations in a range of 0.05–10 μM. After incubation for 4 days at 37 °C in 5% CO₂, cells were washed with PBS, and the cell viabilities were measured by MTT assay as we reported

previously². The cell viabilities after the treatment of nanoprobe or G5 dendrimer were normalized to the value without any treatment (Figure S5).

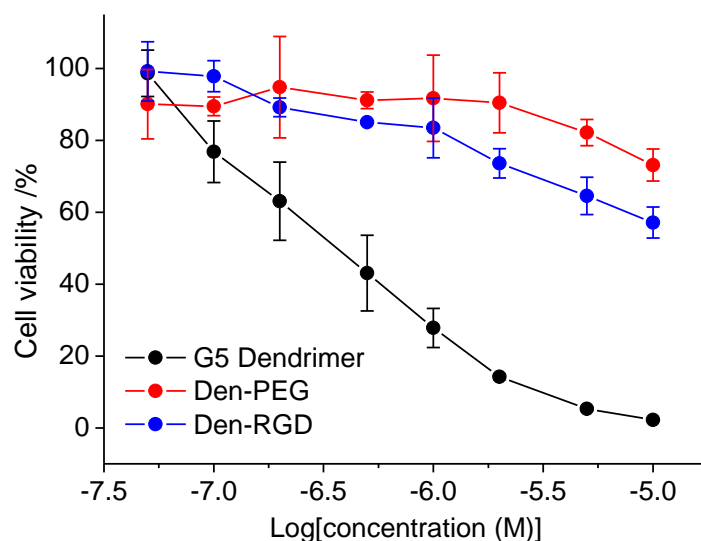


Figure S5. Both **Den-PEG** and **Den-Angio** demonstrated lower cytotoxicity than the unmodified G5 dendrimer in human brain U87MG cancer cell culture. Data are expressed as mean \pm SD (n = 8 for each concentration tested).

Mouse Model and Tumor Implantation

All animal experiments were carried out in accordance with guidelines evaluated and approved by the ethics committee of Fudan University. Wild type U87MG human glioblastoma cancer cells (1.0×10^6 suspended in 5 μ l PBS) were inoculated into the right striatum (1.8 mm lateral, 0.6 mm anterior to the bregma and 3 mm of depth) of male Balb/c nude mice by using a stereotactic fixation device with mouse adaptor. The intracranial tumors with a diameter of 0.2–0.5 mm were ready for imaging experiments after the inoculation for 14–18 days.

In Vivo and *Ex Vivo* Optical Imaging Studies

Optical imaging was performed on a Kodak In Vivo Multispectral Imaging System equipped with a 630 nm excitation filter and a 700 nm emission band pass filter set. Before the imaging, mice were anesthetized with a mixture of ketamine (25 mg/kg)/acepromazine (2.5 mg/kg) and placed face-

up in an imaging plate. X-ray images (exposure time: 30 s), white light photography (exposure time: 0.2 s) and NIR fluorescence images (exposure time: 15.0 s) with the same field of view (FOV) were acquired (FOV = 12.8 cm, f/stop = 4, Bin = high resolution) respectively before and at selected time-points after systemic injection of nanoprobe (4.0 nmol based on dendrimer). The X-ray images were co-registered with the NIR fluorescence images to define the intracranial volume. The time-dependent fluorescence intensity ratio between tumor and surrounding normal brain tissues (*T/N* ratio) were calculated and normalized to the value measured pre-injection of nanoprobe. At the end of *in vivo* imaging, the mouse was anesthetized and perfused with saline and 4% paraformaldehyde (PFA) in succession *via* heart to douche blood and pre-fix the mouse. Brain was carefully isolated and the main organs including liver, kidney, lung, spleen, heart and muscle were sectioned with a thickness of 1–2 mm by a tissue slicer (Braintree Scientific Inc., Braintree, MA). The microscopic fluorescence intensities in tumor and surrounding healthy brain tissue were quantified by ImageJ (National Institutes of Health, Bethesda, MD) software.

***In Vivo* Magnetic Resonance Imaging**

In vivo MR imaging was carried out on a Bruker Biospec 47/30 MRI scanner. Before the experiment, mouse tail vein was catheterized with a home-built catheter system using a small T-junction (Cole-Parmer, Vernon Hills, IL) devised to minimize the dead volume of nanoprobe solution (less than 50 μ L). The head of the anesthetized mice was placed into a home-built solenoid coil. Body temperature of the animals within the magnet was maintained by a thermostat-regulated heating pad and the respiration was continuously monitored by a Bruke PhysioGard system. Isoflurane (0.5–2%) in 100% oxygen was administered upon placement of mouse in the MR coil, and continuously adjusted by monitoring the respiration. Dynamic T1-weighted images of the brain were collected before and after intravenous tail vein injection of a total 0.25 mL PBS solution of nanoprobe with a dose of 0.05 mmol/kg [Gd³⁺]. T1-weighted MR signal enhancements were monitored up 0–120 min and 24 h post

injection. Coronal images of the brain with 1 mm thick slices were acquired with a spin-echo pulse sequence, field of view (FOV) 2 cm × 2 cm, matrix size 128 × 128, TR = 300 ms, TE = 11 ms, and number of average = 8. 3D T1-weighted images were obtained with a fast low-angle shot (FLASH) sequence, flip angle=45°, FOV = 1.5 cm × 1.5 cm × 1.5 cm, matrix size 128 × 128 × 32, TR = 35 ms, TE = 6.2 ms, and number of averages = 8. The intensity enhancement (IE) of region of interest (ROI) at time point t is expressed by $IE = (RI(t) - RI(0))/RI(0) \times 100\%$, where $RI(t)$ corresponds to the normalized signal intensity measured at time point t and $RI(0)$ is the normalized signal intensity before the nanoprobe injection. The time-dependent fluorescence intensity ratio between tumor and surrounding normal brain tissues (T/N ratio) were calculated and normalized to the value measured pre-injection of nanopro

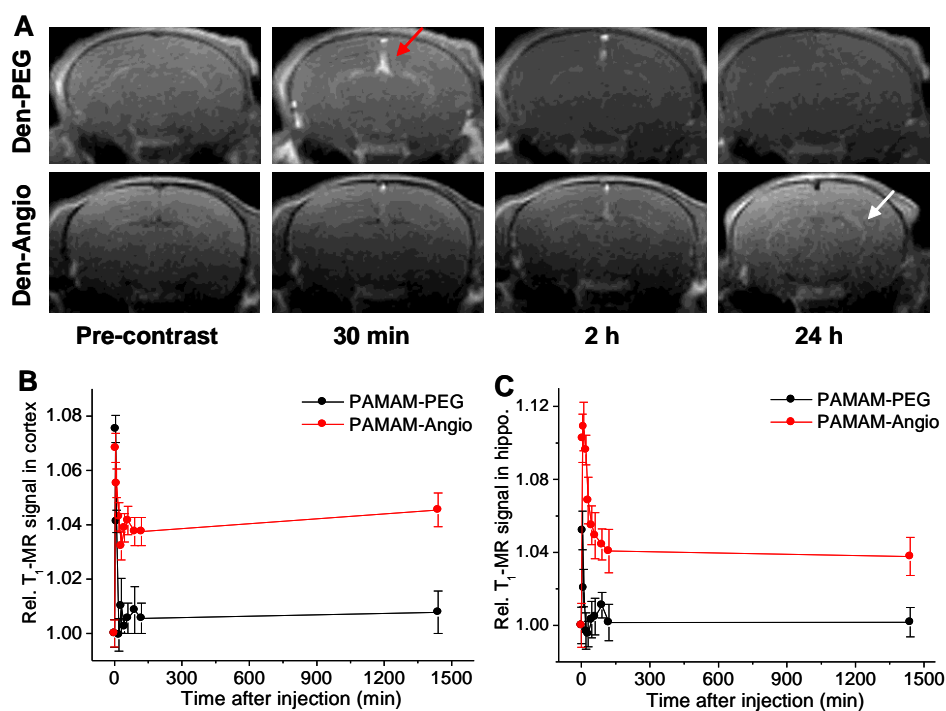


Figure S6. Den-Angio demonstrated higher cerebra uptake than Den-PEG in healthy mouse brain with intact BBB. (A) Representative T1-weighted MR images of the mouse brain before and at 30 min, 2 h and 24 h PI of nanoprobe with a dose corresponding to 0.05 mmol/kg based on Gd³⁺. Red arrow points to the cerebra ventricle and white arrow points to the hippocampus. Time dependent T1-weighted MR signal variations in the cortex (B) and hippocampus (C) area before and after injection of nanoprobe via *i.v.* (n = 4).

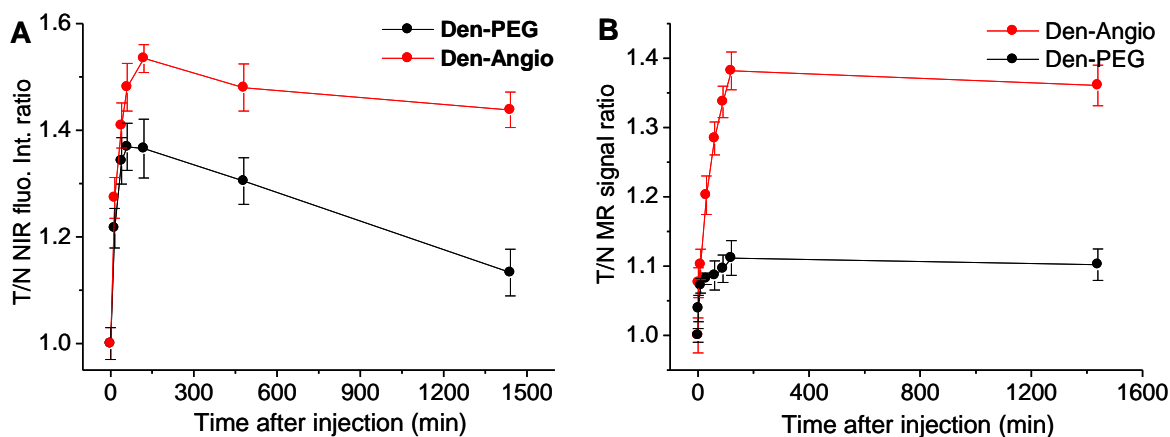


Figure S7. Both *in vivo* MR and optical imaging verified the higher T/N signal ratio of **Den-Angio** compared to **Den-PEG**. *In vivo* time-dependent normalized NIR fluorescence intensity ratio (A) and T1-weighted MR signal ratio (B) between tumor and the surrounding healthy brain tissue (T/N ratio) after the nanoprobe injection via *i.v.*. T/N values were normalized to the value measured before nanoprobe injection. Data were presented as mean \pm SD ($n = 3$). The injection dose is 0.05 mmol $[\text{Gd}^{3+}]$ /mouse for MR studies and 0.02 mmol $[\text{Gd}^{3+}]$ /mouse for the optical imaging studies.

Bio-distribution Studies

Nanoprobes were radiolabeled with ^{125}I isotope on the primary amines of dendrimer by using the Bolton-Hunter reagent (Pierce Biotech., USA) following the purification by the 10 kDa centrifugal tube to remove the unlabeled ^{125}I isotope. Eight U87MG tumor bearing female nude mice (20 g) were divided into two groups randomly. Mice in each groups were injected with $[\text{}^{125}\text{I}]\text{Den-PEG}$ (200 μL , 100 μCi) or $[\text{}^{125}\text{I}]\text{Den-Angio}$ (200 μL , 100 μCi) through the tail vein. The mice were sacrificed at 24 h post-injection and perfused with physiological saline. Selected organs (heart, lung, liver, spleen, kidney, muscle, brain) and tumor were excised and weighted. The radioactivity was counted with an automatic γ -counter. The percentage of the injected dose per gram (%ID/g) was calculated by comparing the tissue counts with the injected radioactivity dose.

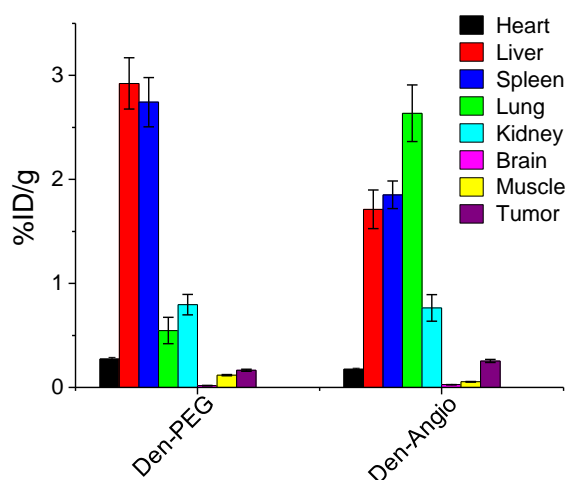


Figure S8. Bio-distribution of radioactive ^{125}I labeled nanoprobe in U87MG brain tumor bearing mice at 24 h post-injection. Averagely the nanoprobe with a radioactivity of 100 μCi was injected via *i.v.* The organs and tumor tissues were excised after the perfusion with PBS to remove the nanoprobe in the circulation system.

***Ex Vivo* Confocal Fluorescence Microscopic Imaging**

At the end of *in vivo* imaging, the mice were anesthetized and perfused with physiological saline and 4% paraformaldehyde (PFA) in succession via heart to douche blood and pre-fix them. The brains were carefully isolated and immersed in 4% PFA for 12 h. Fixed brains were further immersed in 30% sucrose solution for 24 h and cryo-sectioned with a thickness of 5 μm . Rhodamine was excited with a 543 nm laser, and fluorescence emission was detected by a photomultiplier tube using a 560 nm band-pass filter. Simultaneously, DAPI was excited with a 405 nm laser and the fluorescence emission was detected with a second photomultiplier by applying a 490 nm dichroic beam splitter and a 420–480 nm band-pass filter. All the microscopic images were captured with the identical microscopic setting.

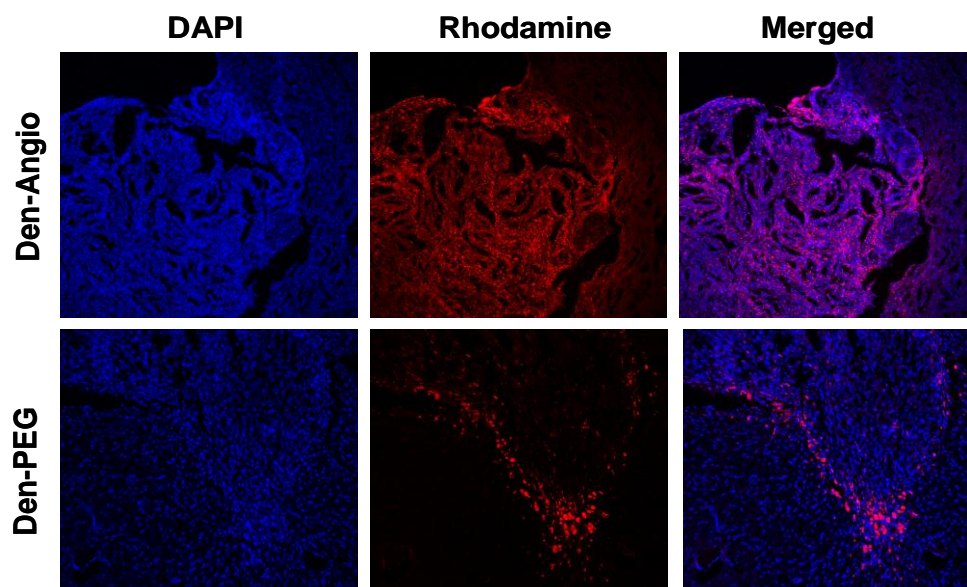


Figure S9. Fluorescence microscopic images of mouse brain bearing U87MG tumor xenograft at 24 h PI of **Den-Angio** (upper panel) or **Den-PEG** (low panel) via *i.v.* (0.02 mmol [Gd³⁺]/mouse). The fluorescence of rhodamine labeled in nanoprobe was displayed in red color and the fluorescence of DAPI count-stained in cell nuclei was displayed in blue color. The nuclear staining in tissue section helps to define the boundary between tumor and surrounding normal brain tissue.

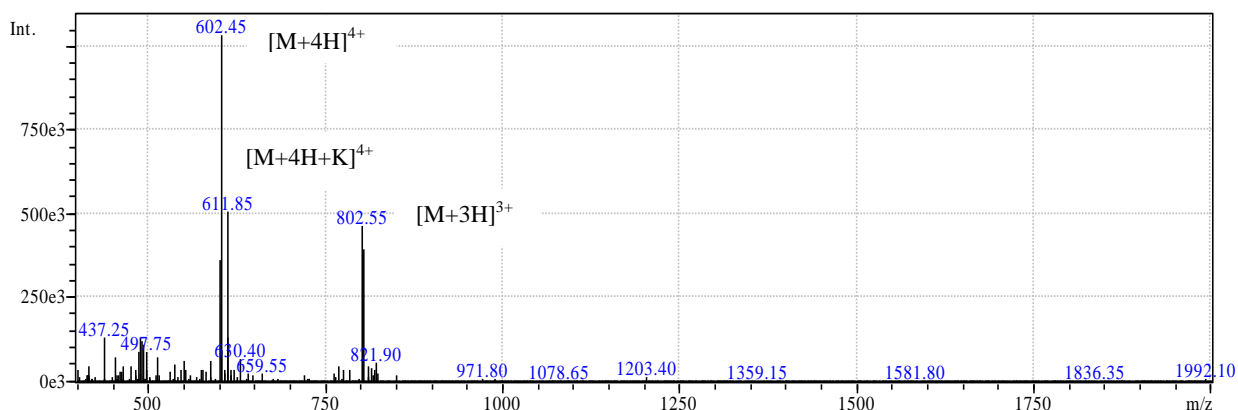
Histological H&E Staining

The brain treated with nanoprobe were excised and immersed in 10% neutral buffered formalin overnight. Fixed tissues were embedded in wax and sectioned with a thickness of 10 μm . The slides were stained with H&E in the Department of Pathology, Shanghai Medical College of Fudan University and imaged by a Leica MZ75 high-performance stereomicroscope equipped with 2.5 X and 5.0 X plano objectives.

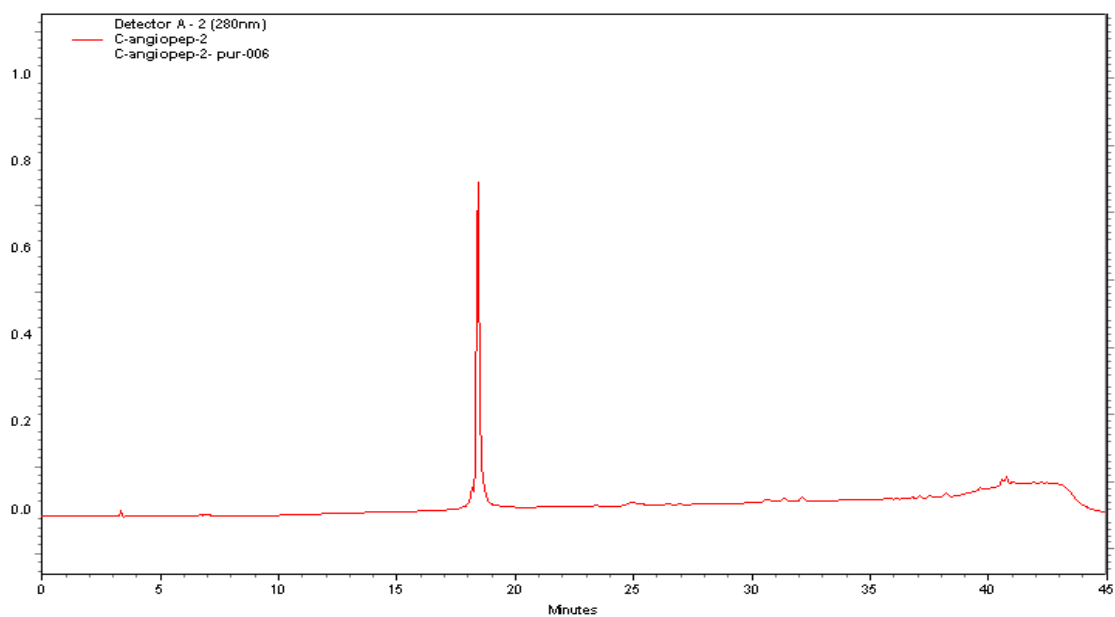
References

1. Li, C.; Winnard, P.; Bhujwala, Z. M., Facile synthesis of 1-(acetic acid)-4,7,10-tris(tert-butoxycarbonylmethyl)-1,4,7,10-tetraaza-cyclododeca ne: a reactive precursor chelating agent. *Tetrahedron Lett.* **2009**, 50 (24), 2929-2931.
2. Li, C.; Winnard, P. T., Jr.; Takagi, T.; Artemov, D.; Bhujwala, Z. M., Multimodal image-guided enzyme/prodrug cancer therapy. *J Am Chem Soc* **2006**, 128 (47), 15072-3.

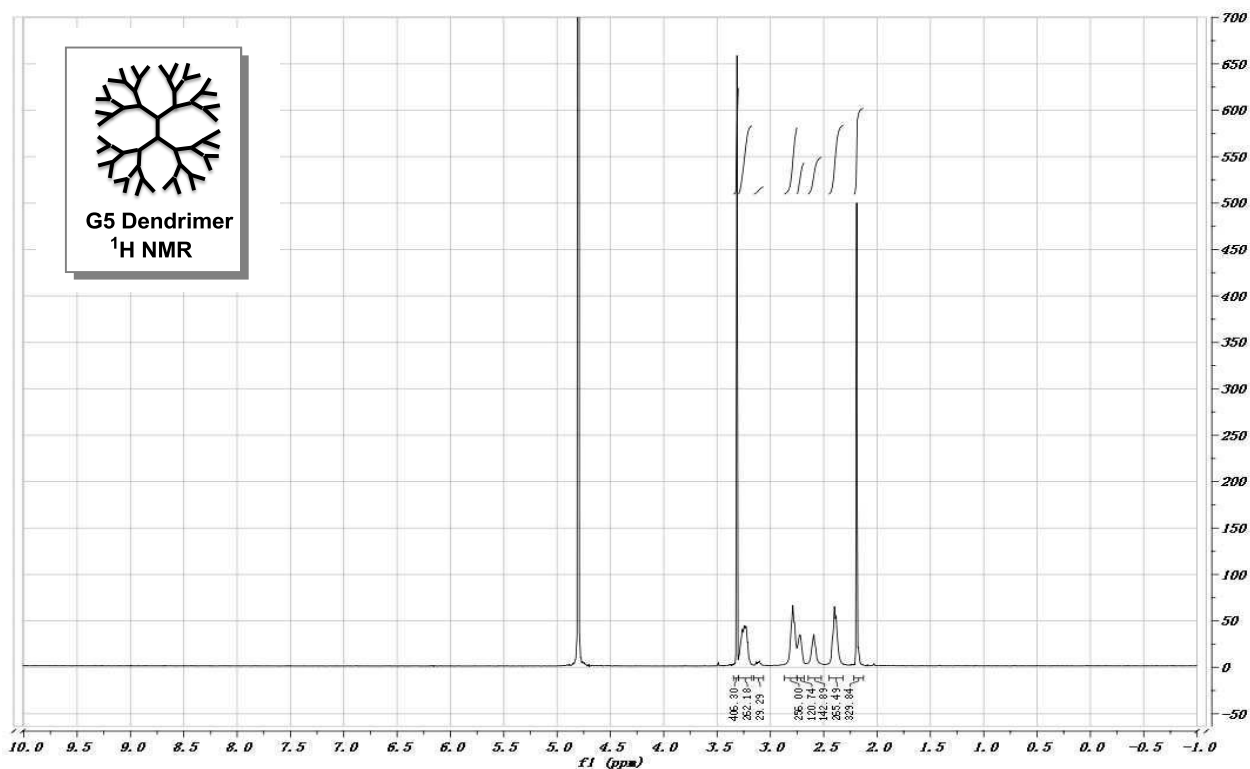
Supplemental Spectra



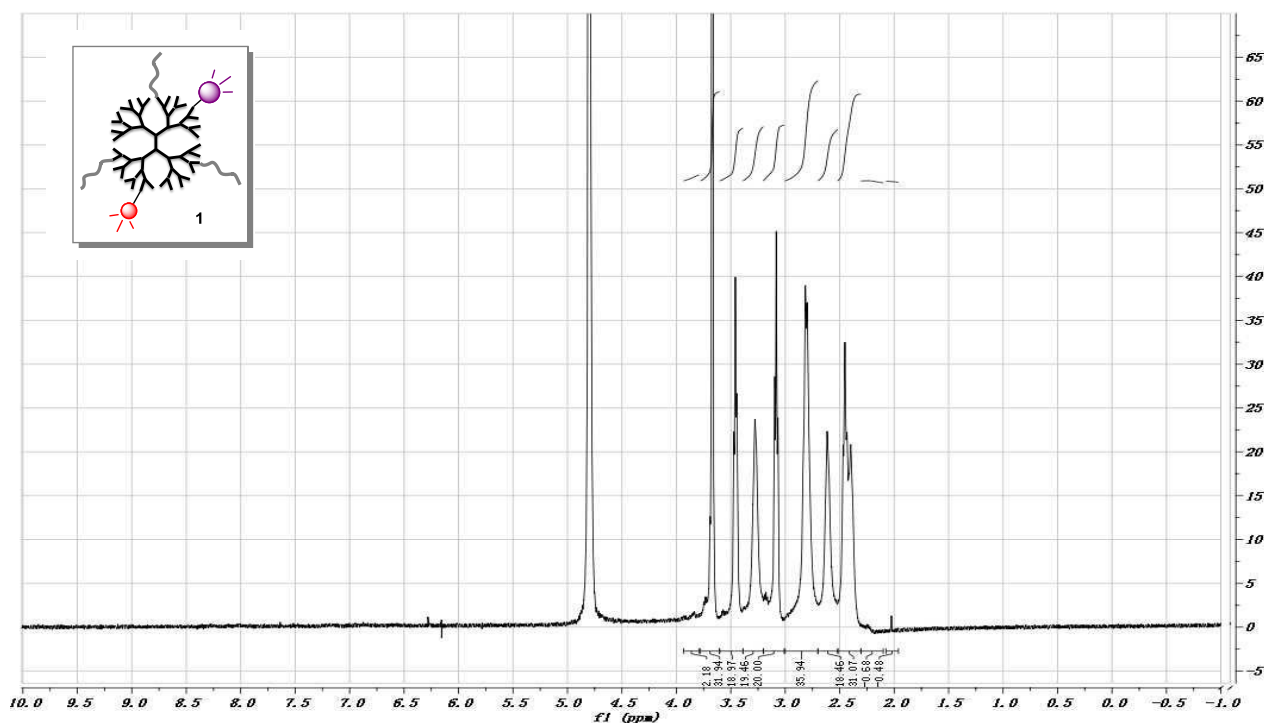
Spectrum 1. Mass spectrum of TFFYGGSRGKRNNFKTEEYC peptide (MW: 2404.6 Da). Found ES-MS 602.45 $[M+4H]^{4+}$; 611.85 $[M+4H+K]^{4+}$; 802.55 $[M+3H]^{3+}$.



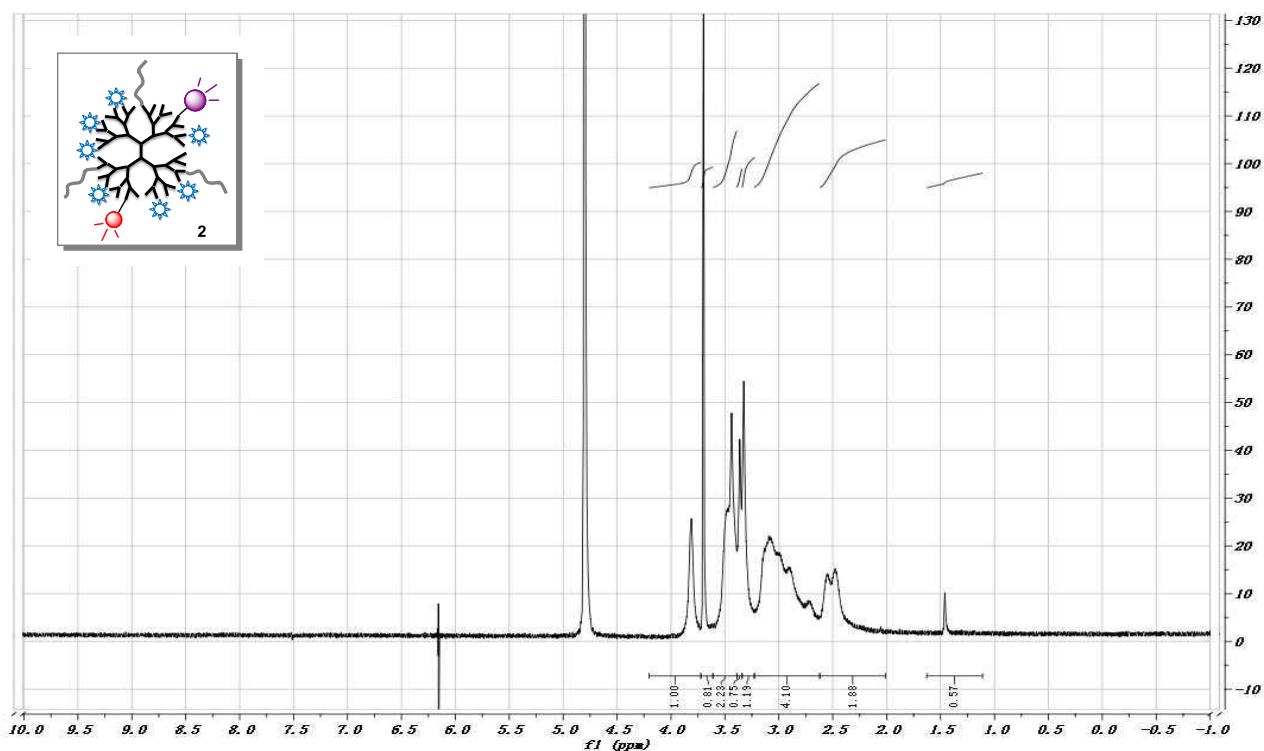
Spectrum 2. Analytical HPLC-Vis chromatography of TFFYGGSRGKRNNFKTEEYC peptide. Flow rate, 0.7 mL/min; solution A: 0.1% TFA in acetonitrile, solution B: 0.1% TFA in H₂O. Running time: 45 min, monitored at 214 and 280 nm. HPLC method: the solvent system was programmed linearly from 90 to 65% solvent B from 0 to 26 min. Then a constant solvent system of 10% B was applied from 27 to 32 min. At last, a constant 90% solvent B was applied to wash the column.



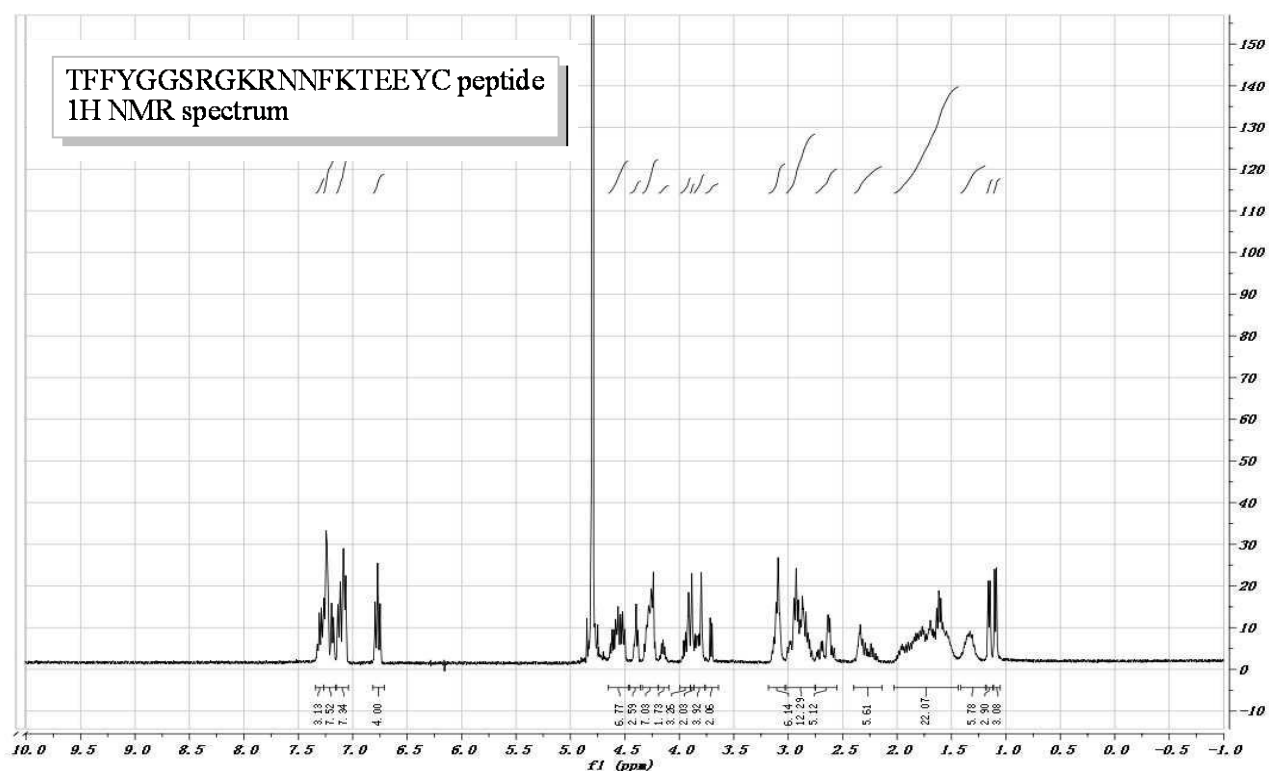
Spectrum 3. ¹H NMR spectrum of G5 dendrimer. ¹H NMR (400MHz, D₂O): δ 3.3–3.1 ppm (778H, m, dendrimer), 2.9-2.65 ppm (420H, m, dendrimer), 2.65-2.5 ppm (158H, m, dendrimer), 2.5-2.25 ppm (296H, m, dendrimer), 2.25-2.1ppm (368H, m, dendrimer).



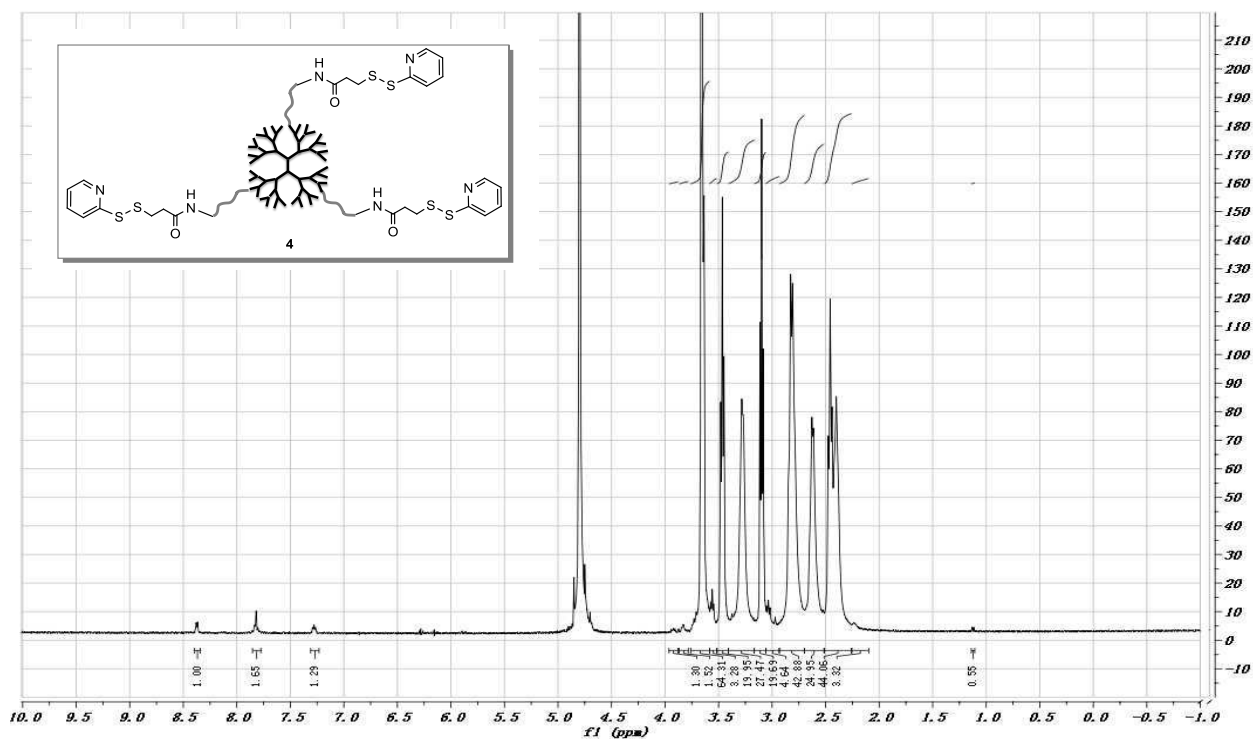
Spectrum 4. ¹H NMR spectrum of **1**. ¹H NMR (400MHz, D₂O): δ 3.7 ppm (180H, m, PEG), 3.6-3.4ppm (266H, m, dendrimer), 3.4–3.15 ppm (273H, m, dendrimer), 3.15-3.0 ppm (281H, m, dendrimer), 3.0-2.7 ppm (505H, m, dendrimer), 2.7-2.5 ppm (259H, m, dendrimer), 2.5-2.2 ppm (436H, m, dendrimer).



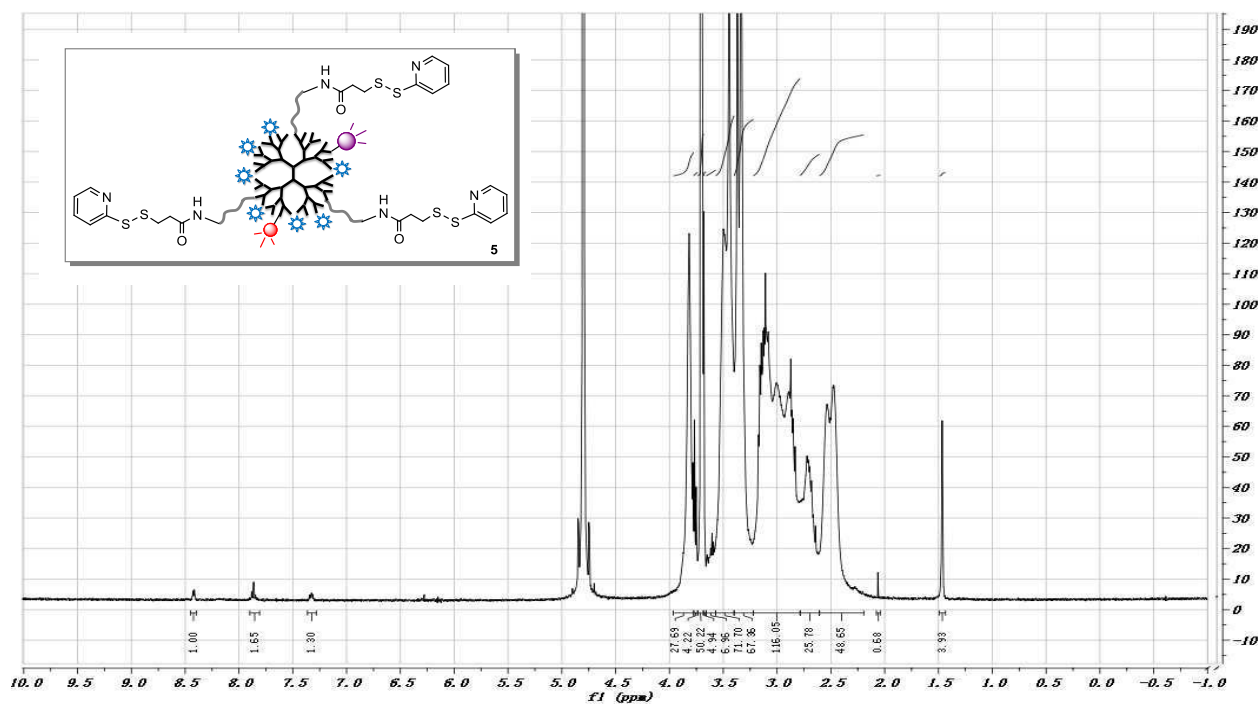
Spectrum 5. ¹H NMR spectrum of **2**. ¹H NMR (400MHz, D₂O): δ 3.7 ppm (180H, m, PEG), 3.5–2.2 ppm (2020H, m, dendrimer and DOTA).



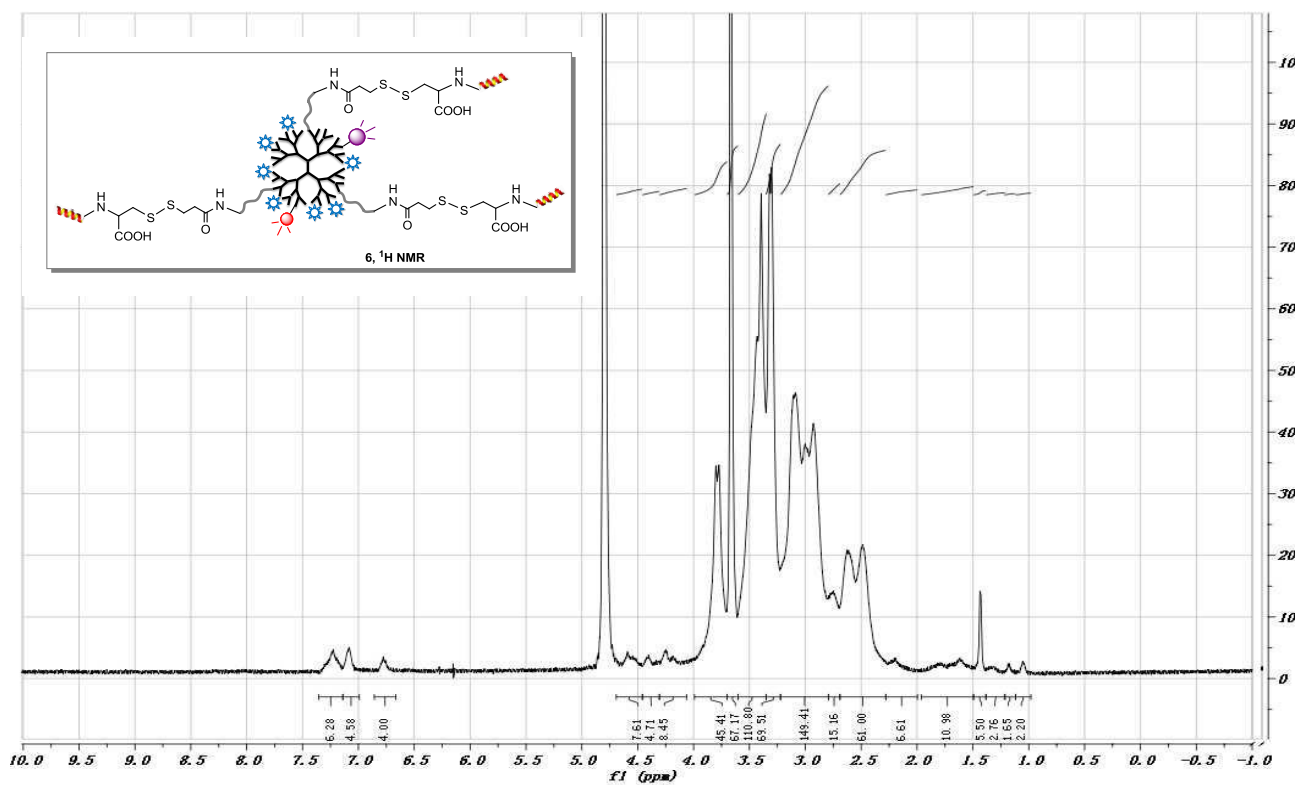
Spectrum 6. ¹H NMR spectrum of angiopep2 peptide. ¹H NMR (400MHz, D₂O): δ 7.4-7.0 ppm (17H, m), 6.75 ppm (4H, m), 4.7-4.5 ppm (7H, m), 4.5-4.0 ppm (11H, m), 4.0-3.7 ppm (8H, m), 3.2-2.5 ppm (22H, m), 2.5-2.0 ppm (5H, m), 2.0-1.5 ppm (21H, m), 1.5-1.0 ppm (11H, m).



Spectrum 7. ¹H NMR spectrum of **4**. ¹H NMR (400MHz, D₂O): δ 8.4-7.2ppm (4H, m, SPDP), 3.7 ppm (180H, m, PEG), 3.6-3.4 ppm (266H, m, dendrimer), 3.4–3.15 ppm (273H, m, dendrimer), 3.15–3.0 ppm (281H, m, dendrimer), 3.0-2.7 ppm (505H, m, dendrimer), 2.7-2.5 ppm (259H, m, dendrimer), 2.5-2.2ppm (436H, m, dendrimer).



Spectrum 8. ¹H NMR spectrum of **5**. ¹H NMR (400MHz, D₂O): δ 8.4-7.2 ppm (4H, m, SPDP), 3.7 ppm (180H, m, PEG), 3.5–2.2 ppm (about 3220H, m, dendrimer and DOTA).



Spectrum 9. ¹H NMR (400MHz, D₂O): δ 7.4-7.0 ppm (17H, m), 6.75 ppm (4H, m), 3.7 ppm (180H, m, PEG), 3.5–2.2 ppm (about 3220H, m, dendrimer and DOTA).