

Two-Order Targeted Brain Tumor Imaging By Using an Optical/Paramagnetic Nanoprobe across the Blood Brain Barrier

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Synthesis

1. Synthesis of cyclic [RGDyK] peptide. c[RGDyK] was prepared by the Fmoc-protected solid-phase peptide synthetic method. The obtained fully protected linear peptide (Fmoc-Gly-2-cl-Trt)-(Fmoc-Arg(pbf)-OH)-(Fmoc-Lys(Boc)-OH)-(Fmoc-D-Tyr(tBu)-OH)-(Fmoc-Asp(OtBu)-OH) was cutting down from the resin by trifluoroacetic acid (TFA). The linear peptide was then cyclized in presence of benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PYBOP) and DIEA (N,N-Diisopropylethylamine). The cyclic peptide was deprotected in 95% TFA. Peptide purification was performed by a preparative reverse-phase Waters SymmetryPrep™ C18 column (7 μ m, 19 \times 300 mm) equipped on a Waters 600 controller with a gradient solvent system of acetonitrile in water (0.1% trifluoroacetic acid) followed lyophilization. Peptide purity was verified by a reverse phase analytic YMC column (150 \times 4.6 mm) equipped on an Agilent 1100 High Performance Liquid Chromatography. The molecular weight of the peptide was determined by Electron Spray Ionization mass spectra (SHIMADZU LCMS-2020, Kyoto, Japan) LCQ quadrupole ion trap mass spectrometer with methanol as the carrier solvent. ESI-MS: found 620.5 [M + H⁺], calculated 619.5.

2. Synthesis of cysteine modified Angiopep-2 peptide. Angiopep-2 peptide: 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 a preparative reverse-phase Waters SymmetryPrepTM300A C18 7 μ m 19 \times 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% TFA) followed lyophilization. ESI-MS: found 802.5 [M^{3+}], calculated 2404.6.

3. Synthesis of control nanoprobe Den-RGD. 5 mg c(RGDyK) (8.06×10^{-6} mol, 13 equiv.) and 12.4 mg Mal-PEG^{2K}-NHS ester (6.2×10^{-6} mol, 10 equiv) were added into 500 μ L anhydrous DMF in presence of 10 μ L triethylamine. The mixture was stirred at room temperature (r.t.) for 2 h and added dropwise into PAMAM G5 dendrimer (17.9 mg, 6.2×10^{-7} mol) in 2.0 mL PBS (pH 7.4). The mixture was stirred for overnight to obtain compound **2**, which was further purified by a centrifugal filter (Amicon ultra-15 centrifugal filter tube, MW 1,500 cut off) from Millipore (Bedford, MA, USA) to remove the unconjugated by-products. Rhodamine-NHS ester (0.62 mg, 1.24×10^{-6} mol, 2.0 equiv.) in 50 μ L anhydrous DMF and Cy5.5-NHS ester (1.86 mg, 1.24×10^{-6} mol, 2.0 equiv.) in 50 μ L DMF were added dropwise into compound **2** in 1.0 mL 0.1 M HEPES buffered solution with pH 8.5. After stirring at 25 $^{\circ}$ C for 1.0 h, the mixture was purified by a centrifugal filter (10,000 MW cutoff). To the solution of dual fluorophores labeled compound **2** in 2 mL 0.5 M HEPES buffer with pH 8.5, DOTA-NHS ester (78.6 mg, 7.94×10^{-5} mol, 128 equiv.) as a white solid was added directly in 15 min and pH of the solution was monitored by pH meter and maintained around 8.5 by adding 1.0 M NaOH. After addition, the mixture was stirred at r.t. for overnight the resulting

product was purified by a centrifugal filter (10,000 MW cutoff) to obtain compound **3**. The complexation of compound **3** with gadolinium ion was conducted according to reported reference (Li, C. et al. *Clin. Cancer Res.* **2008**, *14*, 515). $\text{Gd}_2(\text{CO}_3)_3$ (19.5 mg, 3.97×10^{-5} mol, 64 equiv.) was added to compound **3** in 4 mL 0.1 M HEPES solution (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 and the clear supernatant was purified by centrifugation followed lyophilization to offer nanoprobe **Den-RGD** with an overall yield of 82% (based on dendrimer).

4. Synthesis of two-order targeted nanoprobe Den-RGD-Angio. *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) (2.5 mg, 8.06×10^{-6} mol, 13 equiv.) in 300 μL DMF was added to the solution of $\text{NH}_2\text{-PEG}^{2k}\text{-Mal}$ (12.4 mg, 6.2×10^{-6} mol, 10 equiv) in 1.0 mL PBS (pH 7.4). After reaction at r.t. for 1.0 h, the resulting PEG derivative **4** with maleimide and SPDP [3-(2-pyridyldithio) propionate] modified at its two terminals was added to compound **2** (6.2×10^{-7} mol) in 1.0 mL PBS (pH 7.4) without further purification. After stirring for 1.0 h, compound **5** was purified by centrifugation. Rhodamine-NHS ester, Cy5.5-NHS ester, and DOTA-NHS ester were labeled into G5 dendrimer in succession as described above and obtain multiple imaging reporter conjugated compound **6**. To a solution of compound **6** (4.0×10^{-7} mol) in 2 mL PBS, cysteine labelled angiopep-2 peptide (18.6 mg, 5.2×10^{-6} mol, 13 equiv.) in 200 μL DMF was added. The mixture was stirred at r.t. for overnight and was purified in the centrifugal filter (MW 10,000 cut off). After the complexation with $\text{Gd}_2(\text{CO}_3)_3$, the aiming nanoprobe **Den-RGD-Angio** was obtained with a yield of 84% (based on dendrimer).

Characterization

1. The composition of nanoprobe. The molar ratios between dendrimer, PEG, RGD, SPDP and DOTA in nanoprobe were quantified by ^1H NMR (Varian Mercury400 spectrometer, USA). Compounds dissolved in D_2O were analyzed by integrating the characteristic proton of dendrimer (3.3–2.2 ppm), PEG (3.7 ppm), RGD (7.2–6.7 ppm), SPDP(8.4-7.2 ppm) and DOTA (3.3-2.2 ppm) in the ^1H NMR spectrum.

2. The labeling degree of fluorophores. The absorbance of rhodamine ($\epsilon_{552} = 60,000 \text{ M}^{-1}\text{cm}^{-1}$) and Cy5.5 ($\epsilon_{675} = 250,000 \text{ M}^{-1}\text{cm}^{-1}$) in nanoprobe was measured on UV-2401PC UV-vis Recording spectrophotometer (SHIMADZU, Japan). The fluorophore labeling degree in nanoprobe were calculated according to Lambert-Beer's law: $A = \epsilon bc$ (A : absorbance, ϵ = extinction coefficient, b : thickness of the absorber, c : concentration).

3. The Purity of Nanoprobe. The nanoprobe purify was determined by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Nanoprobe (50 μg) in 30 μL treatment buffer [100 mM Tris, pH 6.8, 2% (w/v) SDS, 12% (v/v) glycerol, 0.01% (w/v) bromphenol blue] was loaded on a 7% polyacrylamide gel. After electrophoresis under a voltage of 100 V for 2 h, white light and fluorescent images of the resolved SDS-PAGE gels were acquired under Kodak In Vivo Multispectral Imaging System (FOV = 12.8 cm, $f/\text{stop} = 4$, bin = high resolution, exposure time = 2 s).

4. Molecular Weight Determination. The MALDI-TOF MS were obtained using an AB SCIEX TOF/TOFTM 5800 mass spectrometer (Applied Biosystems, USA) that was calibrated by BSA (Sigma A-0281) and HSA (Sigma A-3782). The specimens were prepared by

mixing 1.0 μL nanoprobe solution (10 μM in methanol) with 1 μL matrix (10 mg/mL CHCA in a solution of 50% CH_3CN , 50% H_2O and 0.1% TFA) on the measuring plate.

5. Hydrodynamic Size Distribution and Zeta Potentials. Hydrodynamic radius of nanoprobe and unmodified G5 dendrimer was determined by a Malvern Zetasizer (Malvern Instruments Inc., USA) dynamic light scattering instrument (DLS). 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 $\mu\text{g/mL}$ by PBS pH 7.4. The hydrodynamic radius and the size distribution patterns 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 nanoprobe was satirized by filtrating through a 0.45 μm filter and diluted to 200 $\mu\text{g/mL}$ in 10 mM NaCl solution.

6. Gd^{3+} Concentration. Gd^{3+} concentration of nanoprobe was determined by a Hitachi P-4010 (Tokyo, Japan) ICP-AES (Inductively Coupled Plasma Atomic Emission Spectroscopy) system 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.

7. The Relaxivity of Nanoprobes. The longitudinal relaxivities of the nanoprobes and the commercial available MR contrast agent Gd^{3+} -DTPA were determined on a BrukerBiospec 47/30 MRI scanner according to the equation of $r_{1p} = (1/T_{\text{sample}} - 1/T_{\text{PBS}})/[\text{Gd}]$. The T_1 values of selected compounds with four different concentrations in PBS 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 relaxivities.

Cell Culture Studies:

1. Cell Culture. Human glioblastoma U87MG cells, human prostate PC3 cells, human breast MDA-MB-231 cells and Bend 3 mouse brain capillary endothelial cells (BCECs) were purchased from the American Type Culture Collection (Rockville, USA). U87MG cells were cultured in Minimum Essential Medium, Alpha 1X (MEM, Mediatech, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% penicillin and streptomycin in a fully-humidified incubator containing 5% CO_2 at 37 °C. PC3 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 1% penicillin and streptomycin. MDA-MB-231 cells were maintained in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin and streptomycin. The Bend 3 mouse BCECs were maintained in DMEM supplemented with 10% FBS.

2. Cytotoxicity. The MTT cell proliferation assay was applied to determine the viabilities of the cells treated with nanoprobe and the unmodified G5 dendrimer. A U87MG 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 with cell density of 2×10^3 cells/well in 100 μ L 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 0.45 μ m syringe filter with final concentrations in a range of 0.05–10 μ M. 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.

Supplemental Figures

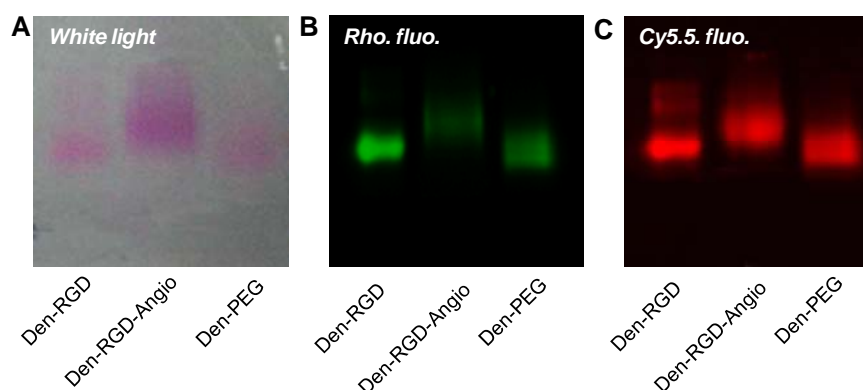


Figure S1. Representative white light and fluorescence images of a resolved SDS-PAGE gel loaded with nanoprobe (40 $\mu\text{g}/\text{well}$). The white light (A), rhodamine (B) and Cy5.5 (C) fluorescence images demonstrate the similar migration pattern of the nanoprobe.

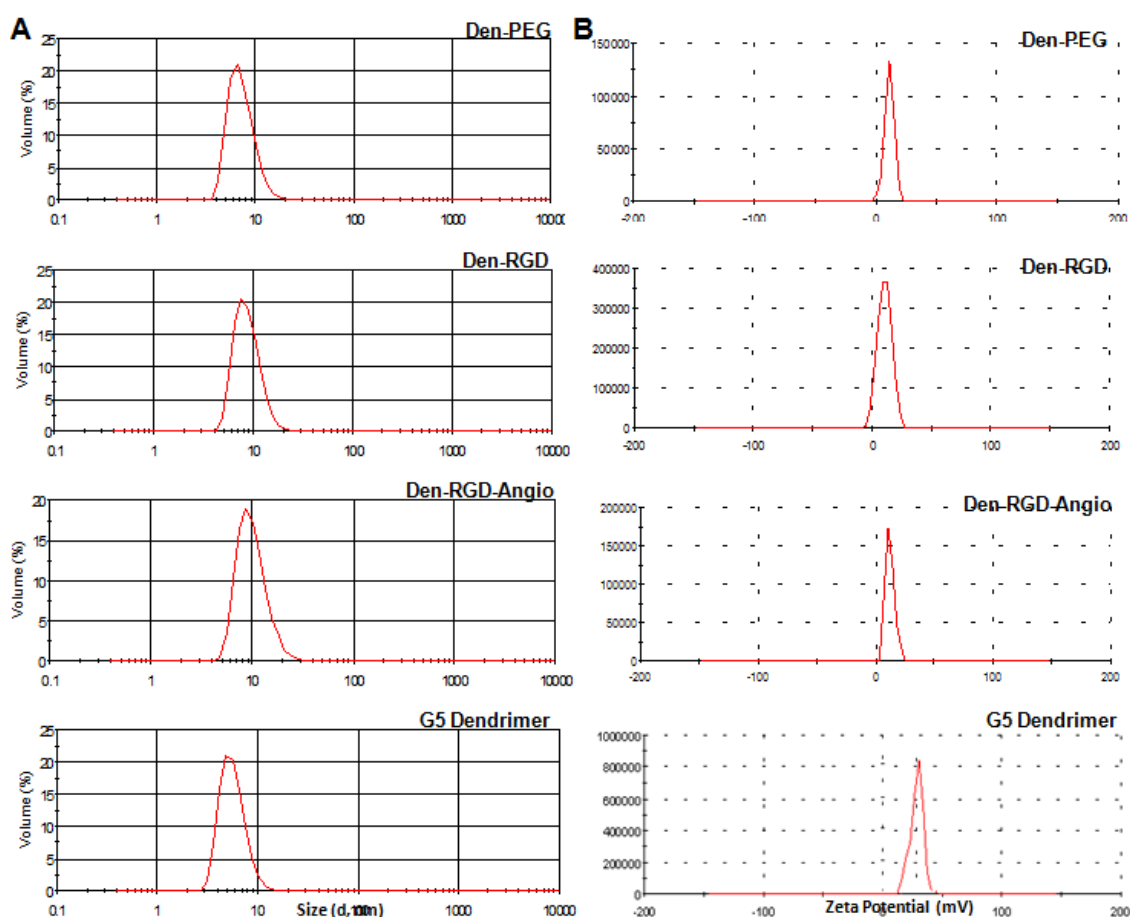


Figure S2. Hydrodynamic size distribution (A) and Zeta potentials (B) of the nanoprobe measured by dynamic light scattering (DLS). The average diameters of unconjugated G5 dendrimer, **Den-PEG**, **Den-RGD** and **Den-RGD-Angio** in PBS were presented as the volume distribution patterns. Their average Zeta potentials were measured in 0.1 M NaCl solution. The corresponding DLS data of **Den-Angio** can be found in the SI of our previous work (*Chem. Commun.*, **2011**, 47, 8130).

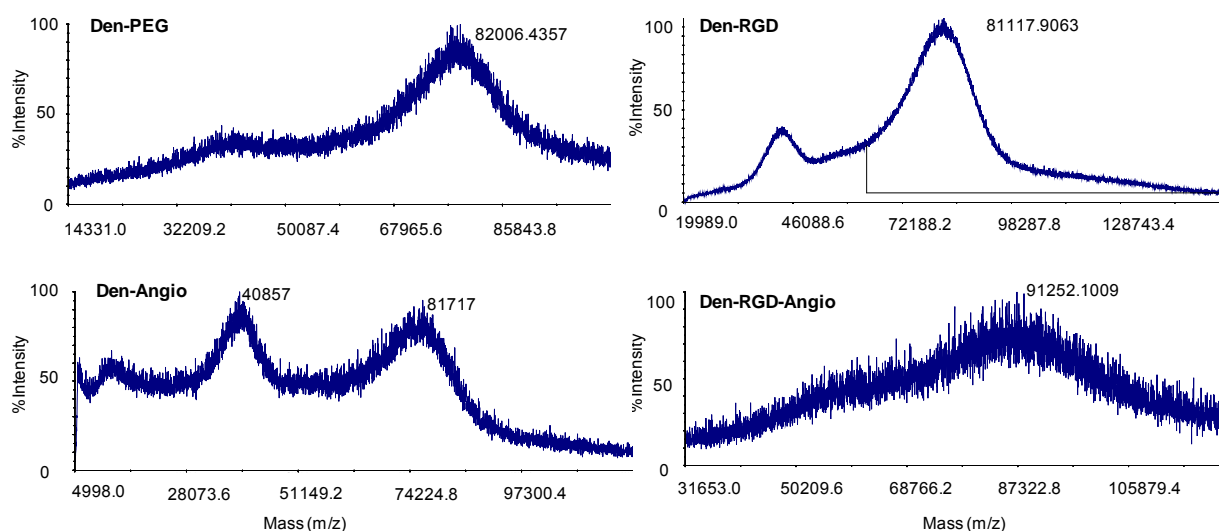


Figure S3. The mass spectra of the nanoprobes measured by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF). The molecular ion peaks of the nanoprobes **Den-PEG**, **Den-RGD**, **Den-Angio** and **Den-RGD-Angio** were measured as 82, 81, 83 and 91 kDa respectively.

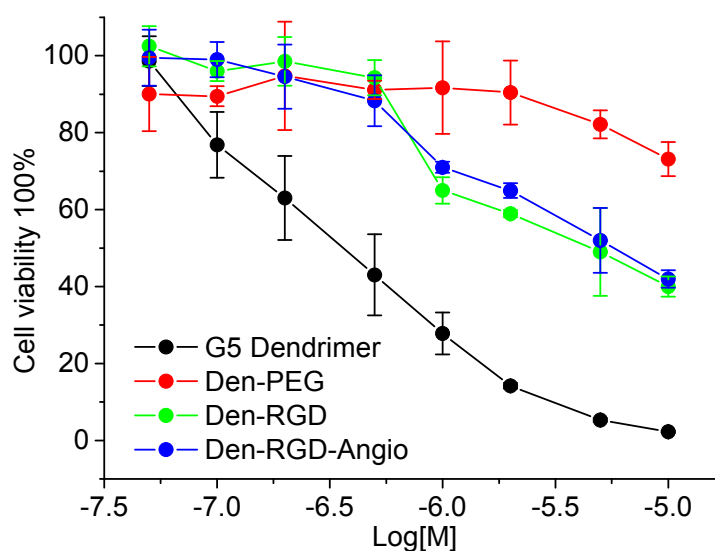


Figure S4. Concentration dependent cell viabilities of U87MG cells at 4 days after the treatment of nanoprobes or unconjugated G5 dendrimer (Points, mean; bars, SD; n = 8). The viabilities were normalized to that of the cells without any treatment. The nanoprobes concentration indicates the molar concentration of dendrimer moiety.

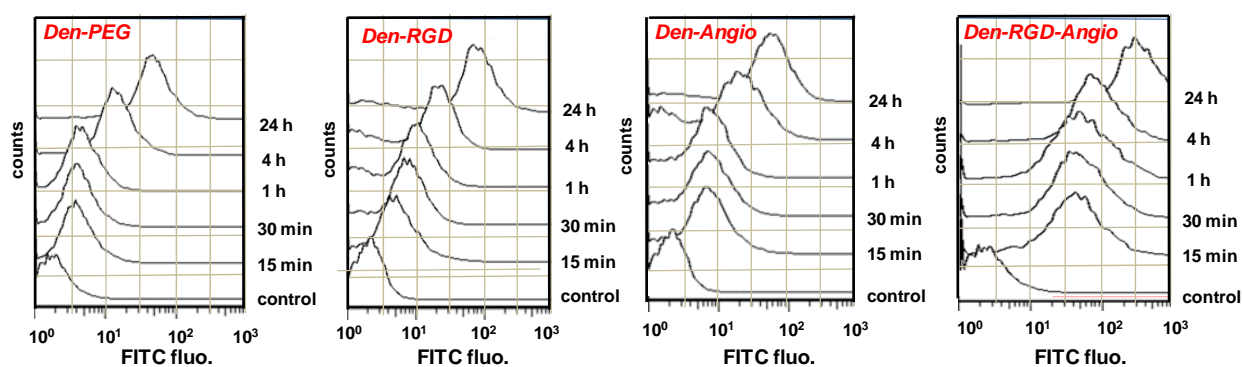


Figure S5. Time-dependent cellular uptakes of the nanoprobes quantified by flow cytometry. The cellular fluorescence of the cells were measured after the treatment of 1.0 μ M nanoprobe at the indicated time points.

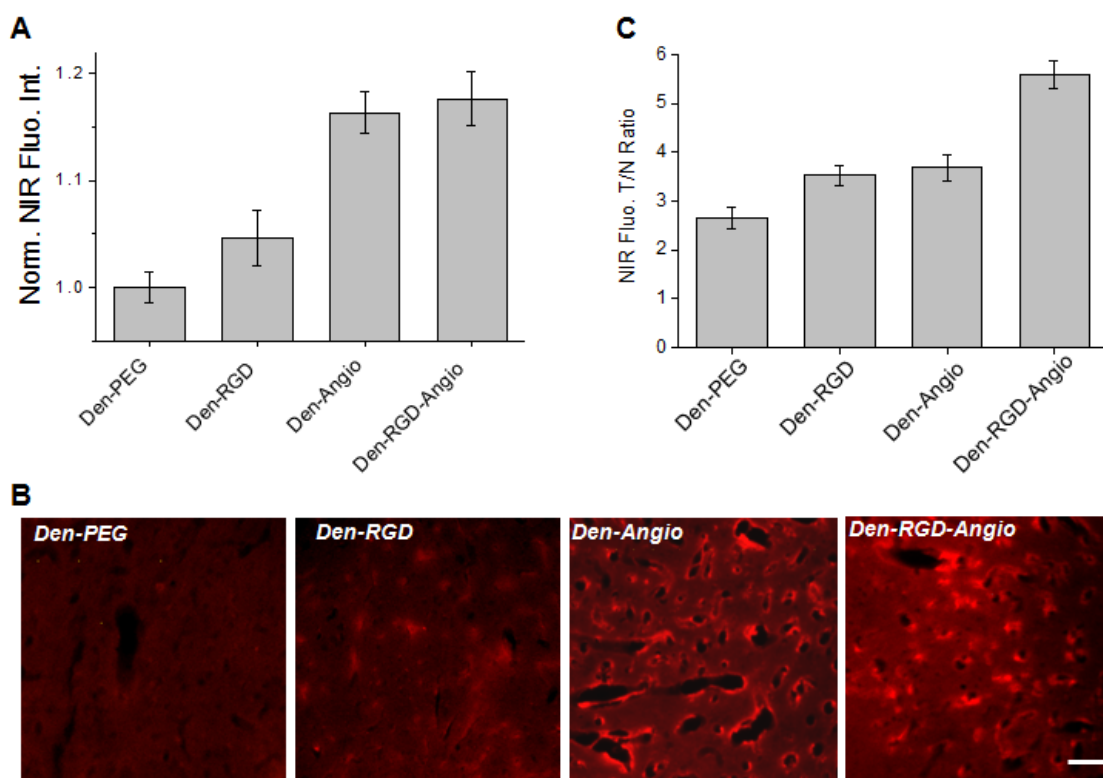
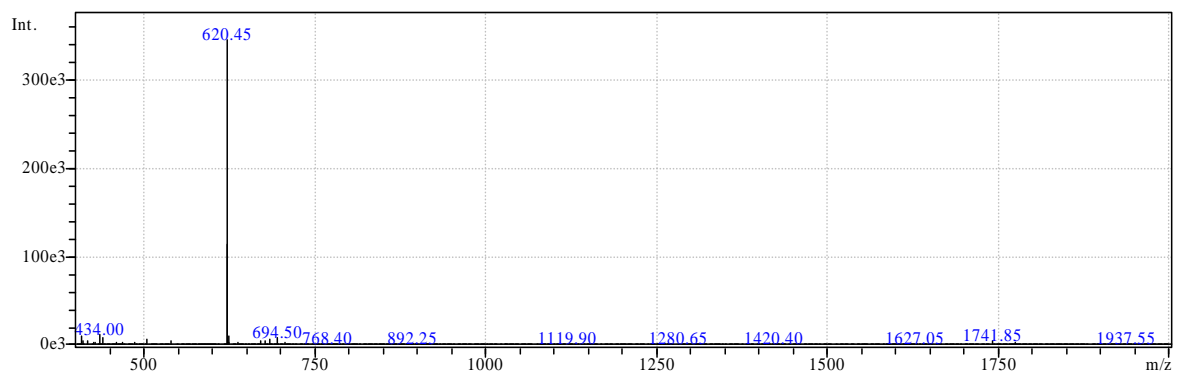
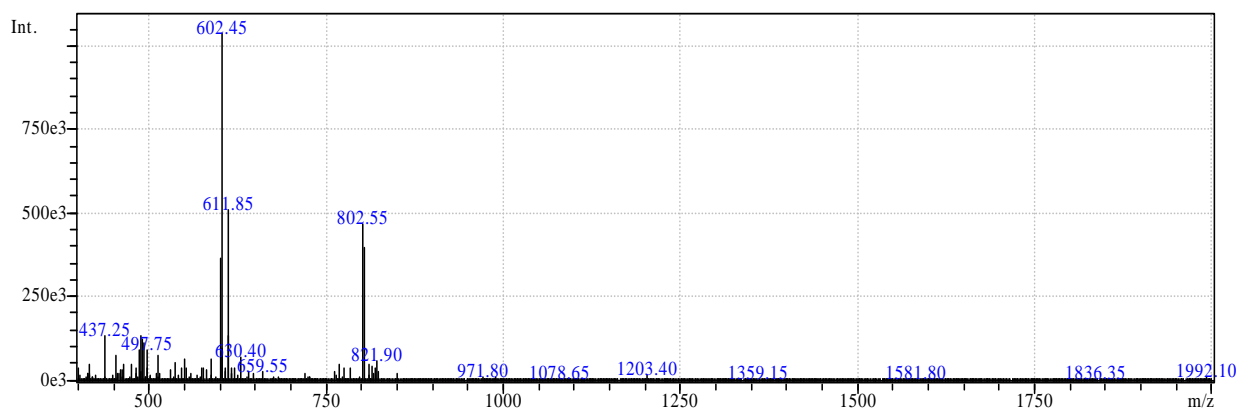


Figure S6. (A) Normalized NIR fluorescence intensities on the excised normal mice brain at 24 h PI of nanoprobe (5.0 nmol/mouse) *via i.v.* Columns present mean values and bars present the data range ($n = 3$). (B) Representative rhodamine fluorescence microscopic images of the normal mouse cortex sections at 24 h PI of the indicated nanoprobes. The scale bar presents 50 μ m. (C) *Ex vivo* NIR fluorescence associated T/N ratio of the nanoprobe at 24 h PI (5.0 nmol/mouse) *via i.v.* Columns present mean values and bars present the data range.

Supplemental Spectra

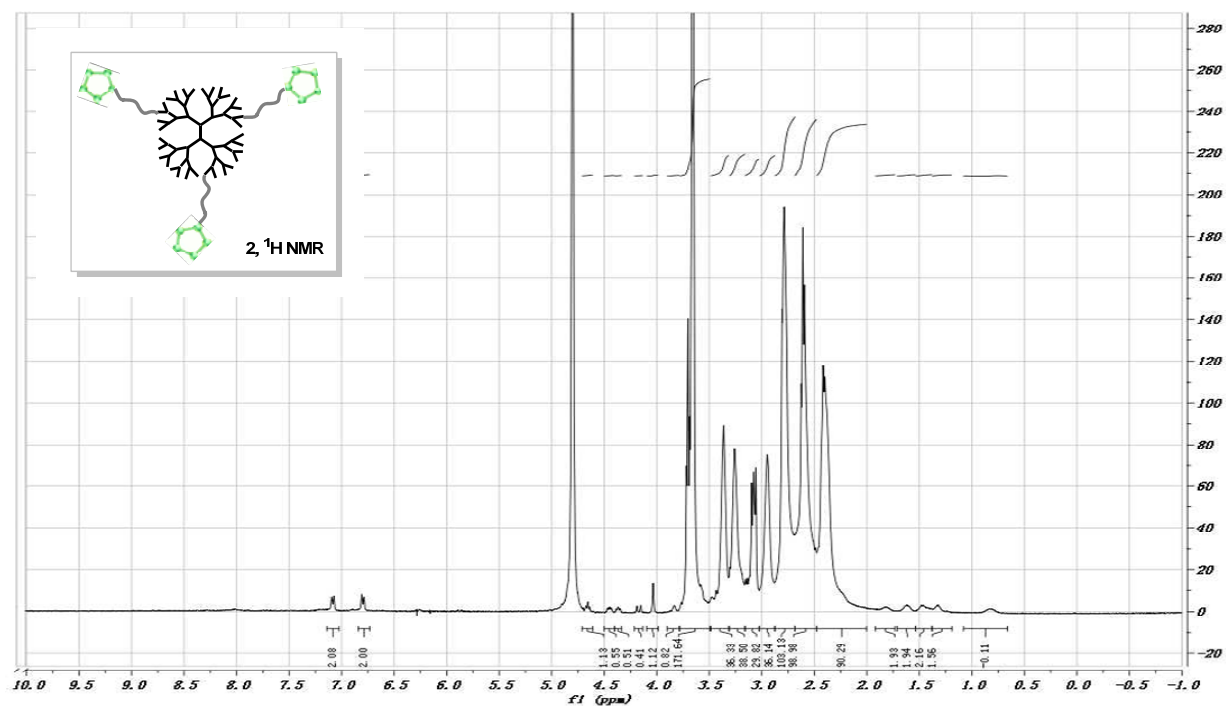


Spectrum 1. Mass spectrum of c[RGDyK] (MW: 619.48 Da). Found ES-MS 620.45 $[M+H]^+$.

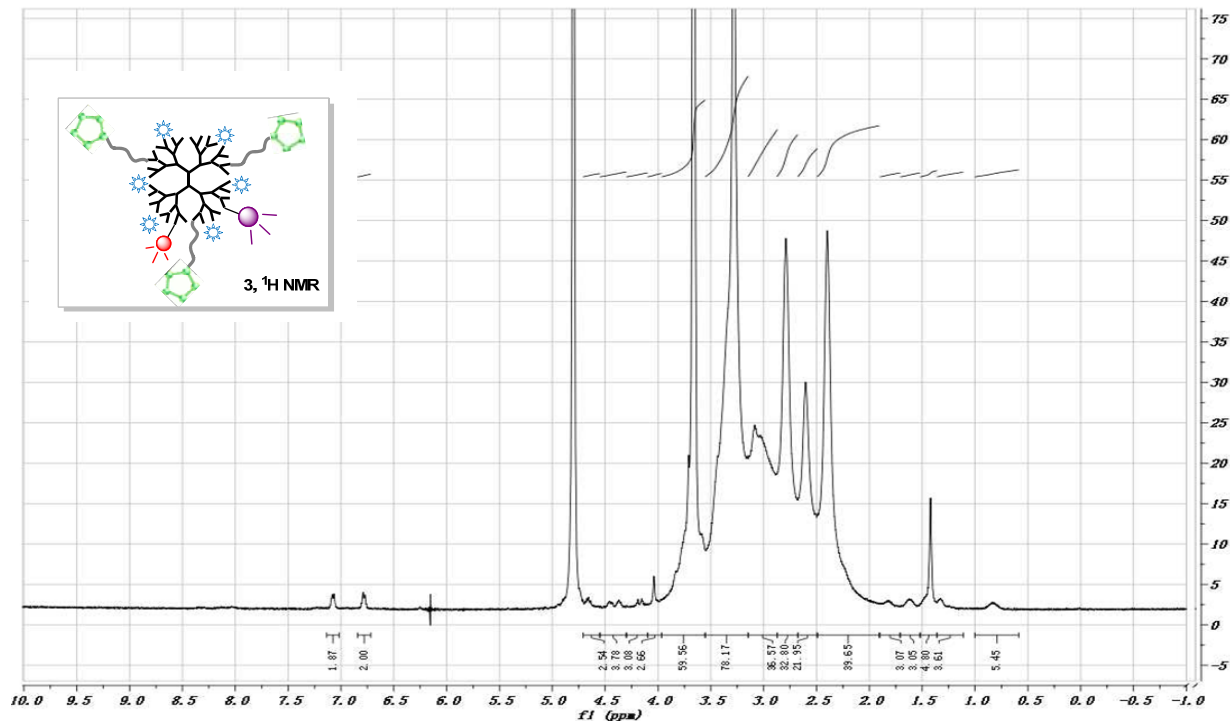


Spectrum 2. Mass spectrum of Angiopep2 peptide (TFFYGGSRGKRNNFKTEEYC, MW: 2404.6 Da).

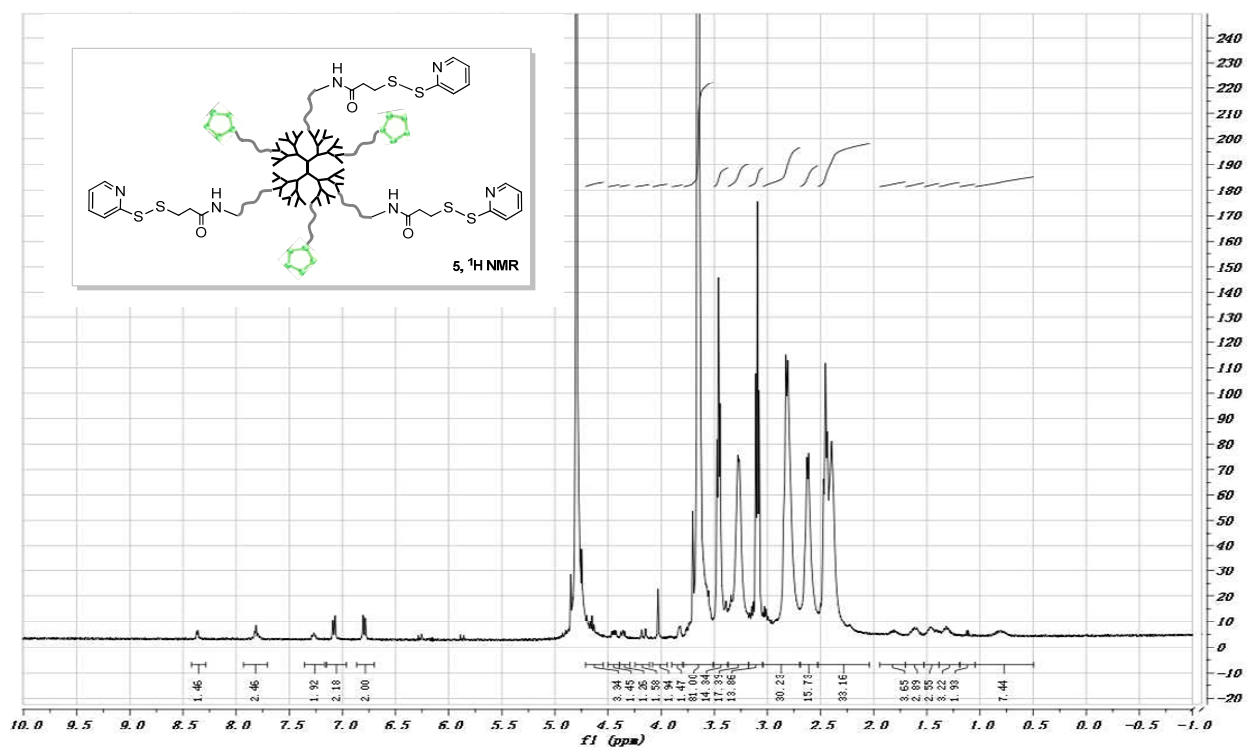
Found ES-MS 602.45 $[M+4H]^{4+}$; 611.85 $[M+4H+K]^{4+}$; 802.55 $[M+3H]^{3+}$.



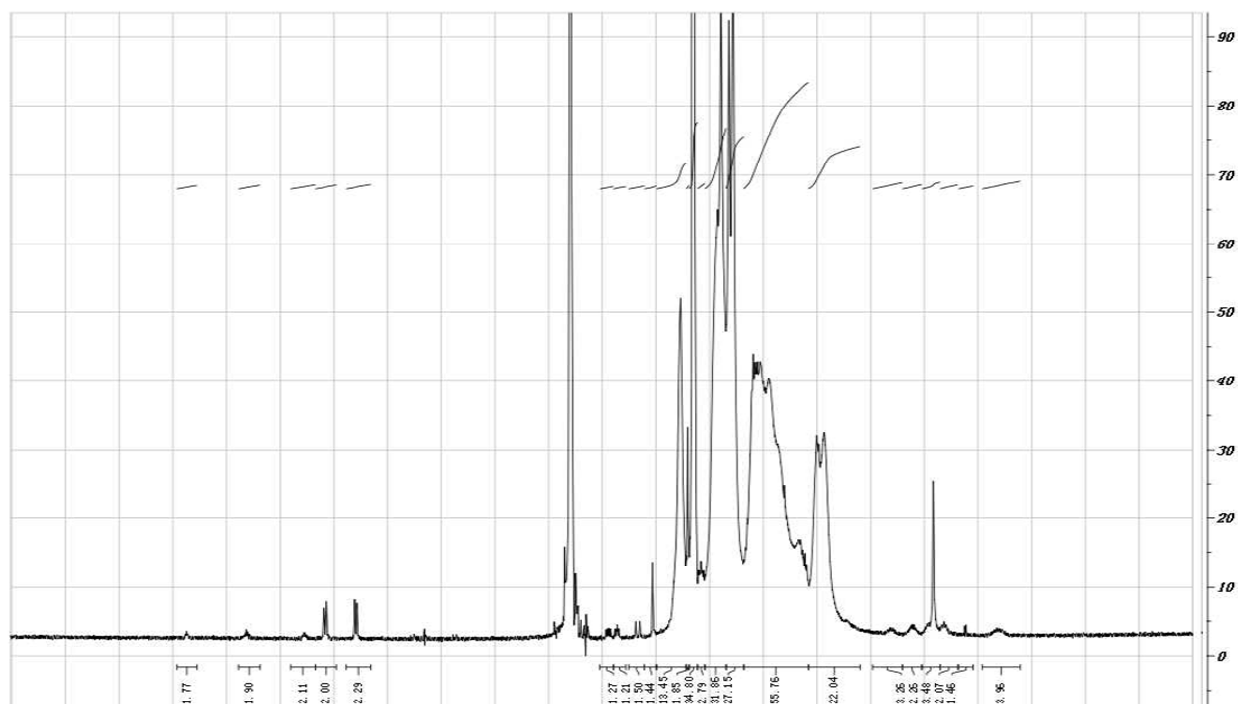
Spectrum 3. ¹H NMR spectrum of compound 2. ¹H NMR (400MHz, D₂O): δ 7.2-6.6 ppm (d, c[RGDyK]), 3.7 ppm (m, PEG), 3.6-3.4 ppm (m, dendrimer), 3.4-3.15 ppm (m, dendrimer), 3.15-3.0 ppm (m, dendrimer), 3.0-2.85 ppm (m, dendrimer), 2.85-2.65 ppm (m, dendrimer), 2.65-2.5 ppm (m, dendrimer), 2.5-2.2 ppm (m, dendrimer).



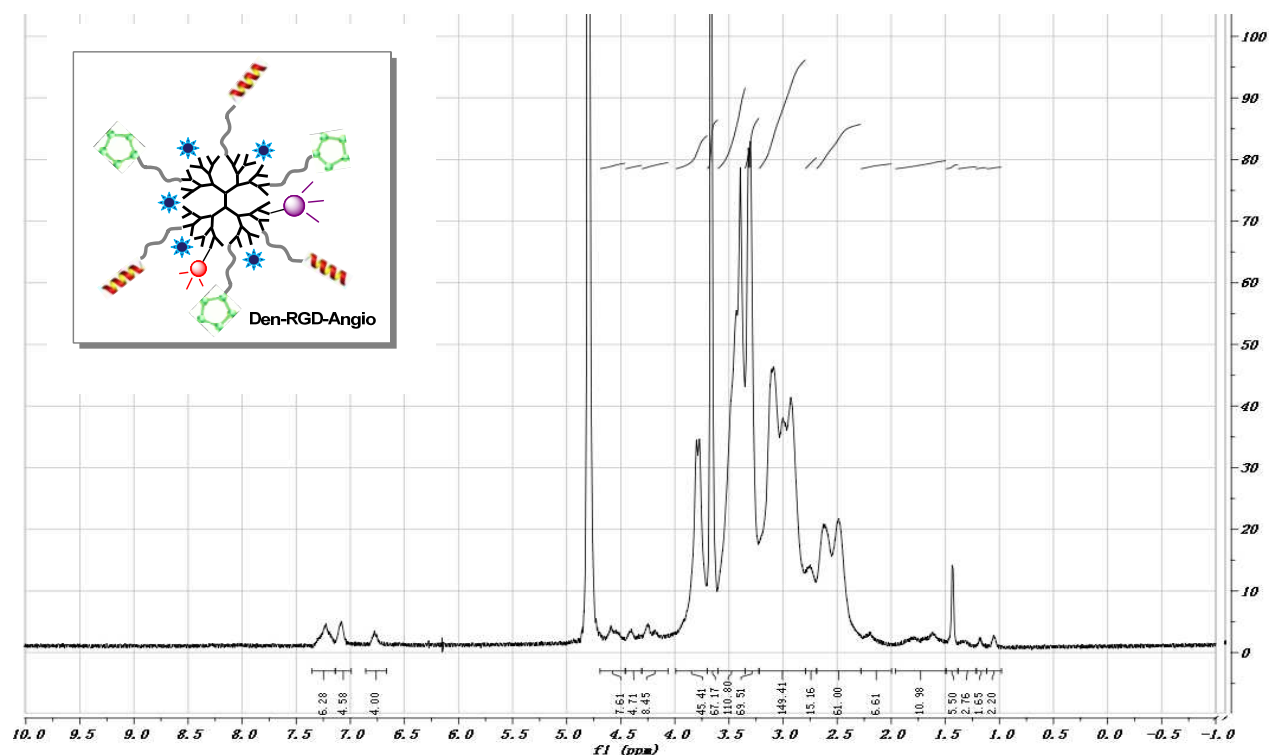
Spectrum 4 ¹H NMR spectrum of compound 3. ¹H NMR (400MHz, D₂O): δ 7.2-6.6 ppm (d, c[RGDyK]), 3.7 ppm (m, PEG), 3.5-2.2 ppm (m, dendrimer and DOTA).



Spectrum 5. ^1H NMR spectrum of compound **5**. ^1H NMR (400MHz, D_2O): δ 8.4-7.2 ppm (m, SPDP), 7.2-6.6 ppm (d, c[RGDyK]), 3.7 ppm (m, PEG), 3.6-3.4 ppm (m, dendrimer), 3.4-3.15 ppm (m, dendrimer), 3.15-3.0 ppm (m, dendrimer), 3.0-2.7 ppm (m, dendrimer), 2.7-2.5 ppm (m, dendrimer), 2.5-2.2 ppm (m, dendrimer).



Spectrum 6. ^1H NMR spectrum of **6**. ^1H NMR (400MHz, D_2O): δ 8.4-7.2 ppm (m, SPDP), 7.2-6.6 ppm (d, c[RGDyK]), 3.7 ppm (m, PEG), 3.5-2.2 ppm (m, dendrimer and DOTA).



Spectrum 7. ^1H NMR spectrum of **Den-RGD-Angio**. ^1H NMR (400MHz, D_2O): δ 7.4-7.2 ppm (m, Angiopep-2), 7.2-6.6 ppm (m, c[RGDyK] and Angiopep-2), 3.7 ppm (m, PEG), 3.5–2.2 ppm (m, dendrimer and DOTA).

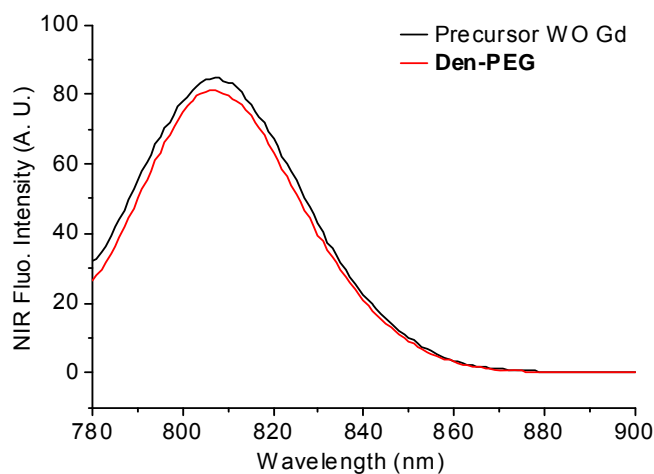


Fig. S7. The effect of labeled paramagnetic Gd^{3+} -DOTA chelators to the NIR fluorescence of nanoprobe. The emission spectra of **Den-PEG** and its precursor without Gd^{3+} -DOTA chelators after excited at their maximal absorbance that was adjusted at the same level. The integrated NIR fluorescence intensity of **Den-PEG** decreased about 6.5% compared to its metal-free precursor, which implies paramagnetic Gd^{3+} ions may slightly quench the NIR fluorescence in aqueous solution.