Supporting Information

A High-performance Imaging Probe with NIR Luminescence and Synergistically Enhanced T_1–T_2 Relaxivity for in Vivo Hepatic Tumor Targeting, Multimodal Imaging

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1. Chemicals.

$HAuCl_4 \cdot 3H_2O$, oleylamine, tert-butylamine-borane (TBAB), 1,2,3,4-tetrahydronaphthalene (tetralin), 1-octadecene, oleic acid, $Fe(CO)_5$, and polyethylene glycol (MW = 4000) were purchased from Sigma-Aldrich. Dicyclohexylcarbodiimide (DCC), N-Hydroxysuccinimide (NHS), Folic acid, 3,4-dihydroxybenzaldehyde (DIB) and $GdCl_3 \cdot 6H_2O$ were from Aladdin in China. Cy5 NHS-ester was purchased from Beijin Fanbo Biochemicals. All chemicals were of analytical grade and used as without further purification, except the solvents of DMF, DMSO, $CHCl_3$ and triethylamine were used as anhydrous. Aqueous solutions were prepared with double-distilled water (ddH_2O) from a Millipore system (>18 MΩcm). All the dialysis bags (MWCO 8000-14000) were obtained from Shanghai Med. 1,ω-Diaminopolyoxyethylene (MW=4000)[1], PEG-3,4-dihydroxy benzyl amine (DIB-PEG-NH_2)[1], PEG-thiol amine 1900 (SH-PEG-NH_2)[1], diethylenetriamine pentaacetic anhydride (DTPAA)[2], and *t*-butyl N-(2-amino-ethyl) carbamate (EDA-Boc)[3] were synthesized according to the published method.

2. Characterization.

The nanoparticle size and morphology were characterized by Tecna i-G2-F30 transmission electron microscope (TEM) at an acceleration voltage of 300 kV. The magnetic hysteresis loops were measured using a Bruker DRX-400MHz high sensitivity vibrating sample magnetometer (VSM) with fields up to 1.5 tesla at room temperature. The hydrodynamic diameter of the magnetite nanoparticles was analyzed by using dynamic light scattering (DLS) at a scatter angle of 90° on a Malvern Nano-ZS90. The fluorescence spectra were recorded on a Hitachi F-7000 spectrofluorophotometer. UV-vis absorbance measurements experiments were carried out on a Hitachi U-3900. Infrared spectra (4000-400 cm^{-1}) were determined with KBr disks on a NICO-LET 170SX FTIR spectrometer. The metal concentration of the nanoparticles was quantified using inductively coupled plasma absorption emission spectroscopy (ICP-AES). ^{1}H-NMR spectra were acquired with JEOL 400 MHz NMR. Fluorescent images were taken on Zeiss Leica inverted epifluorescence/reflectance laser scanning confocal microscope. The relaxivity was determined using MicroMR20-25 at 0.5 ± 0.08 T. MRI was performed on 3.0-T MR imaging system (Signa HDxt GE Healthcare)

3. Experimental section

3.1. Synthesis of Au-Fe_3O_4 nanoparticles.

Uniform 5 nm-14 nm sized Au-Fe_3O_4 nanoparticles, stabilized with oleic acid and oleylamine, were synthesized according to the reported method.[4] Briefly, 5 nm Au NPs were prepared by reduction of $HAuCl_4 \cdot 3H_2O$ according to literature. Briefly, $HAuCl_4 \cdot 4H_2O$ (0.1 g, 0.24 mmol) was added to tetralin (10 mL), followed by oleylamine (10 mL, 30 mmol) to form a yellow solution. The solution was then stirred for 10 min at room temperature. TBAB (0.086 g, 1 mmol), tetralin (1 mL), and oleylamine (1 mL, 3 mmol) were mixed as clear mixture and quickly injected into the above solution. The reaction mixture was further stirred at room temperature for 1 h. Ethanol was added to the solution, and gold particles were collected by centrifugation, washed by ethanol, and then redispersed in hexane and stored at -5 ^{0}C condition.

5 nm Au NP seeds (20 mg) in oleylamine (2 mL, 6 mmol) were added to a solution of 1-octadecene (20 mL) with oleic acid (1 mL, 3 mmol). The mixture was stirred under a gental flow of nitrogen at 120 ^{0}C for 1 h. Then under a blanket of nitrogen, $Fe(CO)_5$ (0.10 mL, 0.76 mmol) was rapidly injected into the solution. The solution was heated to reflux (300 ^{0}C) and left at that temperature for 25 min. After cooled down to room temperature, the particles were separated by adding isopropanol, centrifuged and redispersed into hexane and stored at -5 ^{0}C condition.

3.2. Synthesis of HS-PEG-NH_2.

A solution of thioglycollic acid (0.092 g, 1 mmol) in dry DMF (20 mL) was added DCC (0.40 g, 1.5 mmol) and NHS (0.14 g, 1.2 mmol). After stirring for 14 hours at room temperature in the dark, the white precipitate was removed by centrifugation. The filtrate was mixed with 1,ω-Diaminopolyoxyethylene 4000 (4.0 g, 1.0 mmol) and allowed to react at 25 ^{0}C for 14 hours. The solvent was evaporated to half under reduced pressure and precipitated out with diethyl ether (150 mL), and dried in vacuo. The creamy white powder product was then stored at -20 ^{0}C. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ2.97 (t, 2H, NH_2CH_2), 3.63 (bs, ~373H, PEG_{4000}), 3.68 (t, 2H, $NH_2CH_2CH_2$), 6.45 (d, 1H, Ph), 6.44 (d, 1H, Ph), 6.36 (s, 1H, Ph).

3.3. Synthesis of Au-Fe_3O_4-DIB-PEG-NH_2.

DIB-PEG-NH_2 (200 mg, 0.05 mmol) was dissolved in anhydrous $CHCl_3$ (10 mL), and then Au-Fe_3O_4 (30 mg) dispersed in anhydrous $CHCl_3$ (10 mL) was added. The mixture was stirred overnight at room temperature. The modified Au-Fe_3O_4 nanoparticles were precipitated by adding hexane, and collected by centrifugation at 6000 rpm, and then washed with $CHCl_3$ and hexane (1/5, v/v) 3 times. Finally, the product was redispersed in 20 mL mixture of anhydrous $CHCl_3$ and DMSO (4/1, v/v).

3.4. Synthesis of FA-EDA.

N-(2-aminoethyl) folic acid (EDA-FA) was synthesized according to the reported method with little modification.[3] FA (0.441 g, 1 mmol) was dissolved in anhydrous DMSO (20 mL) into which DCC (0.32 g, 1.5 mmol) and NHS (0.14 g, 1.2 mmol) were added. After stirring for 14 hours at room temperature in the dark, the white precipitate was removed by centrifugation. The filtrate was mixed with EDA-Boc (0.64 g, 4.0 mmol), added three drops of pyridine, and allowed to react at 25 ^{0}C for another 14 hours. The mixture was poured slowly into stirred ice-cold diethyl ether (80 mL). The yellow product was precipitated by centrifugation and washed with DMSO and diethyl ether (1/5, v/v) 3 times, and then dried under vacuum to obtain FA-EDA-Boc. Yield: 86%. ^{1}H-NMR(400 MHz, DMSO-d6) δ H: 1.35 (s, 9H, C24, C25, C26), 1.85~2.39 (m, 4H, C17, C18), 2.95 (m, 2H, C21), 3.06 (m, 1H, C16), 3.36 (m, 2H, C22), 4.28 (m, 1H, N10), 4.48 (d, 2H, C9), 6.64 (d, J = 8 Hz, 2H, C11, C14), 6.80 (d, 1H, N15), 6.95 (t, 2H, N7), 7.66 (d, J = 8 Hz, 2H, C12, C13), 7.82~8.08(m, 2H, N20, N23), 8.61 (s, 1H, C1). ESI-MS (m/z): $[M + H]^+$ 584.3.

TFA (3 mL) was added to dissolve FA-EDA-Boc and stirred at room temperature for 4 h. After removing TFA under reduced pressure, pyridine was added until no more white smoke emerging. The tawny product FA-EDA was precipitated out with diethyl ether (50 mL) by centrifugation, and washed with DMSO and diethyl ether (1/5, v/v) 3 times, then dried in vacuum. Yield: 85%. ^{1}H-NMR(400 MHz, DMSO-d6) δ H: 1.35 (s, 9H, C24, C25, C26), 1.85~2.39 (m, 4H, C17, C18), 2.95 (m, 2H, C21), 3.06 (m, 1H, C16), 3.36 (m, 2H, C22), 4.28 (m, 1H, N10), 4.48 (d, 2H, C9), 6.64 (d, J = 8 Hz, 2H, C11, C14), 6.80 (d, 1H, N15), 6.95 (t, 2H, N7), 7.66 (d, J = 8 Hz, 2H, C12, C13), 7.82~8.08 (m, 2H, N20, N23), 8.61 (s, 1H, C1). ESI-MS (m/z): $[M + H]^+$ 484.3.

3.5. Synthesis of DTPA-EDA-FA.

DTPAA (0.022 g, 0.06 mmol) was dissolved in anhydrous DMSO (3 mL) and triethylamine (15 μL). FA-EDA (0.029 g, 0.06 mmol) was dissolved in anhydrous DMSO (1.5 mL), and then added dropwise into above solution within 10 min. After stirring for 12 hours at 30 ^{0}C in the dark, the product was precipitated by adding diethyl ether, and collected by centrifugation at 4000 rpm. After washing with DMSO and diethyl ethe (1/5, v/v) 3 times, the product was dried in vacuum for further characterization. (**Attention**: In order to prevent ring opening reaction of the residual acid anhydride, the product in anhydrous solvent was directly used for next step reaction without further purification.) ^{1}H-NMR(400 MHz, DMSO-d6) δ H: 1.35 (s, 9H, C24, C25, C26), 1.85~2.39 (m, 4H, C17, C18), 2.95 (m, 2H, C21), 3.06 (m, 1H, C16), 3.36 (m, 2H, C22), 4.28 (m, 1H, N10), 4.48 (d, 2H, C9), 6.64 (d, J = 8 Hz, 2H, C11, C14), 6.80 (d, 1H, N15), 6.95 (t, 2H, N7), 7.66 (d, J = 8 Hz, 2H, C12, C13), 7.82~8.08 (m, 2H, N20, N23), 8.61 (s, 1H, C1). ESI-MS (m/z): $[M + H]^+$ 859.4.

3.6. Synthesis of Au-Fe_3O_4-DIB-PEG-DTPA-FA.

The solution of FA-EDA was gradually added into the mixture solution of Au-Fe_3O_4-DIB-PEG-NH_2, then stirred at room temperature for 12 h. The product was precipitated by adding hexane, and collected by centrifugation at 4000 rpm. After washing with $CHCl_3$ and hexane (1/5, v/v) 3 times, the product was dried in vacuum.

3.7. Synthesis of Gd: FA-DTPA-PEG-Fe_3O_4-Au (1b).

Au-Fe_3O_4-DIB-PEG-DTPA-FA (110 mg) was dispersed in 20 mL $CHCl_3$, and then a solution of the $GdCl_3{\cdot}6H_2O$ (10 mg, 0.027 mmol) in 5 mL ethanol was added. The mixture was stirred at 25 ^{0}C for 24 hours. The product was precipitated by adding hexane, and collected by centrifugation at 4000 rpm. After washing with $CHCl_3$, ethanol and hexane (1/1/5, v/v/v) 3 times, the product was redispersed in DI water.

3.8. Synthesis of HS-PEH-NH-Cy5 (1c).

Cy5-NHS (4 mg, 5.5 μmol) was coupled to the N-terminus of HS-PEH-NH_2 (20 mg, 10.5 μmol) in anhydrous DMF (2 mL) at

room temperature in the dark. After stirring for 6 h, the product was precipitated in ethyl ether (20 mL), dried in vacuum.

3.9. Synthesis of Gd: FA-DTPA-PEG-Fe_3O_4-Au-HS-PEH-NH-Cy5 (1).

1c (40 mg) in $CHCl_3$ (5 mL) was added into **1b** (0.4 g) in $CHCl_3$ (10 mL). The mixture was shaked gently for 8 h in the dark at room temperature. The nanocomposites were then collected by centrifugation, washed with $CHCl_3$ and hexane (1:5 V/V), and then dispersed in water with short time sonication and dialysised with H_2O for 48 h to remove unreacted organic molecules.

4. Cell culture.

BEL7402 (human liver tumor cell line with high FR expression) was cultured in RPMI1640, supplemented with 10% FBS, 100 mg/mL penicillin G and 100 mg/ml streptomycin at 37 ^{0}C in a humidified and 5% CO_2 atmosphere. ECV304 were cultured in DMEM.

5. Cytotoxicity assay.

The cytotoxic consequence of **1** was evaluated by performing methyl thiazolyl tetrazolium (MTT) assay of BEL7402 and ECV304 incubated with the particles. Cells were plated at a density of 8×10^3 cells per well in 96-well plates and kept overnight for cell attachment. Then, the cells were incubated with different concentrations (0, 10, 20, 50, 100 μg/mL in DMEM) for 24 h，48 h and 72 h respectively at 37 ^{0}C and 5% CO_2. The control well was a culture medium with no nanoparticles. Thereafter, MTT (20 μL, 5 mg/mL) was added to each well and the plate was incubated for 4 h at 37 ^{0}C. After the addition of DMSO (200 μL/well), the cell plate was allowed to stand at 37 ^{0}C for 15 min. The optical density was measured at 492 nm using a microplate reader (Shanghai Sanco Instrument Co., Ltd. 318C- microplate reader).

6. Fluorescence imaging-nude mice.

The athymic nude mice used in this study were 4-6 weeks old and weighed at about 20-25 g. 5×10^6 BEL7402 cell in 0.2 ml PBS were inoculated subcutaneously in the right flank of the mice. After the mean diameters of tumor mass reached 2–5 mm in volume, the mice were used for in vivo imaging studies. The subjected mouse (200 mL **1** via tail vein) was firstly anesthetized and imaged at predetermined intervals (0, 0.5, 1, 2, 4, 8, 24 h) post-injection. To confirm the in vivo distribution of **1**, mice were sacrificed 10 h post-injection. The excised organs and tissues (heart, liver, spleen, lung, kidney and tumor) were also non-invasively imaged by the Living image system. (Caliper IVIS LuminaXR, Caliper Life Sciences, USA.

7. Relaxivity measurement.

The relaxivity of the **1** were determined using 0.55 T MR scanner (MicroMR20-25) in combination with 8-channel head coil at 32 ^{0}C. The particles were diluted in distilled water, with iron or gadolinium concentrations in the various range. Samples were transferred to a 96-well plate. Weighted images were acquired using a multi-slice spin echo sequence. The parameters were set as follows: T_2 test：P90 (us) = 4.80, P180 (us) = 9.40, SW (KHz) = 100, TE (ms) = 0.1696, TR (ms) = 5000, RG1 = 20, RG2 = 3, NS = 4, Echo Count = 6000； T_1 test：P90 (us) = 4.80, P180 (us) = 9.40, SW (KHz) = 100,TR (ms) = 15000, RG1 = 20, RG2 = 3, NS = 4, NTI = 20.

8. MRI in vivo.

For tumor induction, 5×10^6 BEL7402 cell in 0.2 mL PBS were inoculated subcutaneously in the right flank of the mice. When the tumor reached a volume of around 5 mm, the nude mice can be subjected to the MRI investigation. MRI was performed on 3.0-T MR imaging system (Signa HDxt GE Healthcare), with a research coil insert designed specifically for imaging rats. The parameters of axial T2-weighted images (T2WI) are following: repetition time (TR) 3000 ms, multiple echo times (TE) 50 ms, field of view (FOV) 60 mm, 6 excitations (NEX), base resolution, 192 × 160, slice thickness 1.5 mm, interval thickness 0.5 mm.

9. Prussian blue staining

Mice were sacrificed 24h postinjection. Tumor tissues were fixed in 10% formalin and cryoprotected with an 18% sucrose solution. Sections were incubated for 30 min in a solution of 2% potassium ferrocyanide and 2% hydrochloric acid at a 1:1 ratio. After Prussian blue staining, the sections were counterstained with a 1% nuclear fast red solution.

10. Calculation the number of gadolinium on the Fe_3O_4 nanoparticles.

The number of Fe ions per a nanoparticle was derived from an equation

$$N_{Fe} = 3N_{Fe3O4} = 3\left(4\frac{V_{NP}}{V_{unit}}\right) = \frac{2\pi d^3}{a^3}$$, where

$\mathbf{V_{unit}}$ is the volume of a unit cell of inverse spinel structure and $\mathbf{V_{NP}}$ is the volume of a spherical nanoparticle. The **a** is the cell parameters of Fe_3O_4, and **a** = 0.835 nm. [1b, 4a] The **d** is the diameter of Fe_3O_4 NPs, and **d** = 14 nm from TEM imaging. Each periodic lattice has 4 unit of Fe_3O_4, and every Fe_3O_4 has three Fe ions.

Corresponding

$$V_{NP} = \frac{4}{3}\pi\left(\frac{d}{2}\right)^2$$, and $V_{unit} = a^3$

The number of Gd on the Fe_3O_4 nanoparticles can be calculated by

$$N_{Gd} = N_{Fe}\frac{n_{Gd}}{n_{Fe}}$$

The molar concentrations of Gd and Fe were measured by ICP-AES, and the Gd/Fe mole percentage (21.7%). Take into the number of Fe atom per NPs, Gd per a Fe_3O_4 nanoparticle is about 6400.

The Characterization of 1

The FT-IR spectrum (Figure S1A) of oleic acid and oleyl amine- coated Au-Fe_3O_4 NPs showed strong absorption bands at 2846 and 2921 cm^{-1}, arising from symmetric and asymmetric C-H stretch in the oleyl chains, respectively.[5] After the attachment of **1a** on Fe_3O_4 side, the characteristic bands of oleic acid remarkably decreased, and a strong characteristic peak at 1600 cm^{-1} for C=O stretch vibrations was observed. Peaks at 624 and 594 cm^{-1} are typical Fe-O absorption bands.[5a, 6] (Figure S1D). The well attachment of **1a** was further supported by a characteristic absorption peak of FA at 282 nm and 356 nm in the UV-vis spectra (Figure S2A, S3).[6] Compared with **1b**, **1** shows obvious fluorescent emission peak at 660 nm (Figure S2B), which indicates that **1c** is well anchored on the Au side.

11. Reference.

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3 O. Aronov, T. Horowitz, A. Gabizon, D. Gibson, *Bioconjugate Chem.,* 2003, **14**, 563-574.

4 a) H. Yu, M. Chen, P. M. Rice, S. X. Wang, R. L. White, S. H. Sun, *Nano Lett.* **2005**, *5*, 379–382; b) C. Wang, C. J. Xu, H. Zeng, S. H. Sun, *Adv. Mater.* **2009**, *21*, 3045–3052.

5 a) G. Pyrgiotakis, C. O. Blattmann, P. Demokritou. *ACS Sustainable Chem. Eng*., 2014, 2, 1681−1690; b) G. R. Shao, G. H. Chen, W. L. Yang, T. Ding, J. Zuo, Q. Yang., *Langmuir* 2014, 30, 2863−2872.

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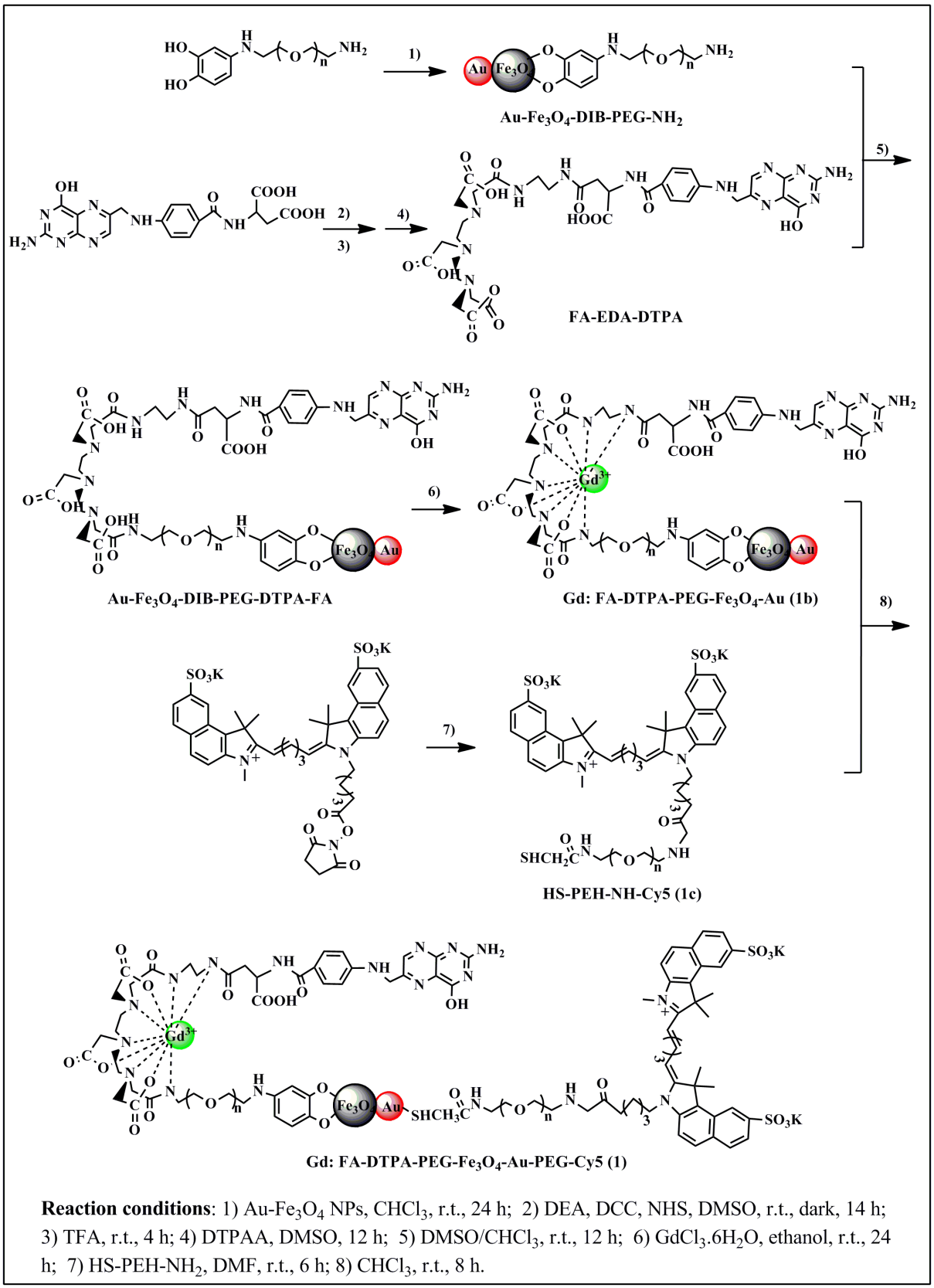
12. Synthetic routes.



Reaction conditions: 1) Au-Fe_3O_4 NPs, $CHCl_3$, r.t., 24 h; 2) DEA, DCC, NHS, DMSO, r.t., dark, 14 h; 3) TFA, r.t., 4 h; 4) DTPAA, DMSO, 12 h; 5) DMSO/$CHCl_3$, r.t., 12 h; 6) $GdCl_3.6H_2O$, ethanol, r.t., 24 h; 7) HS-PEH-NH_2, DMF, r.t., 6 h; 8) $CHCl_3$, r.t., 8 h.

Scheme S1. The Synthetic routes of Gd:FA-DTPA-PEG-DIB-Fe_3O_4-Au-PEG-Cy5 (**1**).

13. FT-IR, Uv-vis and fluorescence spectra.

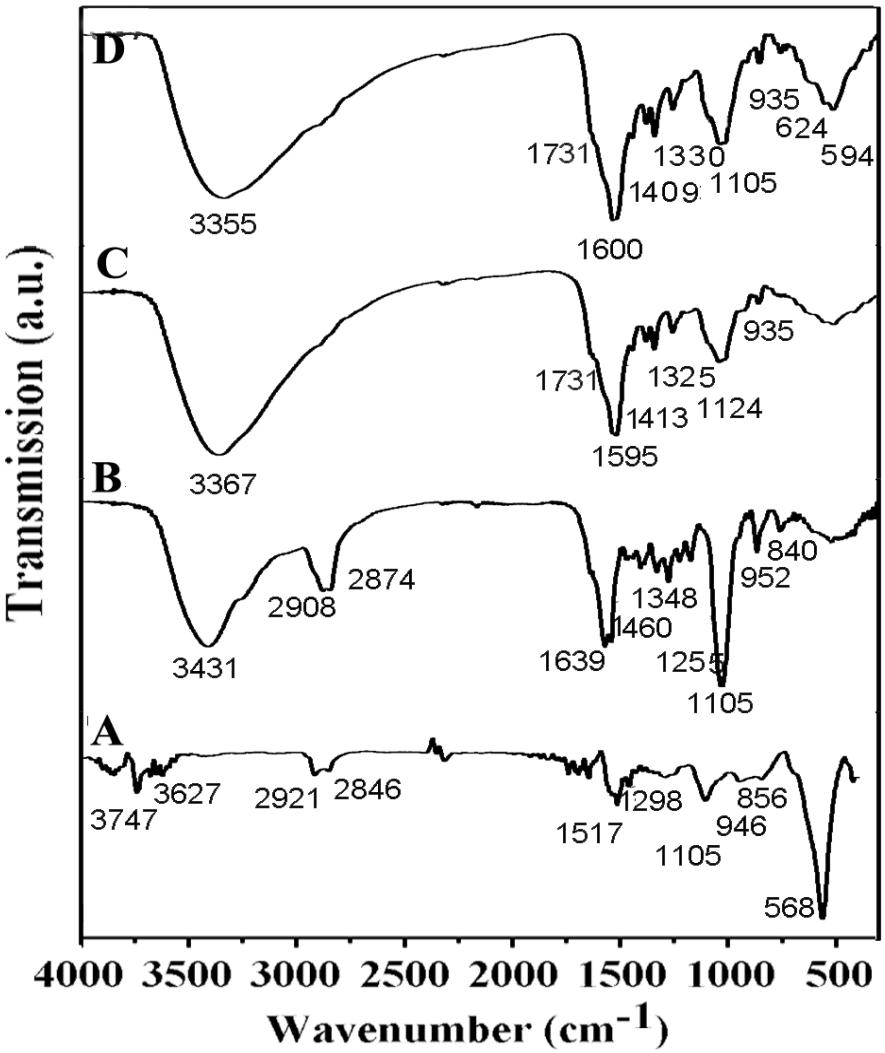


Figure S1. The IR spectra of (A) the as-synthesized Au-Fe_3O_4 nanoparticles ; (B) FA-DTPA-PEG-DIB; (C) Gd:FA-DTPA-PEG-DIB (**1a**); (D) Gd:FA-DTPA-PEG-DIB-Fe_3O_4-Au (**1b**).

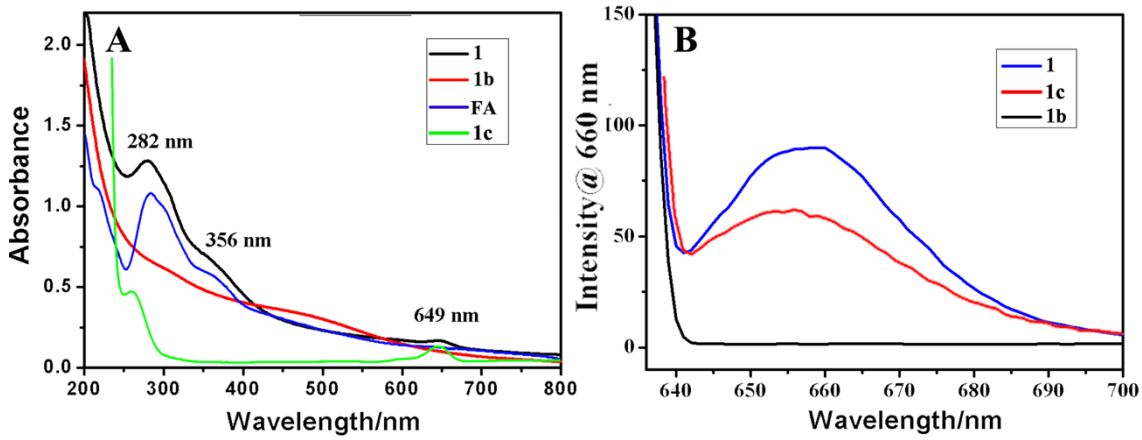


Figure S2. (A) UV-vis detection of **1**. The absorbance peaks at 282 nm and 356 nm belong to FA, and 649 nm to Cy5. (1b, FA and 1c as control) (B) Fluorescence spectra of **1**. Ex = 620 nm, Em = 660 nm, belonging to Cy5. (**1b** and **1c** as control)

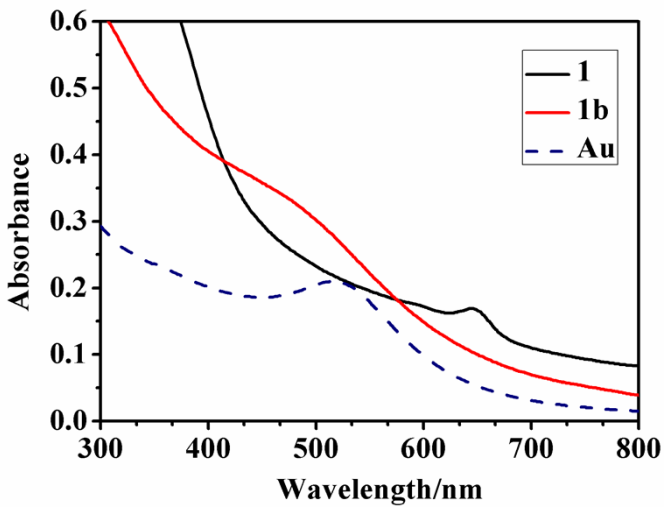


Figure S3. The UV-vis spectra with partial enlarged drawing of **1**, 1b, and Au nanoparticles.

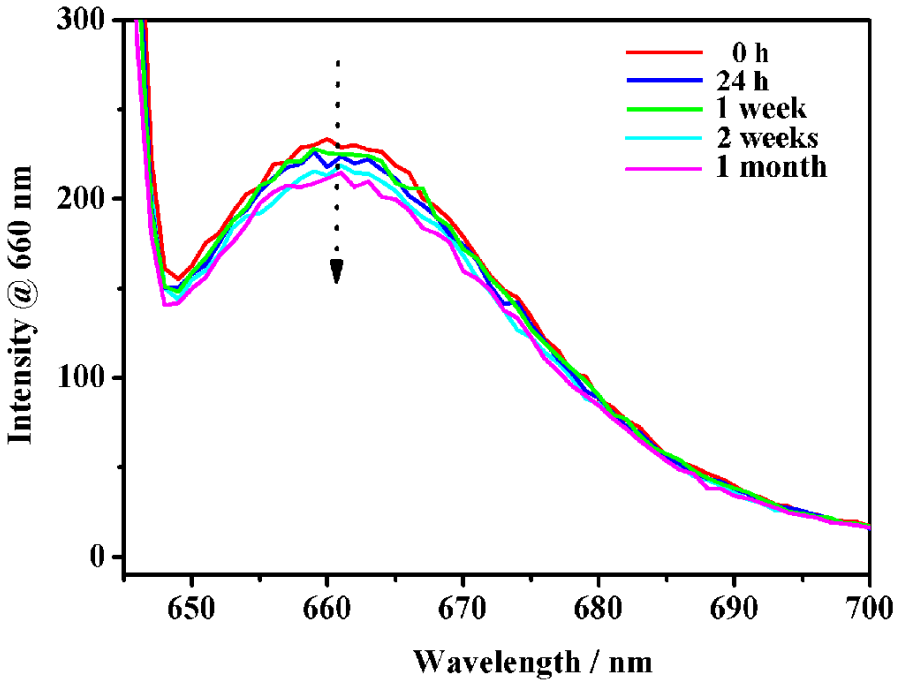


Figure S4. Fluorescence spectra of **1** at different time periods within one month. Ex = 620 nm, Em = 660 nm, belonging to Cy5.

14. Magnetic property measurements.

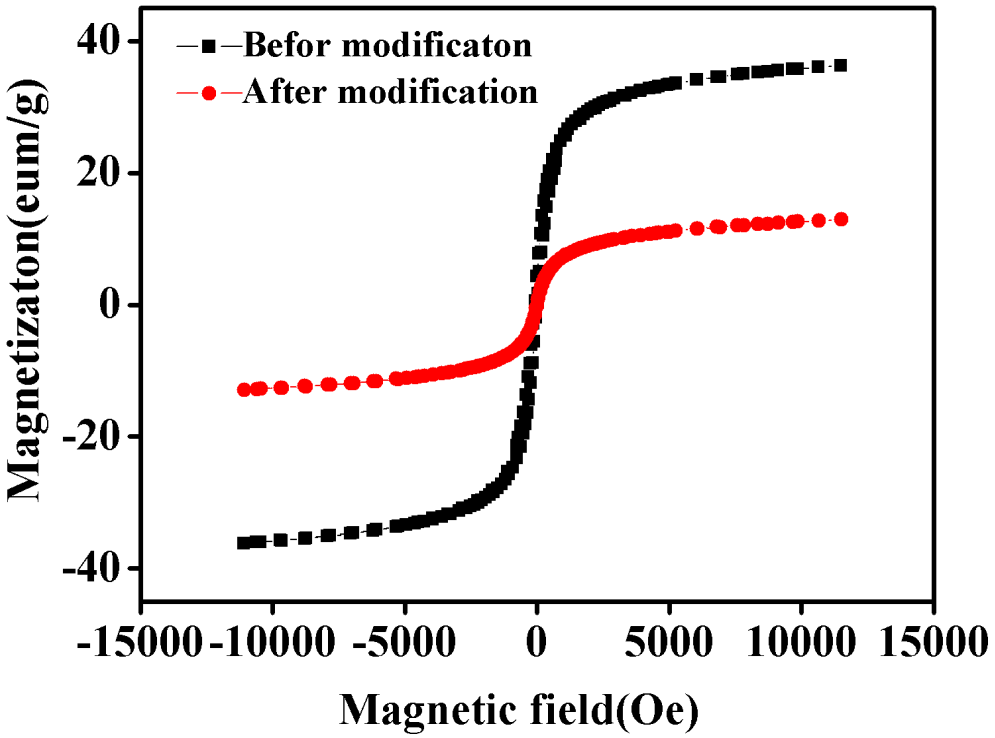


Figure S5. Magnetization measured at 300 K. The saturated magnetization (*Ms*) of Au–Fe_3O_4 NPs and **1** are 36.2 and 12.9 emu/g.

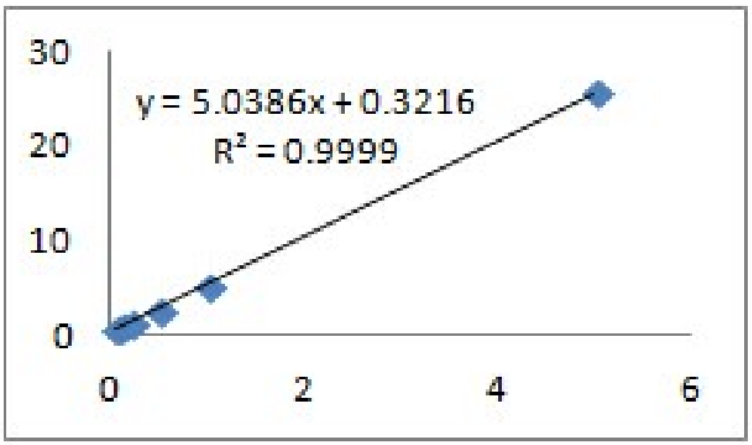


Figure S6. T_1 relaxation rates ($1/T_1$, s^{-1}) of Magnevist® as a function of Gd (mM) in water (0.55 T, 25 ^{0}C).

15. Cytotoxicity.

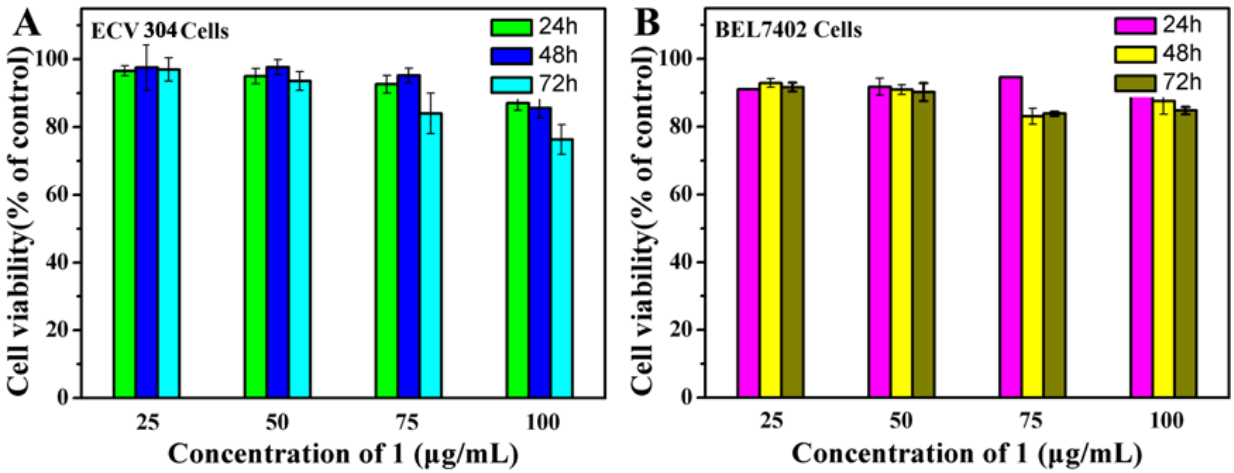


Figure S7. In *vitro* cell viability of (A) ECV304, (B) Bel7402 cells incubated with **1** with different concentrations (0, 25, 50, 75, 100 μg/mL) incubated for 24, 48 and 72 h at 37 ^{0}C.

16. ESI-MS and ^{1}H NMR spectra of important functional molecules.

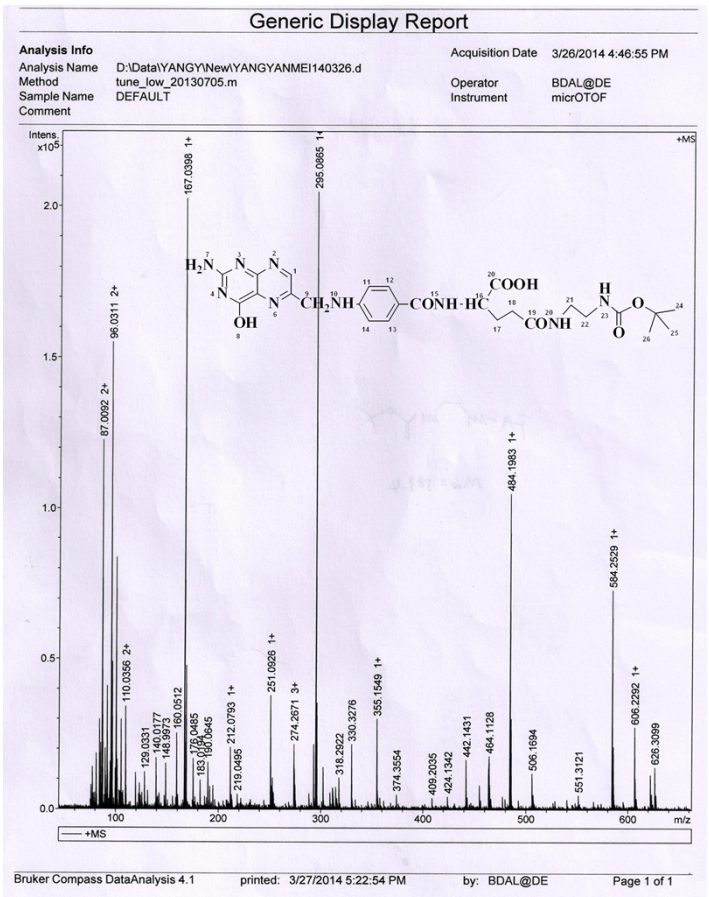


Figure S8. ESI-MS of **FA-EDA-Boc**. (m/z): $[M + H]^{+}$ 584.3.

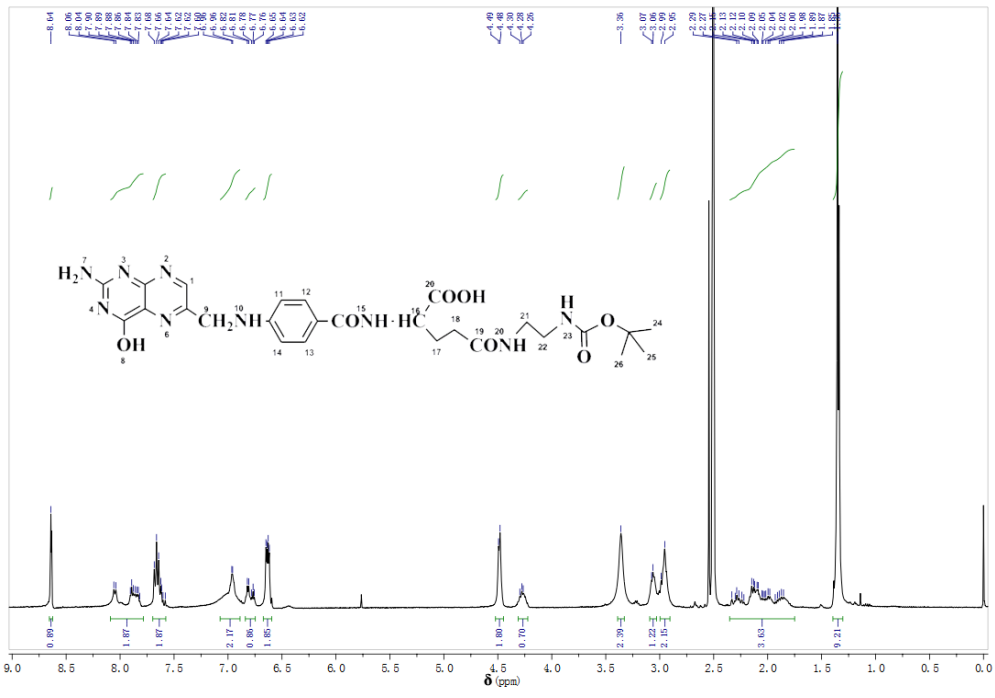


Figure S9. [1]H NMR Spectrum of **FA-EDA-Boc**. (400 MHz, DMSO-d6)

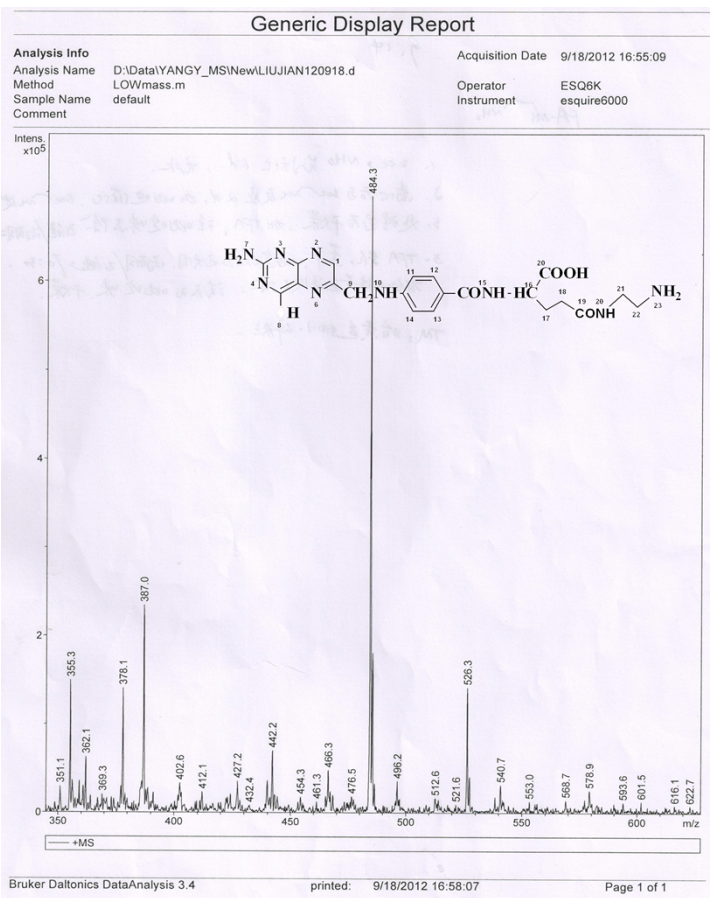


Figure S10. ESI-MS of **FA-EDA**. (m/z): $[M + H]^+$ 484.3.

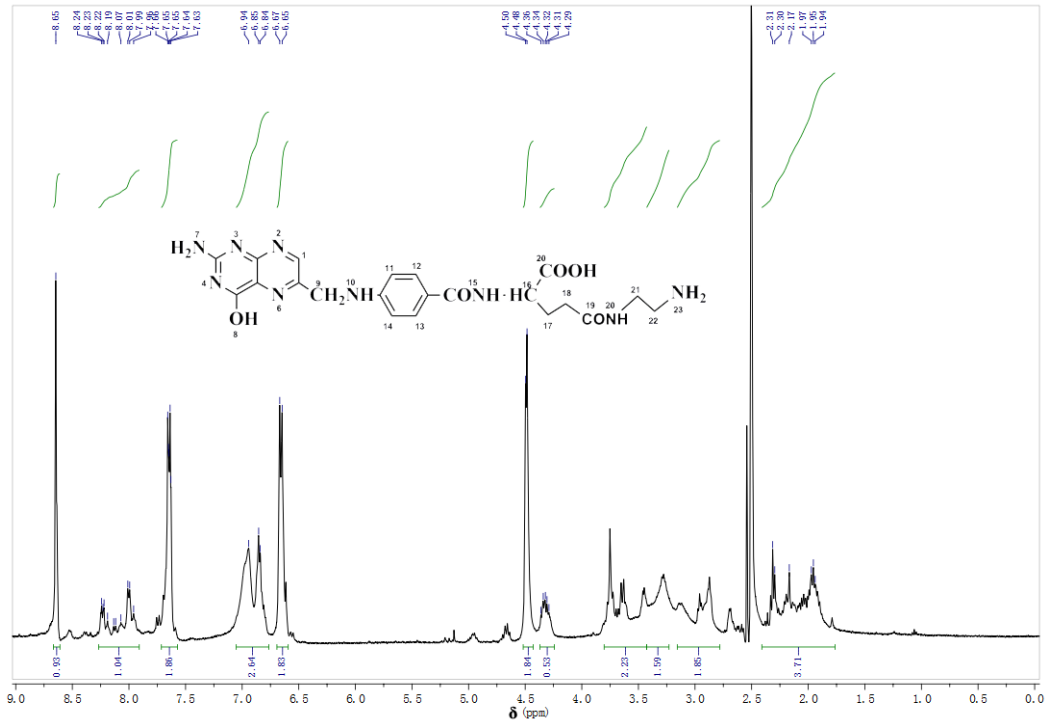


Figure S11. ^{1}H NMR Spectrum of **FA-EDA**. (400 MHz, DMSO-d6)

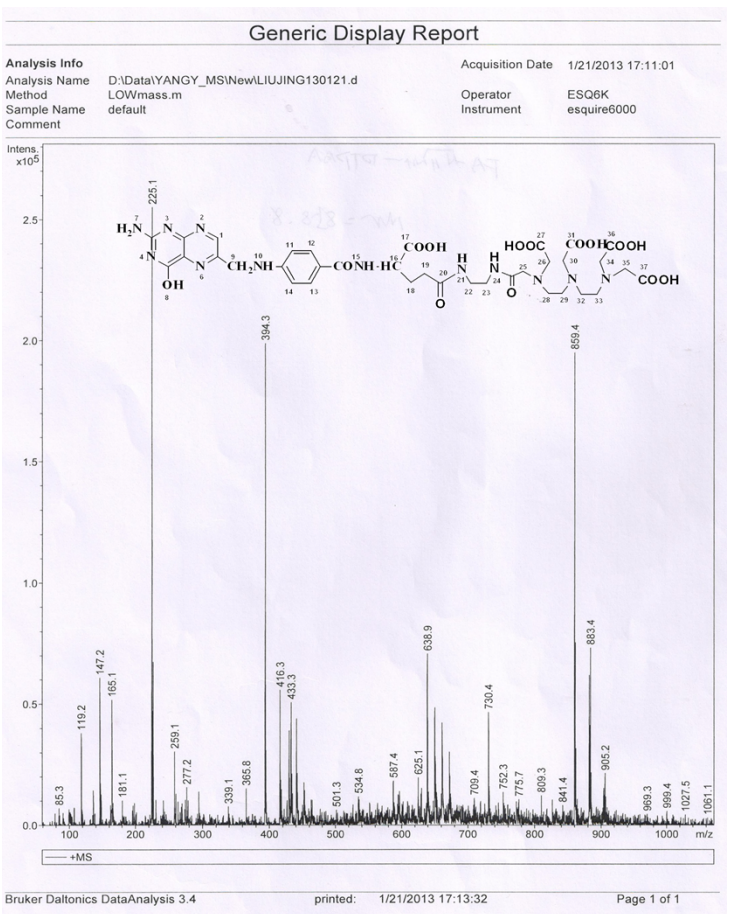


Figure S12. ESI-MS of **FA-EDA-DTPA**. (m/z): $[M + H]^{+}$ 859.4.

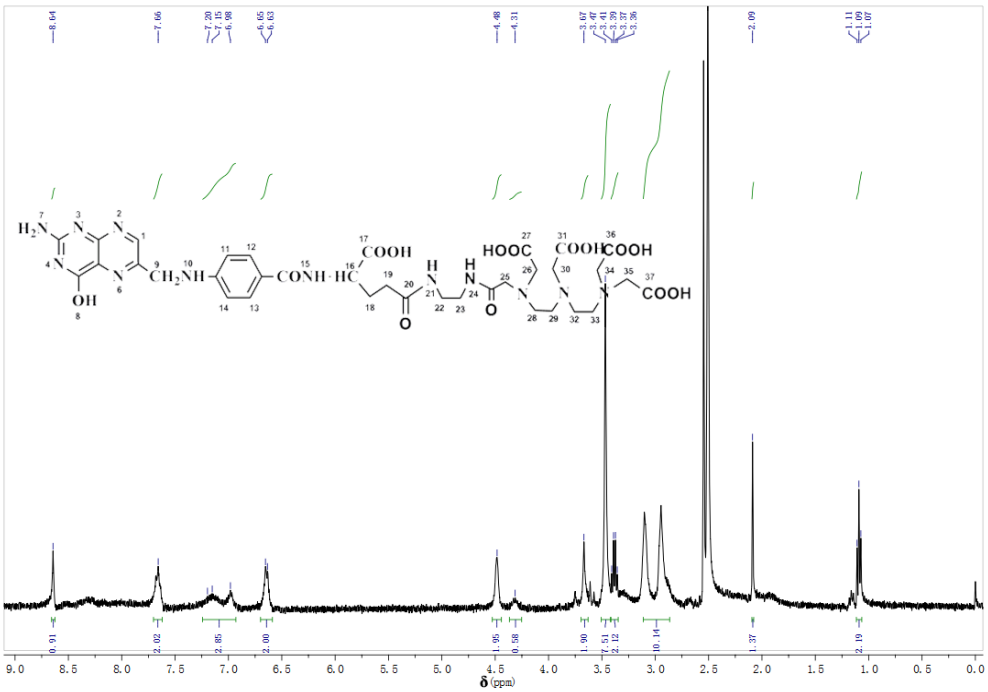


Figure S13. ^{1}H NMR Spectrum of **FA-EDA-DTPA**.