Electronic Supplementary Material (ESI) for New Journal of Chemistry.

Poly(HPMA)-DTPA/DOTA-Gd Conjugates for Magnetic Resonance Imaging

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Experimental

Materials

2,2-Azobis(2-methylpropionitrile (AIBN, Aldrich) was recrystallized from ethanol and dried at room temperature under vacuum. Diethylenetriaminepentaacetic (DTPA) and 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetate (DOTA) (>98%), Thiazolyl blue tetrazolium bromide (MTT, 98%), thionyl chloride, dimethyl sulfoxide (DMSO, 99.8%), dichloromethane, acetone and isopropanolamine were all purchased from Aldrich. NMR solvents (D_2O, $CDCl_3$ and DMSO) were purchased from Fisher. Regenerated cellulose dialysis membrane (Spectra/Por 6, molecular weight cutoff 1000 and 2000 Da) was purchased from Fisher. MilliQ water (resistivity>18.2 MΩ cm^{-1}) was generated using a Millipore MilliQ Academic Water Purification System.

Synthesis of Poly(HPMA)

HPMA was synthesized according to the literatures.[1] Poly(HPMA) was prepared as following: HPMA (0.14g) and AIBN (0.01g) were dissolved in DMSO (2.0 ml). The mixture was degassed with nitrogen gas in an ice bath for 30 min. Then the Schlenk flask was placed into a constant temperature oil bath at 70 °C for 24 h. The final mixture was precipitated into acetone to give a white powder (0.10 g, yield: 66%). The compound was dissolved in 20 ml of deionized water and dialyzed against water for

48 h in a dialysis bag. The final product was obtained by lyophilizing the dialysis solution.

Synthesis of Poly(HPMA)-DTPA and Poly(HPMA)-DOTA

DTPA dianhydride (DTPAda) was synthesized according to the literatures.[2] The operations were carried out under N_2 atmosphere *via* standard Schlenk techniques. Poly(HPMA)-DTPA was prepared as following: Poly(HPMA) (0.14g, 0.01 mol) was dissolved in DMSO (2 ml). The solution was degassed with nitrogen gas in an ice bath for 30 min. Then DTPAda (0.71 g, 0.002 mol) was added. The mixture was stirred for 24 h and was precipitated into acetone to give poly(HPMA)-DOTA conjugate as a white powder (0.10 g, yield: 66%). DOTA was activated according to the literatures.[3] Poly(HPMA)-DOTA was prepared as following: Poly(HPMA) (0.14g, 0.01 mol) was dissolved in DMSO (2 ml). The solution was degassed with nitrogen gas in an ice bath for 30 min. Then The activated DOTA was dissolved in DMSO (1 mL) was added. The mixture was stirred for 48 h at room temperature. The solution was added slowly to cooled(ice-salt bath) acetone (20 mL) with stirring. The resulting polymer was purified by dialysis *via* 3000 M_w cut-off dialysis tubing, and lyophilized to dryness, giving poly(HPMA)-DOTA conjugate as pale yellow powder (0.9 mg, 51%).

Synthesis of Poly(HPMA)-DTPA-Gd and Poly(HPMA)-DOTA-Gd

Poly(HPMA)-DTPA-Gd and Poly(HPMA)-DOTA-Gd was prepared as

following: Poly(HPMA)-DTPA or Poly(HPMA)-DOTA (0.14 g) was dissolved in 20 mL H_2O. $GdCl_3 \cdot 6H_2O$ (155 mg, 0.42 mmol) dissolved in 1 mL H_2O was added, at the same time the pH value of the system was maintained around 5.5 using 1 M NaOH. Afterwards, the reaction solution was placed in an oil bath thermostated at 70 ^oC for 12 h. The resulting reaction solution was cooled down to room temperature and was dialyzed against water for 48 h in a dialysis bag. The final product was obtained by lyophilizing the dialysis solution.

Characterization

Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Mercury plus-400 instrument. The chemical shifts are reported in ppm relative to the solvent residual peak. A Bruker IFS 66v/s IR spectrometer (Bruker, Karlsruhe, Germany) was used for the Fourier transformed infrared (FTIR) analysis in the range of 400–4000 cm^{-1} with the resolution of 4 cm^{-1}. The morphologies were characterized by JSM-6701F Field Emission Scanning Electron Microscope (JEOL, FE-SEM), when the samples were coated with gold film. The molecular weights and distributions were obtained by Gel Permeation Chromatography (Waters Alliance GPCV 2000 chromatograph). The composition and structure were characterized by TGA (Perkin Elmer STA6000).

***In Vitro* MRI Research**

For the measurement of T_1 and T_2 relaxivity (r_1 and r_2) and T_1-weighted

MR images, 200 μL Poly(HPMA)-DTPA-Gd and Poly(HPMA)-DOTA-Gd solution with different Gd concentration were transferred to tubes, and the data were acquired with spin-echo acquisition on 7.0 T NMR-analyzer (Molecular imaging center, institute of materia medica, Chinese Academy of Medical Sciences, PharmaScan 70T/16 US, Bruker, Germany). The concentration of the Poly(HPMA)-DTPA-Gd was selected to be 1.1, 0.55, 0.28, 0.14 and 0.07 mmol/L. The concentration of Poly(HPMA)-DOTA-Gd was set to be 0.72, 0.36, 0.18, 0.09 and 0.045 mmol/L. The parameters were set as follows: TR (repetition time) = 100.0 ms, TE (echo time) = 8.6 ms, NS (number of scan) = 1. The r_1 and r_2 values were calculated from the slop of curve-fitting result of $1/T_1$ and $1/T_2$ (s^{-1}) versus the Gd concentration (mM).

Hemolysis assay

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Northwest Normal University and approved by the Animal Ethics Committee of Northwest Normal University. The fresh blood of 5.0 mL was drawn from rabbit and immediately placed into saline containing centrifuge tube. The tube was centrifuged at 2000 rpm for 10 min and the supernatant was discarded. The obtained precipitate was repeatedly washed and centrifuged until the supernatant was substantially colorless. The precipitated red blood cells were mixed with physiological saline to prepare a 2% (v/v) cell

suspension. Hemolysis tests were performed based on previously published procedures.[4,5] A series of solutions (1.0 mL) of different mass concentrations were placed in the centrifuge tube to which an equal volume of 2% (v/v) red blood cell suspension was added. Another 2% (v/v) red blood cell suspension (1 mL) was added to an equal volume of distilled water and physiological saline as a positive control and a negative control, respectively. The all tubes were thoroughly mixed and incubated for 1 h in a 37 °C incubator. Then, the all tubes were centrifuged at 3000 rpm for 10 min and the supernatant (100 uL) was pipetted into a 96-well plate. The OD value of each well at a wavelength of 450 nm on a 96-well plate was measured using a microplate reader. The hemolysis rate is calculated using the following formula: Hemolysis rate % = [(sample OD -negative OD) / (positive OD - negative OD)] × 100%. Each concentration was set to three parallel groups.

Cytotoxicity Assay

The MTT assay was introduced to study the cytotoxicity of the conjugate-based nanoparticles against MCF-7, HepG2 and normal lung cells MRC-5 cells according to previous reports. MCF-7, HepG2 and normal lung cells MRC-5 cells cultures were carried out in 96-well plates (cell density: 5×10^3). After 24 h, the Poly(HPMA)-mustard conjugate and Nitrogen mustard (equivalent mustard dose, 5, 10, 20 and 40 μg mL^{-1}) were added, respectively. The additional culture lasted for 24 h at 37°C. Before being

incubated with MTT (5 mg/mL^{-1}, 20 μL per well), the medium was removed and cells were washed twice with ice-cold PBS. Then the solution was removed before adding dimethyl sulfoxide (150 μL per well) into the wells to dissolve the formazane of MTT. The cell viability was calculated via ELISA plate reader (Thermo Fisher Scientific, MA, USA) with the ultraviolet absorbance at 570 nm. The optical density was used to calculate cell viability.

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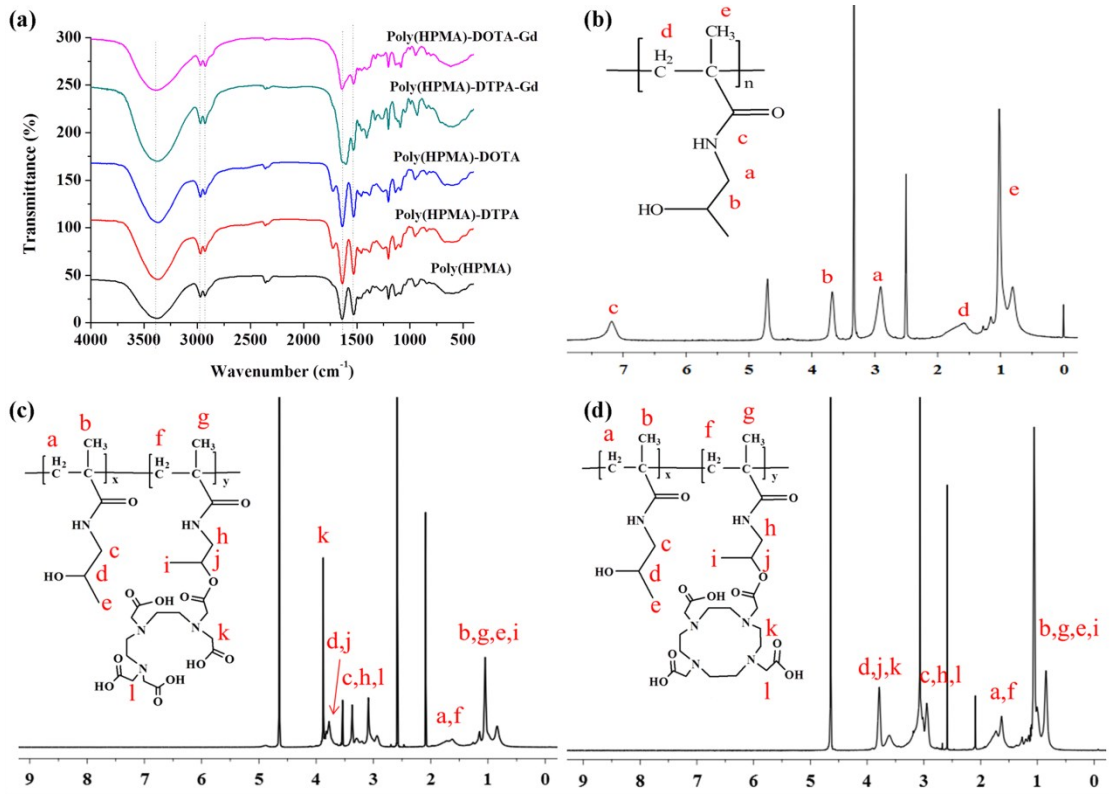

Fig. S1 The FTIR spectrum of poly(HPMA), poly(HPMA)-DTPA/DOTA and poly(HPMA)-DTPA/DOTA –Gd (a), the NMR spectra of poly(HPMA) (b), poly(HPMA)-DTPA (c) and poly(HPMA)-DOTA (d).

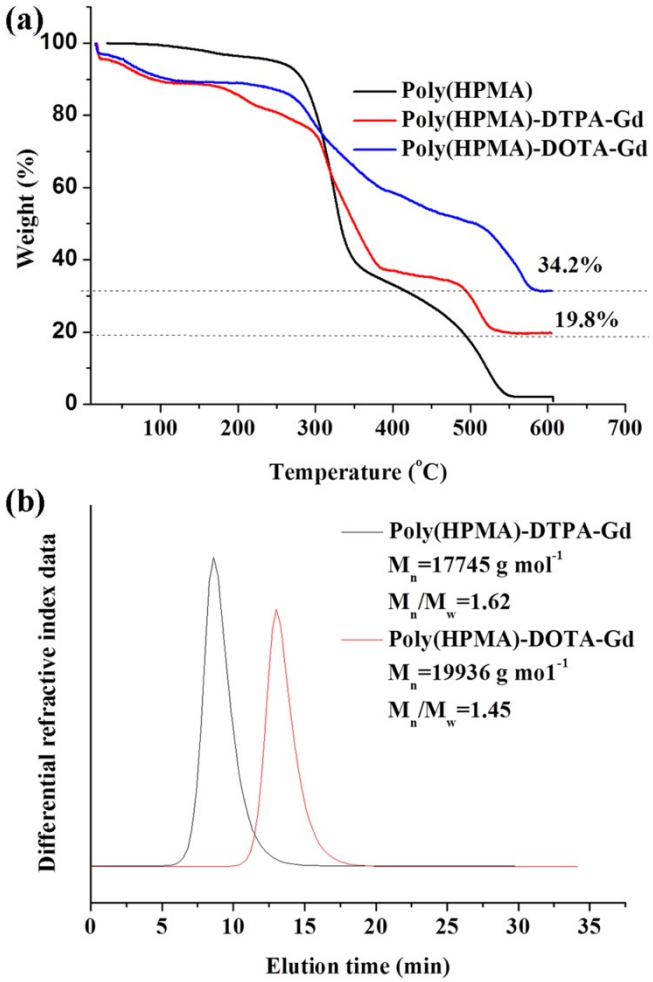

Fig. S2 The TGA (a) and GPC (b) curves of the poly(HPMA), poly(HPMA)-DTPA/DPTA-Gd conjugate

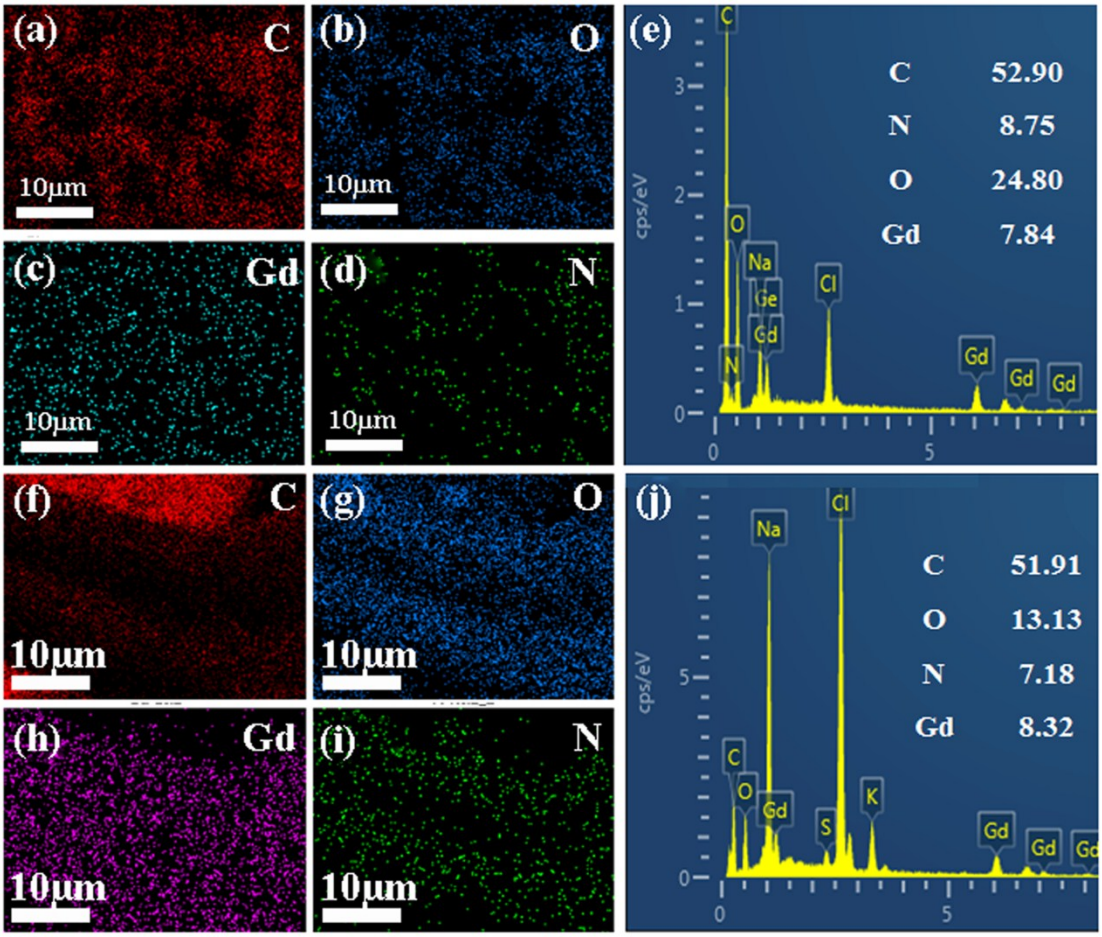

Fig. S3 the element mapping of poly(HPMA)-DTPA-Gd (a-d) and poly(HPMA)-DOTA-Gd (f-i), the EDS curves of poly(HPMA)-DTPA/DOTA-Gd (e and j).

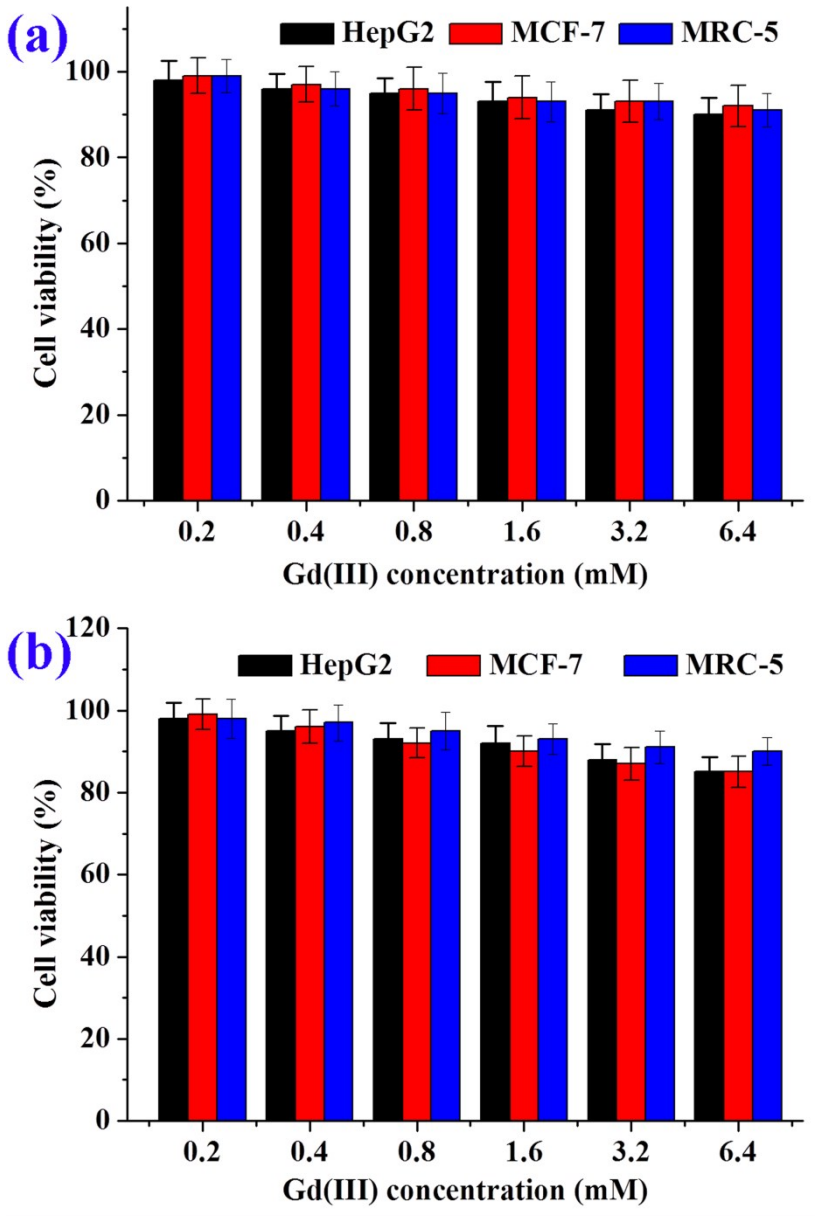

Fig. S4 *In vitro* cell viability of HepG2 cells, MCF-7 cells and normal lung cells MRC-5 incubated with poly(HPMA)-DTPA-Gd (a) and poly(HPMA)-DOTA-Gd (b) at different concentrations.

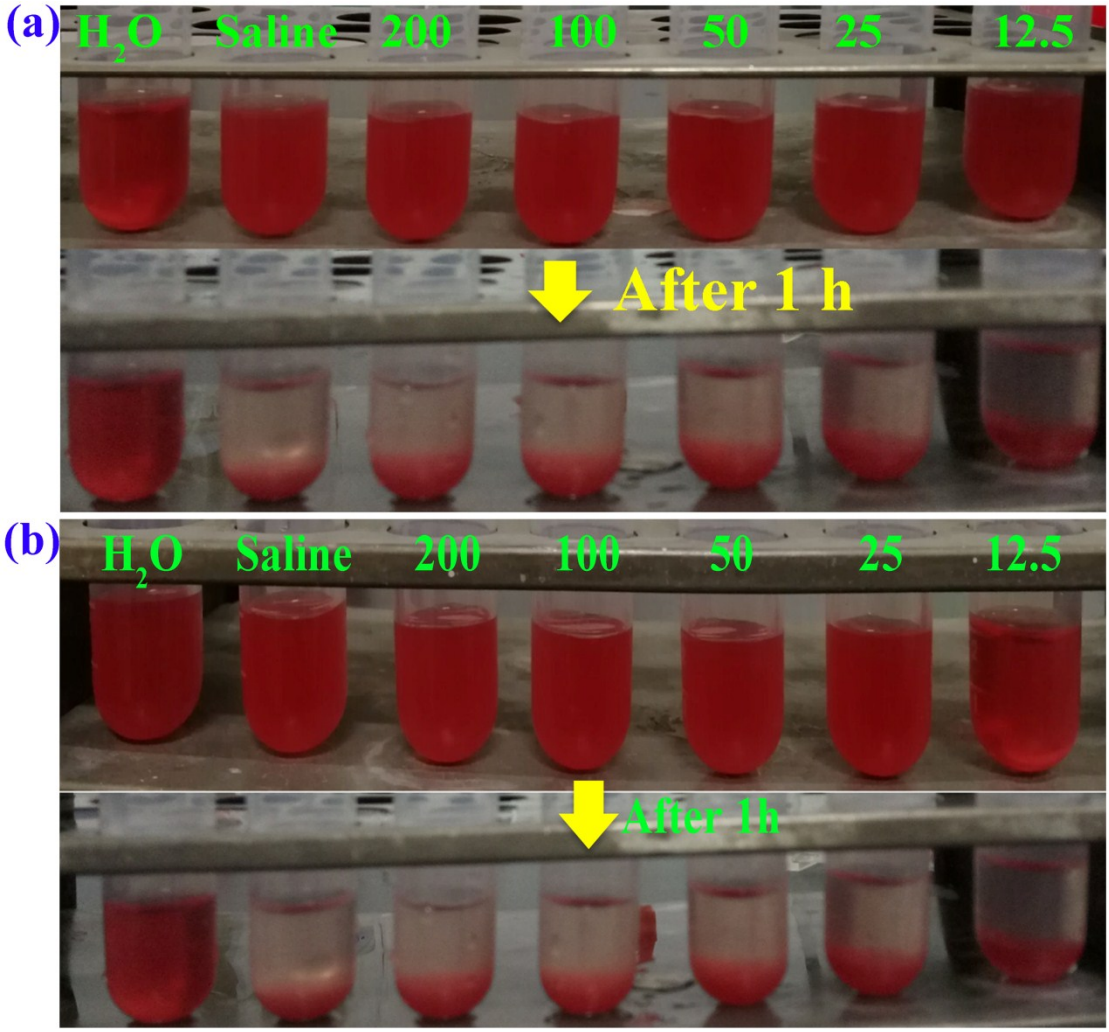

Fig. S5 Erythrocyte hemolysis resulted in the presence of poly(HPMA)-DTPA-Gd (a) and poly(HPMA)-DOTA-Gd (b) at different concentrations.

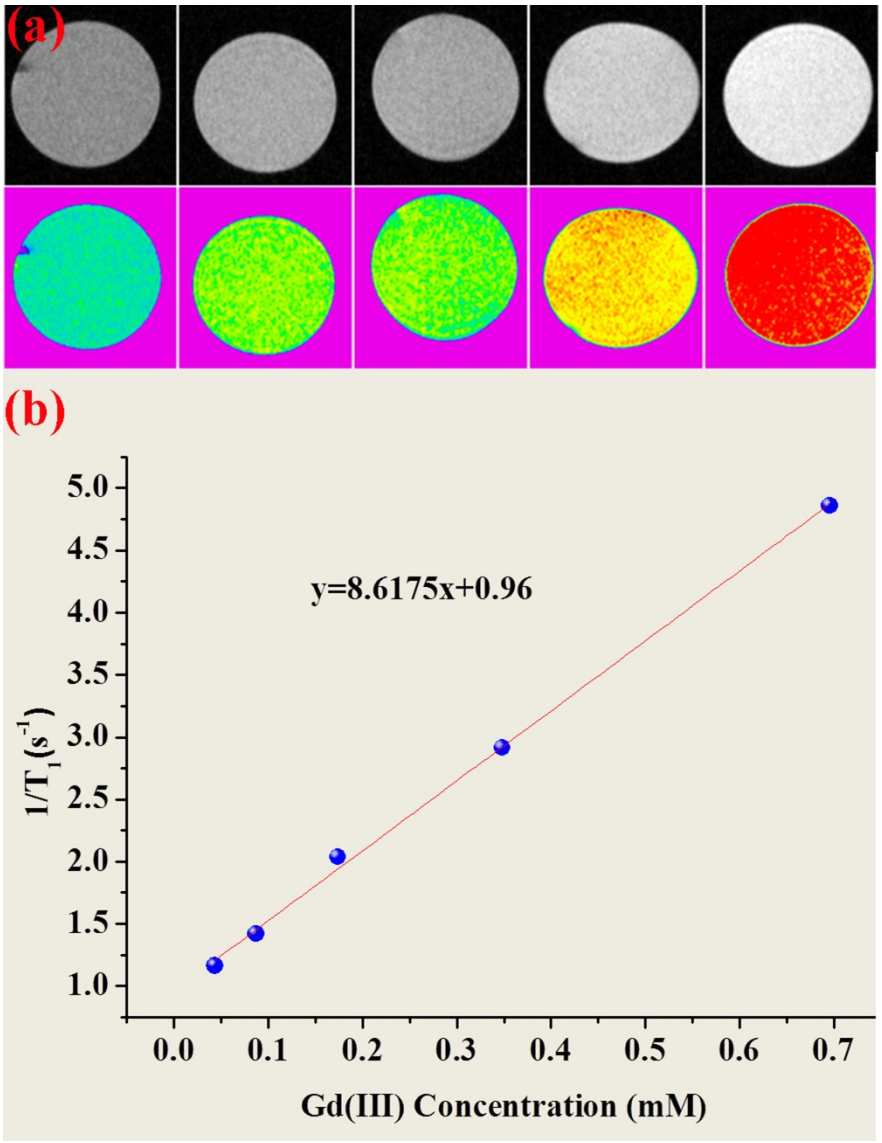

Fig. S6 The T_1-weighted MRI images of poly(HPMA)-DOTA-Gd (a) and the T_1-relaxation rate of poly(HPMA)-DOTA-Gd (b).

Table S1 The comparison of Poly(HPMA)-DTPA/DOTA-Gd conjugate with DTPA-Gd, DOTA-Gd.

	r_1 [mM^{-1} s^{-1}] (Gd)	r_2 [mM^{-1} s^{-1}] (Gd)	r_2/r_1
DTPA-Gd	3.85	13.2	3.44
DOTA-Gd	3.81	13.9	3.64
Poly(HPMA)-DTPA -Gd	8.6	23.9	2.78
Poly(HPMA)- DOTA-Gd	7.4	21.6	2.92

The r_1 and r_2 are measured on a MRI scanner system (7.0 T, PharmaScan 70T/16 US, Bruker, Switzerland).

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