Supporting Information

Cancer Theranostic Nanoparticles Self-Assembled from Amphiphilic Small Molecules with Equilibrium Shift-Induced Renal Clearance

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1. Experiment section

Critical aggregation concentration (CAC): The CAC value of Gd(DTPA-CPT) was measured by fluorescence spectroscopy using pyrene as a hydrophobic florescence probe. 20 μL of pyrene solutions in acetone (3×10^{-5} mol L^{-1}) were added into 2 mL of aqueous Gd(DTPA-CPT) solutions with serial concentrations. Thereafter the solutions were kept at room temperature for 24 h. Then the fluorescence spectra were recorded at an excitation wavelength of 310 nm, and the fluorescence emission was collected at 397 nm. The CAC value was determined by extrapolating the inflection point of fluorescence intensity.

Model and simulation method: The relative stability of Gd(DTPA-CPT) and PEG-*b*-PCL nanoparticles was investigated by using molecular dynamics simulations. Here, the MARTINI force field[1] with four-to-one mapping strategy is used to represent the molecules in the simplified model; that is, on average, four atoms are represented by a single interaction center. Based on the mapping rule of the MARTINI force field, we constructed coarse-grained (CG) model for Gd(DTPA-CPT) and PEG-*b*-PCL, respectively. There are four main types of beads: polar (P), nonpolar (N), apolar (C) and charged (Q) to represent the chemical nature of the underlying atomistic structures. The bead types for smaller particles representing 2-3 atoms including those in ring are labeled by a prefix "S". Gd(DTPA-CPT) and PEG-*b*-PCL were respectively represented by 19 and 113 CG beads (Figure S15).

Table S1 provides a summary of the detailed simulation setup used in this work. Firstly, Gd(DTPA-CPT) and PEG-*b*-PCL nanoparticles were pre-assembled by the PACKMOL[2] program (Figure S16). Secondly, the configurations were optimized using the L-BFGS algorithm with an energy tolerance of 10.0 kJ mol^{-1} nm^{-1}. A cutoff value of 1.0 nm was used for non-bond interactions. Lastly, 100 ns long CG molecular dynamics simulations of Gd(DTPA-CPT) and PEG-*b*-PCL

nanoparticles in aqueous solution were performed with the cubic box, respectively. The last 10 ns equilibrated trajectory was further used to assess the structures and energies. All the calculations were employed by using GROMACS 4.5.5[3] in the NPT ensemble. A 40 fs time step is used to integrate the equations of motion with the leap-frog algorithm. The periodic boundary is used in the x, y and z directions. The neighbor list is updated every 10 steps with a cutoff 1.2 nm. The standard shift function of GROMACS is used to deal with the LJ and Coulomb potentials. The LJ potential is shifted from 0.9 nm to a cutoff of 1.0 nm. The Coulomb potential is shifted from 0.0 nm to a cutoff of 1.0 nm. The initial velocities of beads are generated based on the Maxwell distribution at system temperature. The temperature and pressure are controlled at 303 K and 1 atm by the Berendsen thermostat and barostat method.

Dynamic dialysis experiments: 2.0 mL of Gd(DTPA-CPT) nanoparticles, free CPT and pyrene-labeled PEG-*b*-PCL nanoparticles aqueous solutions (1.0 mg mL^{-1}) were placed in the dialysis bag, respectively. Dialysis experiments were conducted after immersion of the dialysis bag in 50 mL of PBS (pH 7.4). Aliquots (2 mL) were withdrawn from outside the dialysis bag at different time intervals. 50 mL of phosphate buffer (pH 7.4) was added outside the dialysis bag at different time intervals (16 h, 32 h, 48 h). After dilution, small molecular Gd(DTPA-CPT) could pass through the dialysis bag, which was verified by HPLC. The amount of small molecular Gd(DTPA-CPT), free CPT and pyrene-labeled PEG-*b*-PCL unimer was determined by UV-vis spectrophotometry.

In vitro drug release from Gd(DTPA-CPT) nanoparticles: The *in vitro* drug release behavior of Gd(DTPA-CPT) nanoparticles in different environments was detected using high-performance liquid chromatography (HPLC, Agilent 1260 Infinity system, Agilent Inc.). The Gd(DTPA-CPT) nanoparticles (0.4 mg mL^{-1}) were dispersed in different environments (phosphate buffer (pH 7.4),

acetate buffer (pH 5.0), phosphate buffer (pH 7.4) containing 17 U mL^{-1} esterase and acetate buffer (pH 5.0) containing 17 U mL^{-1} esterase). The solutions were incubated at 37 °C in a laboratory shaker with gentle shaking (100 rpm) and sampled at 2, 4, 6, 8, 10, 12, 24, 36, 48 and 60 h, respectively. Subsequently, these samples were freeze-dried and the residue was redissolved with 1 mL of DMSO. The amount of CPT was determined by HPLC using acetonitrile and water as mobile phases. Each experiment was replicated three times and the results presented are the average values.

Cell culture: LoVo cells (a human colon cancer cell line) were cultured in Ham's F12K medium with 2 mM L-glutamine adjusted to contain 1.5 g L^{-1} sodium bicarbonate. HepG2 cells (a human hepatocarcinoma cell line) were cultured in DMEM. The culture mediums contained 10% FBS and antibiotics (50 units mL^{-1} penicillin and 50 units mL^{-1} streptomycin). All of the cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

In vitro anticancer effects of Gd(DTPA-CPT) nanoparticles: The LoVo cells and HepG2 cells were chosen to evaluate the anticancer effects of Gd(DTPA-CPT) nanoparticles by MTT assay. Meanwhile, the free drug CPT, Gd(DTPA) and CPT/Gd(DTPA) mixture were used as controls. The cells were seeded in 96-well plates at a density of 1×10^4 cells per well in 200 μL medium. After 24 h incubation, the cells obtained 70-80% confluence. Then the culture medium was removed and replaced with 200 μL fresh medium containing serial dilutions of Gd(DTPA-CPT) nanoparticles, CPT, Gd(DTPA) and CPT/Gd(DTPA) mixture. The cells without any treatment were set as control. For Lovo cells, the cells were cultured for another 24 h, 48 h and 72 h at 37 °C under 5% CO_2, respectively. For HepG2 cells, the cells were cultured for another 48 h at 37 °C under 5% CO_2. Thereafter, 20 μL of MTT assay stock solution (5 mg mL^{-1} in PBS) was added to each well and the cells were incubated for another 4 h. Then culture medium was removed and 200 μL DMSO was added into each well to dissolve the formazan crystals. Then the plates were vibrated for 10 min and

the absorbance of each well was measured in a BioTek® Synergy H4 at a wavelength of 490 nm.

Cellular uptake studies of Gd(DTPA-CPT) nanoparticles: Confocal laser scanning microcopy (CLSM) was chosen to study the cellular uptake of Gd(DTPA-CPT) nanoparticles by LoVo cells. LoVo cells were seeded in glass-bottom culture dishes at a density of 5.0×10^5 cells per dish in 1.5 mL of complete Ham's F12K medium and cultured for 24 h. Then the medium was replaced with 1.5 mL medium containing Gd(DTPA-CPT) nanoparticles at a concentration of 30 μM. The cells were incubated at 37 °C for 3 h. Then the culture medium was removed and the cells were rinsed with PBS three times and fixed with 4% paraformaldehyde for 30 min at room temperature. Subsequently, the cells were treated with 0.5% Triton X-100 in ice bath for 15 min, and then treated with RNAse (100 μg L^{-1}) at 37 °C for 30 min. Finally, the cells were stained with 2.4 nM YOYO-1 at room temperature for 20 min and rinsed with PBS three times. The cells were observed with Nikon Ti-E inverted motorized microscope.

Flow cytometry: For flow cytometry, LoVo cells were seeded in 6-well plates at a density of 5.0×10^5 cells per well in 1.5 mL complete Ham's F12K medium and cultured for 24 h. Then the medium was replaced with 1.5 mL medium containing Nile-red loaded Gd(DTPA-CPT) nanoparticles at a concentration of 30 μM. Then, the cells were incubated at 37 °C for predetermined intervals, 5, 15, 30, 45 and 60 min. Thereafter, the culture medium was removed and the cells were washed with PBS three times and then harvested. The amount of intracellular fluorescent signal of Nile-red was quantified using BD Accuri™ C6 flow cytometer, indicating the amount of Gd(DTPA-CPT) nanoparticles internalized by LoVo cells.

Cell apoptosis assay: LoVo cells were seeded in 6-well plates at a density of 5.0×10^5 cells per well and cultured for 24 h. Then the LoVo cells were treated with Gd(DTPA-CPT) and CPT at the same concentration (3 μM) for 24 h. The untreated LoVo cells were used as control. The

apoptosis experiments were performed using a combined staining with FITC-Annexin V/PI (FITC, fluorescein isothiocyanate, PI, propidium iodide) flow cytometry assay. Briefly, untreated and treated cells (1.0×10^5) were suspended in 1 × annexin binding buffer, with the addition of FITC-labeled Annexin V and PI, and incubated at room temperature for 15 min. After incubation, the samples were analyzed by BD Accuri™ C6 flow cytometer to acquire 10,000 events per sample. Finally, the cell populations were analyzed with the FlowJo software.

Hemolysis assay: 10 mL of blood from the ear artery of a male New Zealand white rabbit was collected. The blood was subsequently centrifuged at 2000 rpm for 10 min at 4 °C. The plasma supernatant was removed, and the erythrocytes were resuspended in ice cold PBS. The cells were again centrifuged at 2000 rpm for 10 min at 4 °C. This was repeated more than two times to ensure the removal of any released hemoglobin. After the supernatant was removed, the cells were resuspended in PBS to achieve a 2% w/v red blood cell (RBC) solution. The Gd-DTPA-CPT and the reference compounds were also prepared at serial concentration (0.02, 0.1, 0.2, 1 and 2 mg mL^{-1}) with PBS (pH=7.4). Then 2 mL of the Gd-DTPA-CPT or the reference compounds (PEI and Dextran) prepared in PBS was added to 2 mL of the 2% w/v RBC solution in centrifuge tubes and incubated for 1 h at 37 °C, respectively. Complete hemolysis was attained by treatment with 2% v/v Triton-X, yielding the 100% hemolysis value. After incubation, the centrifuge tubes were centrifuged, and the supernatants were collected. The release of hemoglobin was determined by spectrophotometric analysis of the supernatant at 545 nm. Results were expressed as the amount of hemoglobin release induced by the compounds as a percentage of the total.

Tumor xenografts: All animal experiments were conducted in agreement with the guidelines of the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University. LoVo cells were harvested and pelleted by centrifugation and re-suspensed in sterile PBS. 2×10^6 cells were

injected into male Balb/c nude mice (4 weeks old, Chinese Academy of Sciences of Shanghai). The studies were performed when the tumor volume reached approximately 200 mm^3.

In vitro MRI relaxivity measurements: The longitudinal relaxation rates ($1/T_1$) of Gd(DTPA-CPT) nanoparticles in aqueous solution were measured on 3 T Siemens Magnetom Trio Tim, using a T_1-weighted MR image (repetition times (TR) were 24, 100, 200, 400, 600, 900, 1200, 2000, 3000, 5000 ms, echo time (TE) = 11 ms). T_1 values of Gd(DTPA-CPT) nanoparticle solutions and Gd(DTPA) (Magnevist®, Bayer Healthcare) solutions with different Gd concentrations ranging from 0.5 to 2.5 mM in water were measured. The Gd concentration was measured using ICP-AES. Longitudinal relaxivity (r_1) was calculated as the slope of $1/T_1$ vs the concentration of Gd.

According to the theory for MRI contrast agent, the overall measured relaxivity (R_1^{obs}) is a result of different contributions as indicated by:

$$R_1^{obs}=R_{1p}^{IS}+R_{1p}^{OS}+R_1^{W}$$

where R_{1p}^{IS} and R_{1p}^{OS} are the inner-sphere and outer-sphere relaxation enhancement in the presence of the paramagnetic complex at 1 mM concentration, respectively, and R_1^{W} is the relaxation rate of the water solvent in the absence of the paramagnetic complex. In addition, after calculation, the relaxivity of classical gadolinium complexes is related to the rotational correlation time τ_R, the coordination number q, the exchange rate τ_M and the electronic relaxation time $T_{iE.}$ The rotational correlation time τ_R is strictly related to the size and the rigidity of the contrast agent.[4] Gd(DTPA-CPT) self-assembly into nanoparticles can reduce the molecular tumbling of contrast agents and thus increase the rotational correlation time τ_R. Hence, the relaxivity of Gd(DTPA-CPT) nanoparticles is slightly higher than that of Gd(DPTA).

In vivo MRI experiments: In vivo MRI experiments were performed on anesthetized Balb/c male nude mice bearing LoVo tumors with 4% chloral hydrate under authorization of the regional

ethic committee for animal experiments. The experiments were conducted on a 3 T Siemens Magnetom Trio Tim, using a T_1-weighted MR image (repetition time (TR) = 500 ms, echo time (TE) = 14 ms, slice thickness = 1.0 mm, field of view (FoV): 49 × 99 and 50 × 50 respectively and matrix = 192 × 384 and 512 × 512, respectively). The Gd(DTPA-CPT) nanoparticles and Gd(DTPA) solutions were intravenously injected into the mice via tail vein at a dose of 0.10 mmol kg^{-1}, respectively. The mice were scanned before and after the administration of the contrast agent.

Pharmacokinetic studies: Sprague-Dawley (SD) rats (~200 g) were chosen to study the pharmacokinetics of Gd(DTPA-CPT) nanoparticles and free Gd(DTPA). Rats were randomly divided into two groups (n = 4). The Gd(DTPA-CPT) nanoparticles and free Gd(DTPA) solutions were intravenously injected via tail vein at a dose of 0.10 mmol kg^{-1}. Then, blood samples (0.5 mL) were collected from each animal at 5 min, 15 min, 30 min, 1 h, 2.5 h, 4 h, 5 h, 6 h, using retro-orbital blood collection method. Before inductively coupled plasma mass spectrometry (ICP-MS) analysis, the blood samples were digested with nitric acid for 24 h. After cooling down, the digestion solutions were filled up to 25 mL with water and analyzed by ICP-MS (Agilent Inc.).

Biodistribution studies: The biodistribution studies of Gd(DTPA-CPT) nanoparticles and Gd(DTPA) were performed on Balb/c male nude mice as mentioned above. The mice were randomly divided into two groups (n = 4), and intravenously injected via tail vein with Gd(DTPA-CPT) nanoparticles and Gd(DTPA) aqueous solution at a dose of 0.10 mmol kg^{-1}. Then the mice were sacrificed at 0.5 h post-administration., and the heart, liver, spleen, lung, kidney, and tumor were excised, washed, and weighted. Thereafter, the organs and tumors were digested in aqua regia at 70 °C until the solution was colorless and clear. Thereafter, the Gd content was analyzed with ICP-MS.

In vivo anticancer experiments: When the tumor size reached ~50 mm^3, the LoVo tumor-bearing mice were randomly divided into five groups (6 mice/group). Therefore, the mice in different treatment groups were intravenously injected via the tail vein with PBS, CPT, Gd(DTPA), Gd(DTPA-CPT) nanoparticles and Gd(DTPA)/CPT mixture at doses of 0.029 mmol kg^{-1} (low dose) or 0.10 mmol kg^{-1} (high dose) once every 3 days, respectively. The tumor sizes and the body weights of mice were measured every other day after treatments. Tumor volume (V) was determined by the following equation: $V = ab^2/2$, where a is the length and b is the width of the tumor. The tumor volume was normalized to its initial size before any treatment. After 27 days postinjection, the mice were sacrificed, and tumors were separated for histopathological analysis.

Histological examination (H&E) and immunohistochemical analysis: 3 days after the last treatment, the mice were sacrificed, and tumors were separated and cut into small pieces, fixed in 4% paraformaldehyde for 8 h. Then the tissues were embedded in paraffin and cut into 5 μm sections for H&E staining and immunohistochemical analysis. The sections were immunostained with an antibody against PCNA (proliferating cell nuclear antigen) to detect proliferating cells. Typically, the sections were treated with 3% hydrogen peroxide aqueous solution for 15 min to quench endogenous peroxidase activity, and then heated to boiling in EDTA antigen retrieval solution for 10 min in water bath for antigen retrieval. Then the sections were allowed to cool down, rinsed with PBS three times. Subsequently, the sections were treated with goat serum in TBS for 20 min to block non-specific binding sites, followed by incubation with PCNA antibody (1:100) for 1 h at room temperature. Then, the sections were rinsed with PBS three times, incubated with goat anti-mouse HRP secondary antibody (1:1000) followed by colorimetric detection with diaminobenzidine (DAB) as chromogen. Finally, the sections were counterstained with hematoxylin and prepared for mounting. Finally, images were taken by using a Nikon ECLIPSE Ti microscope.

2. Supplementary Figures and tables

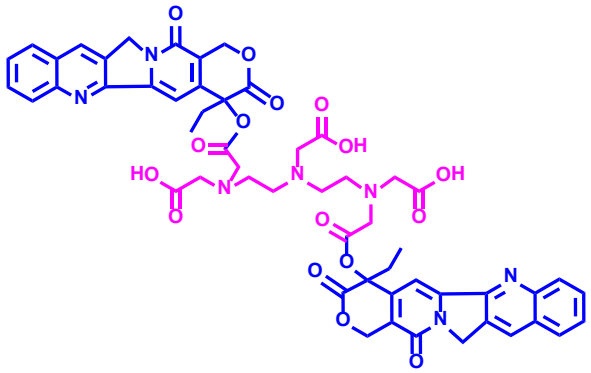

Figure S1. The side reaction was negligible for DTPA-CPT_2.

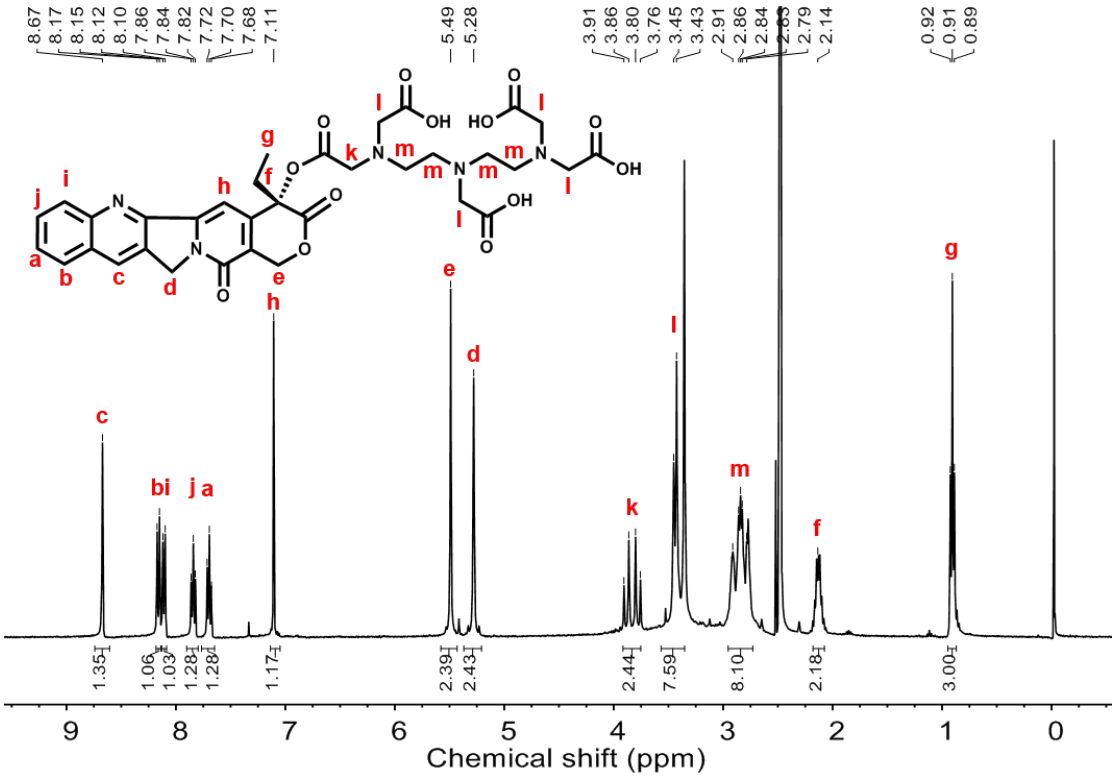

Figure S2. ^{1}H NMR spectrum of DTPA-CPT in DMSO-d_6.

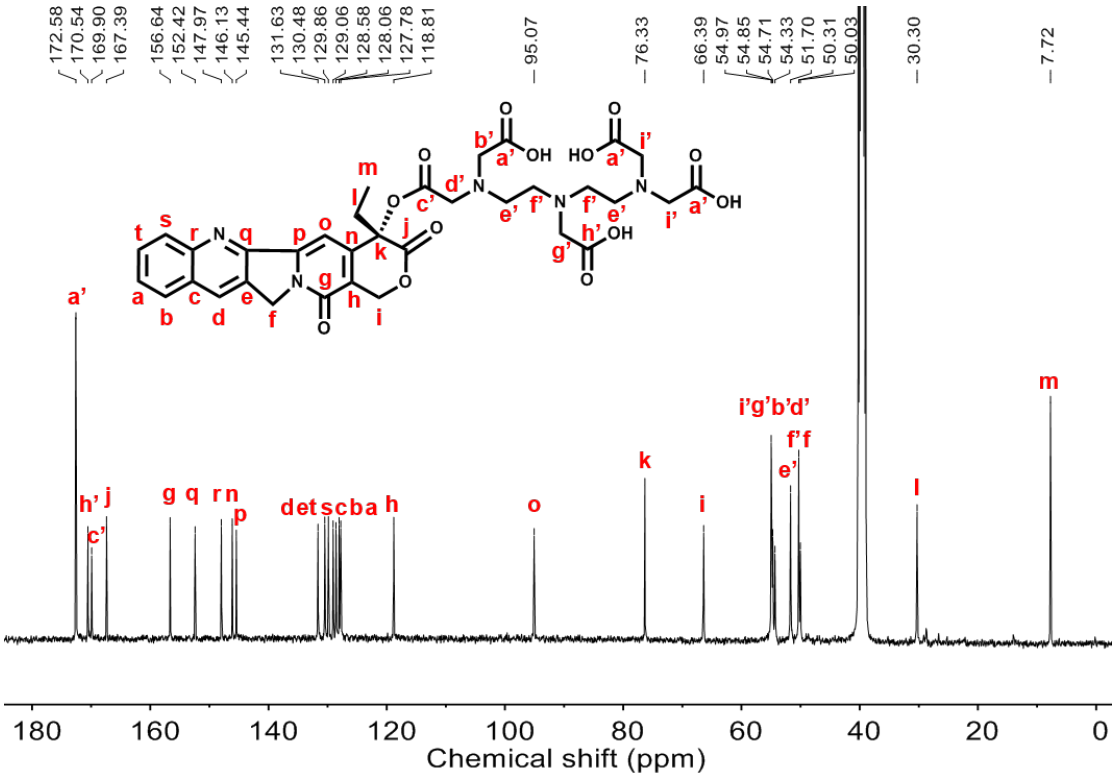

Figure S3. ^{13}C NMR spectrum of DTPA-CPT in DMSO-d_6.

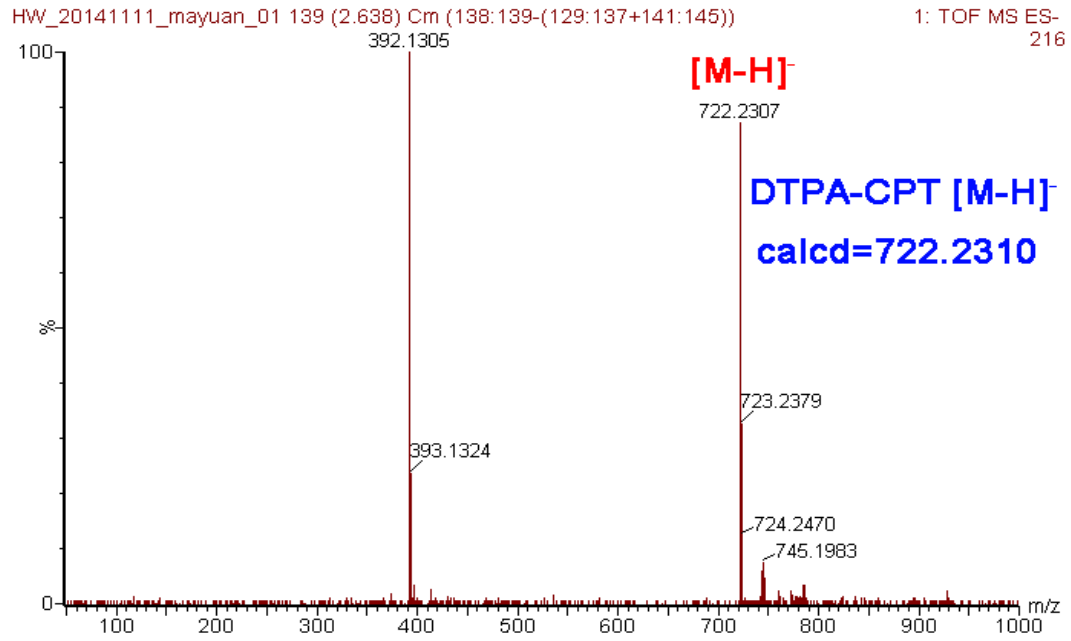

Figure S4. Mass spectrum of DTPA-CPT.

Figure S4 indicates that the molecular weight of DTPA-CPT (*m/z*, [M-H]$^-$) is 722.2307, which is consistent with the calculated value (*m/z*, [M-H]$^-$, 722.2310).

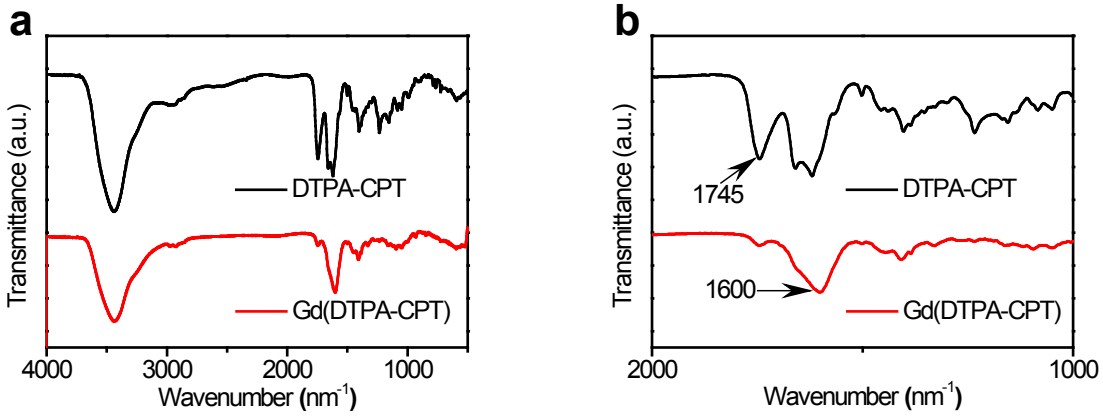

Figure S5. (a) FTIR spectra of DTPA-CPT and Gd(DTPA-CPT). (b) A partial FTIR spectra of DTPA-CPT and Gd(DTPA-CPT) in the range of 2000 ~ 1000 cm^{-1}.

In the FTIR spectrum of DTPA-CPT, a strong C=O stretching absorption band at 1745 cm^{-1} can be ascribed to the carboxyl stretching vibration. In the FTIR spectrum of Gd(DTPA-CPT), the band at 1600 cm^{-1} corresponds to the stretching bands of C=O of COO^-. The shift of the bands indicates the coordination between the Gd^{3+} and carboxylic ion.

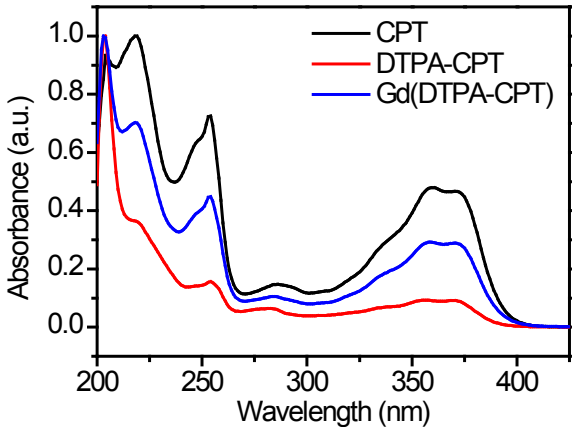

Figure S6. UV-vis spectra of CPT, DTPA-CPT, and Gd(DTPA-CPT) in methanol.

Compared with the UV-vis absorption of free CPT and DTPA-CPT at 359 nm, a 3 nm blue-shift is observed in the absorption of the Gd(DTPA-CPT) at 356 nm. The UV-vis results demonstrate the coordination between the Gd^{3+} and carboxylic ion.

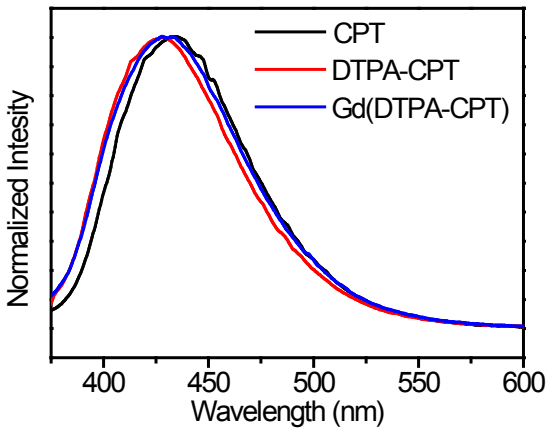

Figure S7. Fluorescence emission spectra of CPT (λ_{ex} = 359 nm, λ_{em} =434 nm), DTPA-CPT (λ_{ex} = 359 nm, λ_{em} = 428 nm), Gd(DTPA-CPT) (λ_{ex} = 359 nm, λ_{em} = 430 nm) in methanol.

The maximum emission peaks of free CPT and DTPA-CPT are 428 nm. For the Gd(DTPA-CPT), the maximum emission red-shifts to 435 nm, which confirms that the Gd(DTPA-CPT) is synthesized successfully.

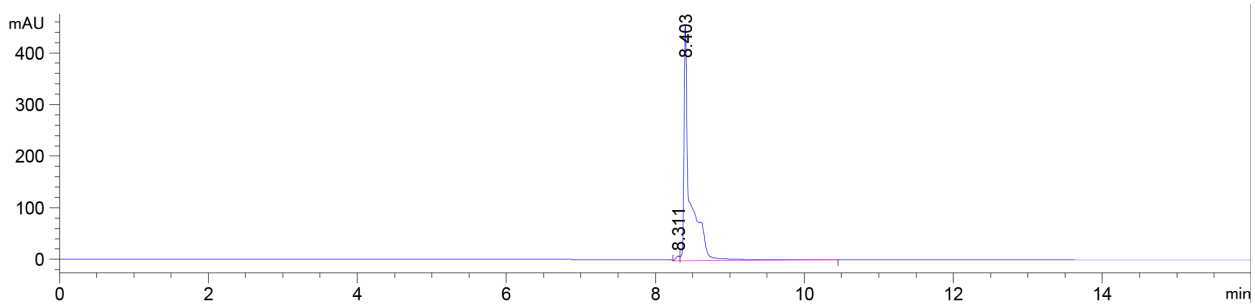

Figure S8. HPLC analysis of DTPA-CPT.

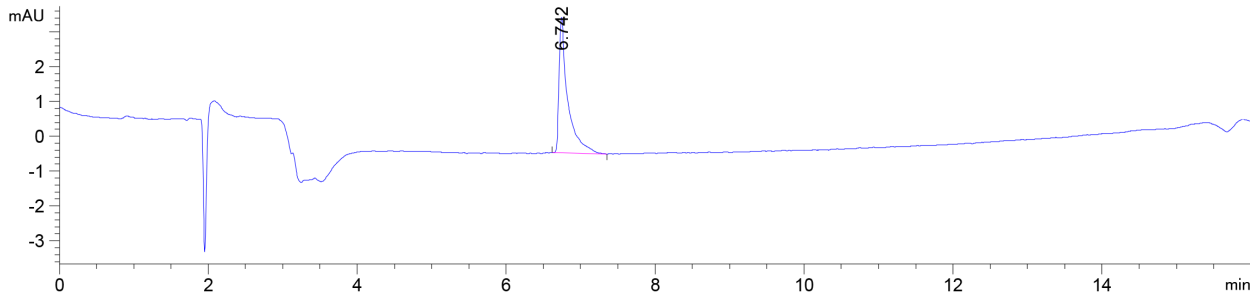

Figure S9. HPLC analysis of Gd-DTPA-CPT.

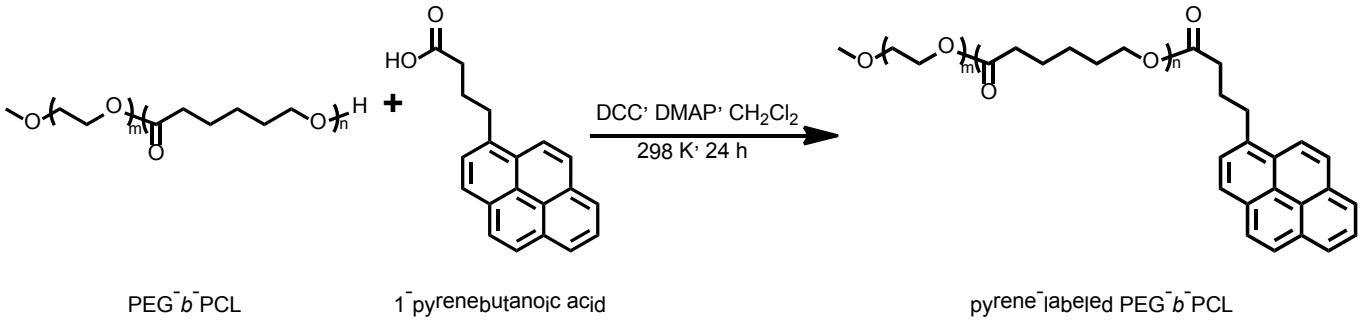

Figure S10. The synthesis route of pyrene-labeled PEG-*b*-PCL.

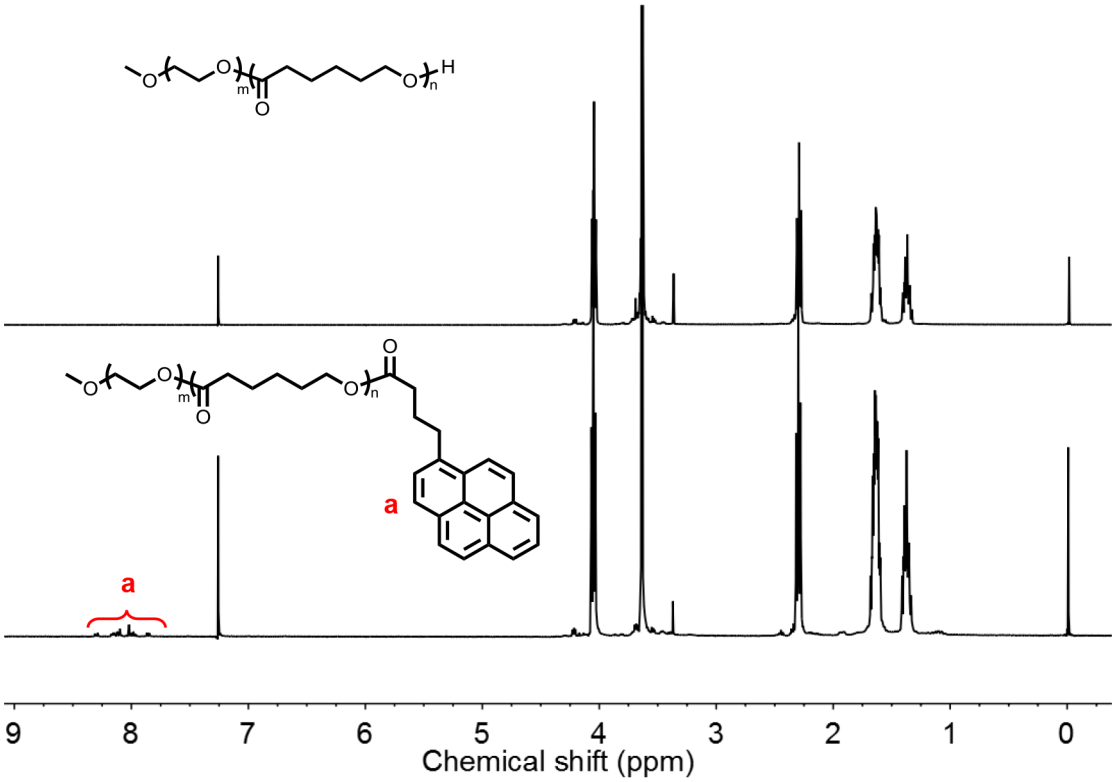

Figure S11. ^{1}H NMR spectrum of PEG-*b*-PCL and pyrene-labeled PEG-*b*-PCL in $CDCl_3$. The signals (a) belong to the protons of pyrene.

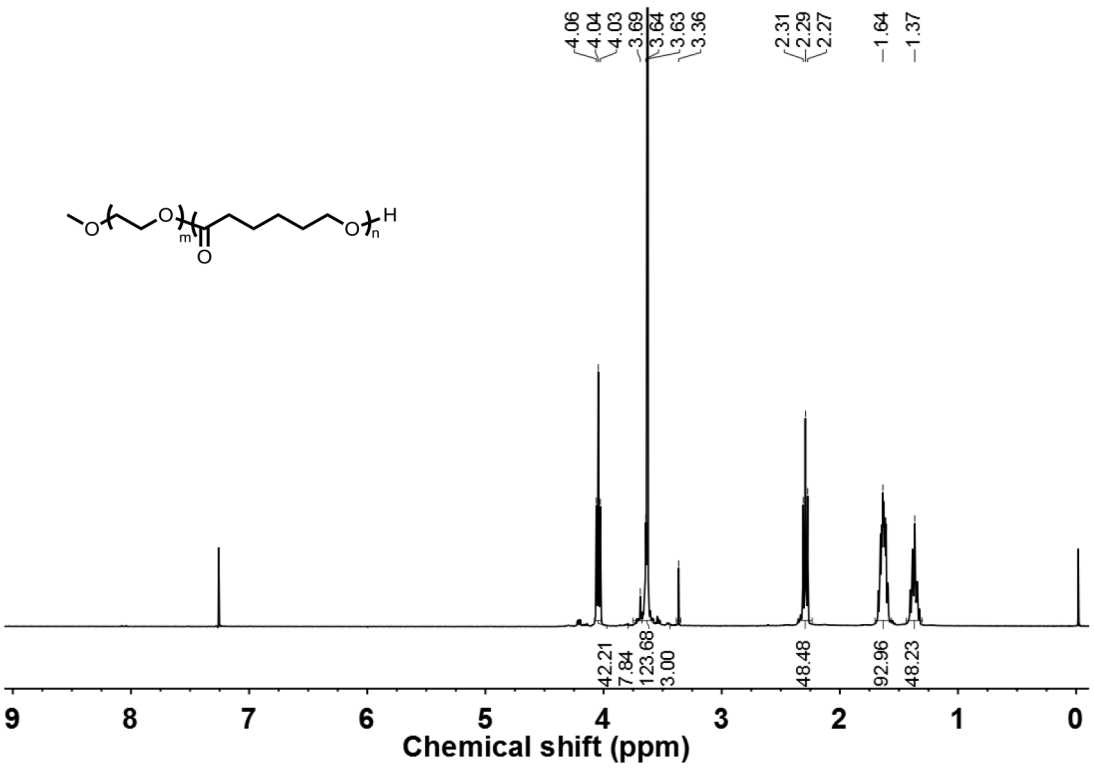

Figure S12. ^{1}H NMR spectrum of PEG-*b*-PCL in $CDCl_3$.

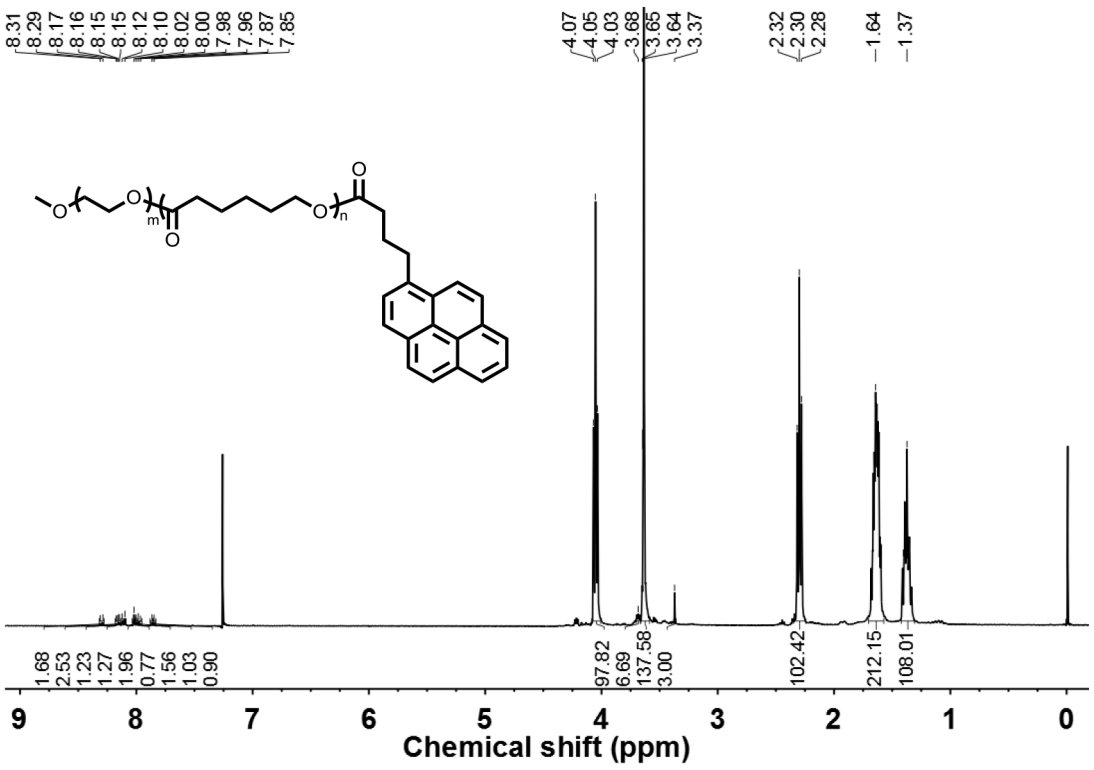

Figure S13. ^{1}H NMR spectrum of pyrene-labeled PEG-*b*-PCL in $CDCl_3$.

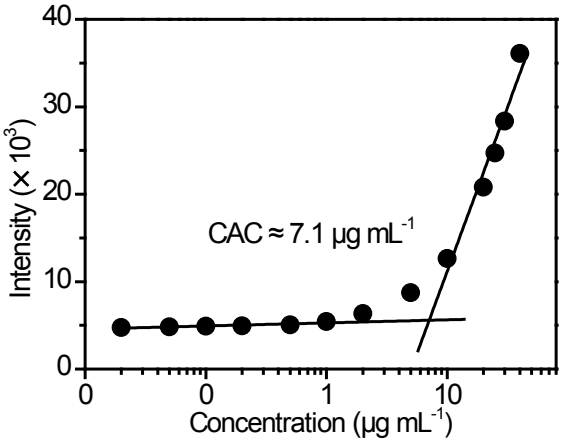

Figure S14. The fluorescence intensity of pyrene at 397 nm under excitation as a function of the logarithm of Gd(DTPA-CPT) concentration.

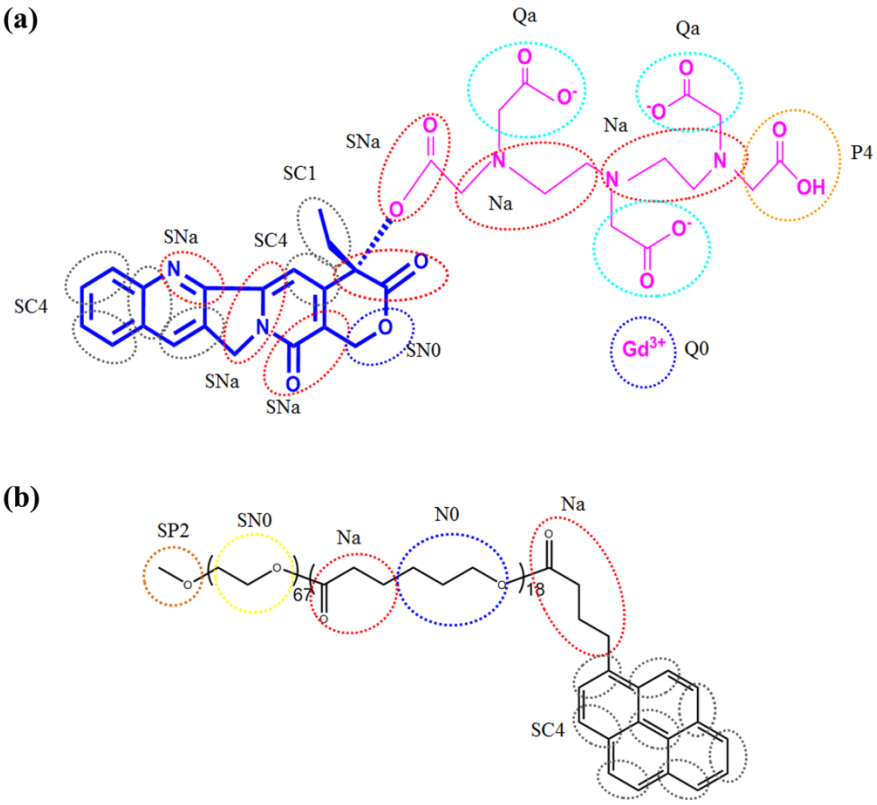

Figure S15. Mapping from atomistic structures to CG bead for (a) Gd(DTPA-CPT) and (b) PEG-*b*-PCL models.

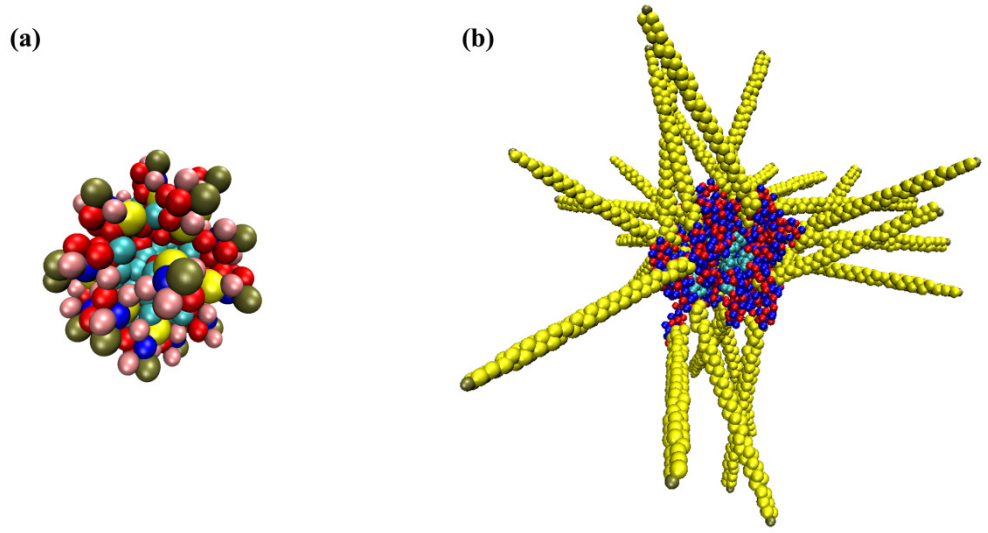

Figure S16. The pre-assembled nanoparticle structures for (a) Gd(DTPA-CPT) and (b) PEG-*b*-PCL models.

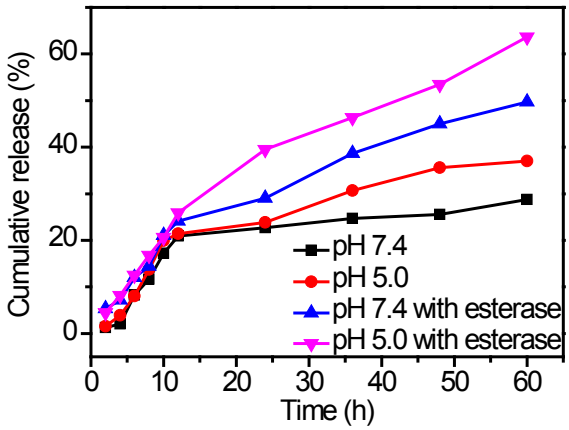

Figure S17. *In vitro* CPT release kinetics from Gd(DTPA-CPT) nanoparticles at 37 °C in different environments (phosphate buffer (pH 7.4), acetate buffer (pH 5.0), phosphate buffer (pH 7.4) containing 17 U mL^{-1} esterase and acetate buffer (pH 5.0) containing 17 U mL^{-1} esterase).

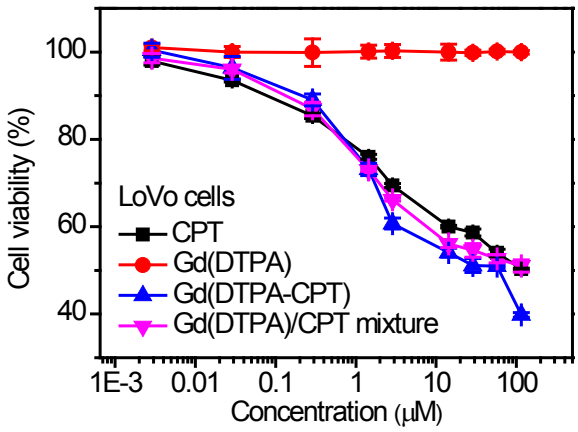

Figure S18. Cell viability assay of CPT, Gd(DTPA), Gd(DTPA-CPT) nanoparticles and Gd(DTPA)/CPT mixture to LoVo cell line determined by MTT assay after 24 h incubation, respectively.

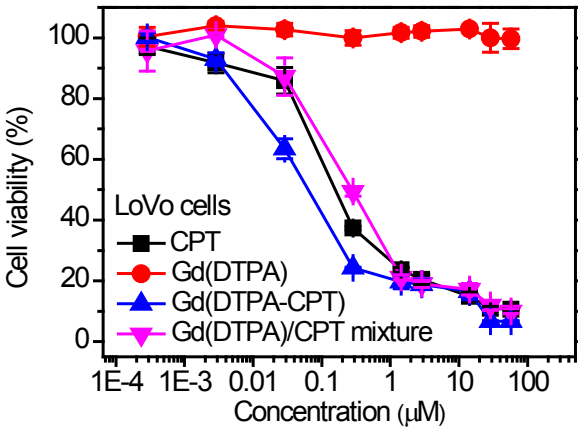

Figure S19. Cell viability assay of CPT, Gd(DTPA), Gd(DTPA-CPT) nanoparticles and Gd(DTPA)/CPT mixture to LoVo cell line determined by MTT assay after 72 h incubation, respectively.

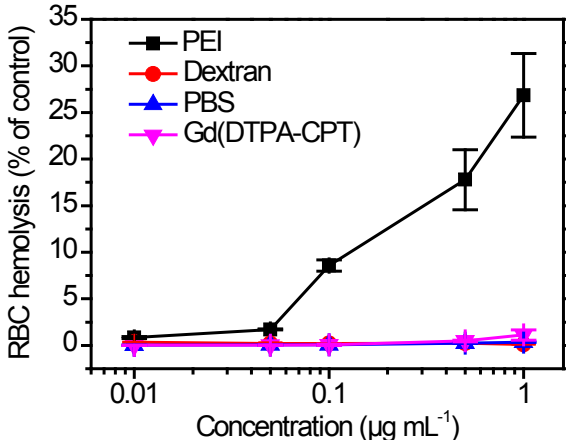

Figure S20. Hemolysis assay of Gd-DTPA-CPT compared with PEI, dextran and PBS with different concentrations (Triton X-100 was used as a 100% hemolysis value). Data are presented as the average ± standard deviation (n = 3).

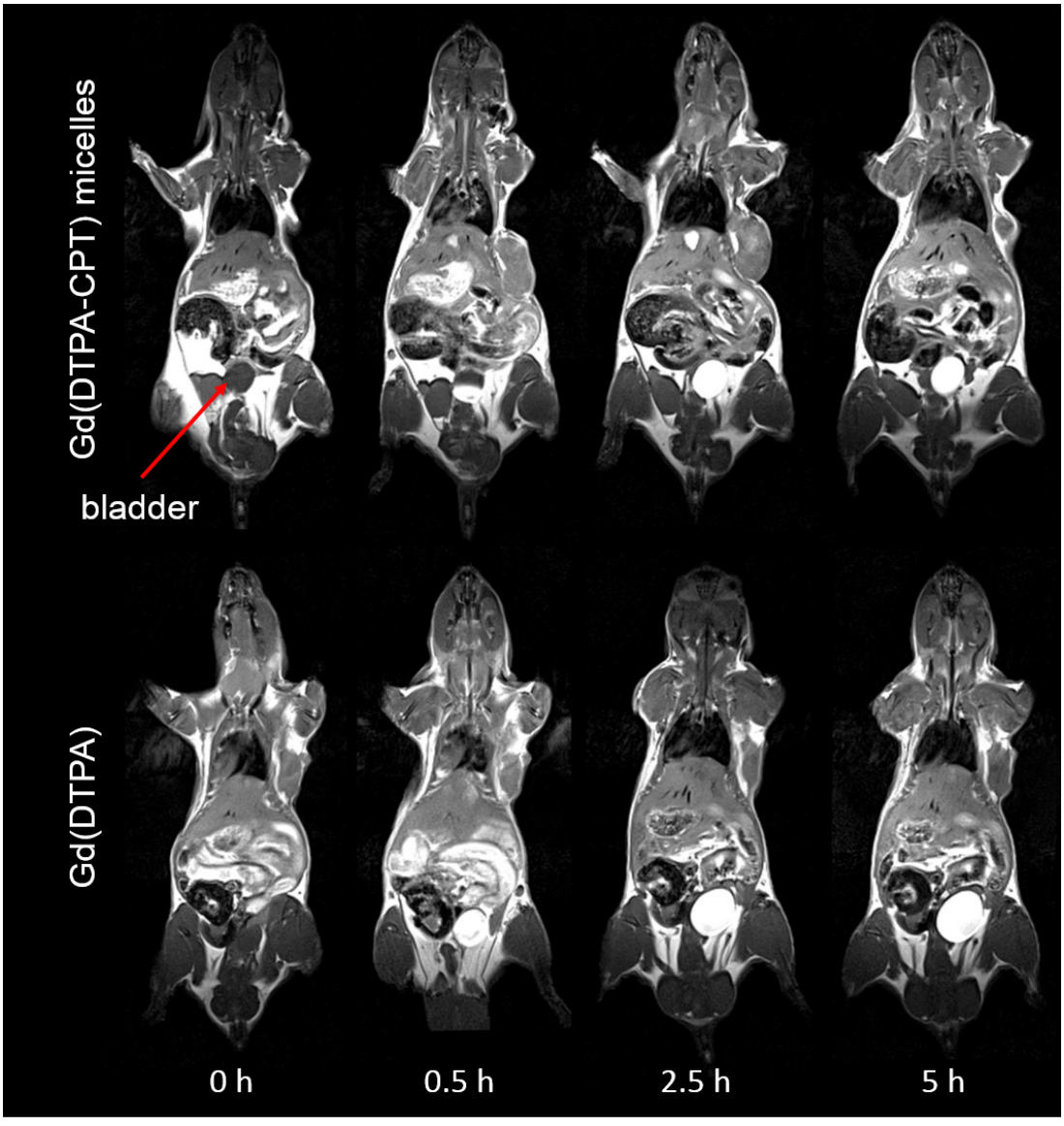

Figure S21. *In vivo* T_1-weighted MR images of nude mice bearing LoVo tumors at pre-injection and 0.5, 2.5, 5 h after intravenous injection of Gd(DTPA-CPT) nanoparticles and Gd(DTPA) at a dose of 0.10 mmol kg^{-1}. The bladder is represented in the coronal T_1-weighted MR images of nude mice.

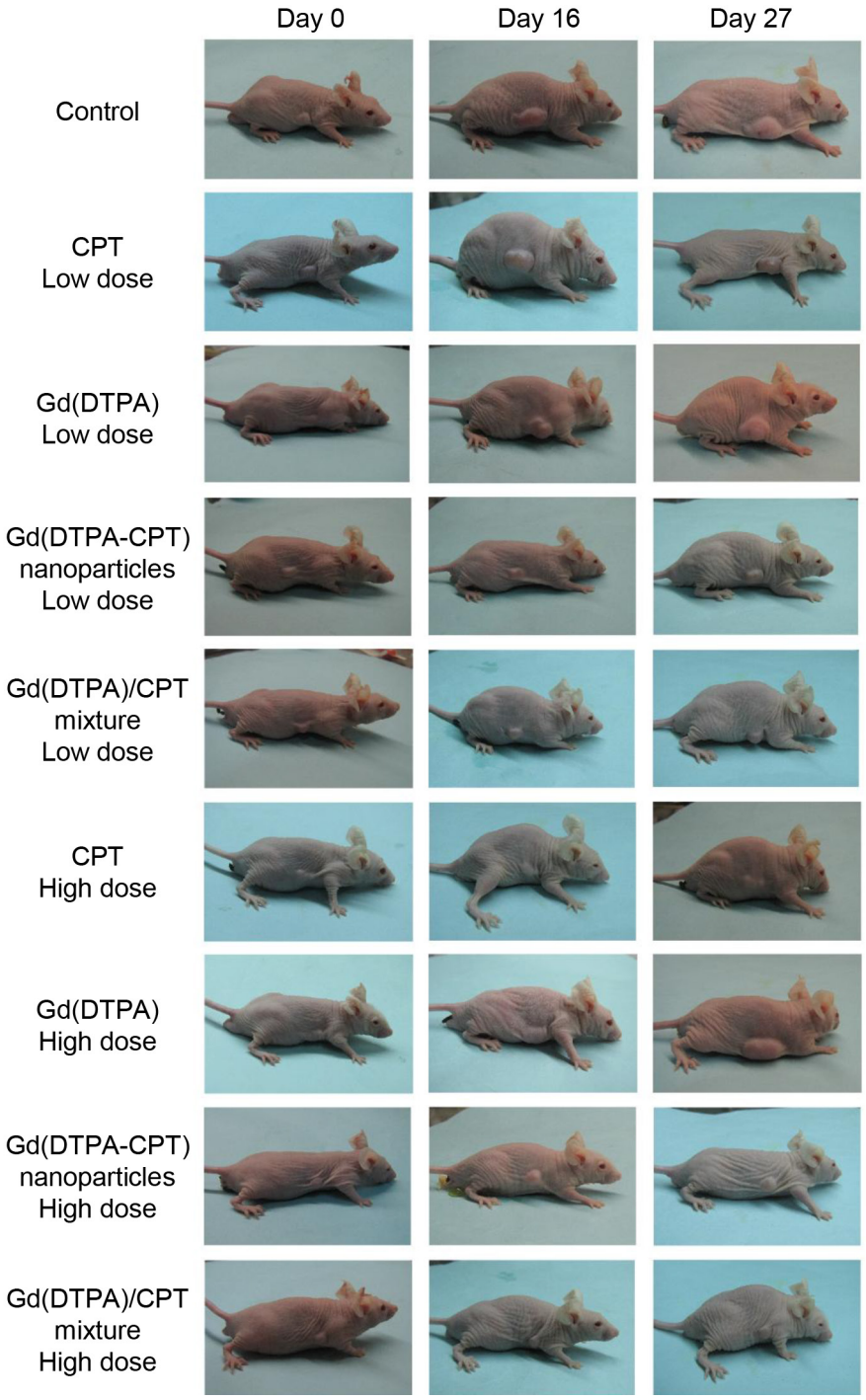

Figure S22. The mice are treated with different formulations and the tumor size is real-time monitored during the 27-day evaluation period.

Table S1. Summary of simulations conducted.

Systems	$N_{molecules}$	N_{water}	L(nm)	Time(ns)
Gd(DTPA-CPT)	25	7614	10	100
	50	29551	15	100
PEG-*b*-PCL	25	225126	30	100
	50	343837	35	100

Table S2. The IC_{50} values of free CPT, Gd(DTPA-CPT) nanoparticles and Gd(DTPA)/CPT mixture to Lovo cell line after 24 h, 48 h and 72 h incubation.

Incubation time	CPT	Gd(DTPA-CPT)	Gd(DTPA)/CPT mixture
24 h	117.37 μM	60.84 μM	116.49 μM
48 h	0.26 μM	1.71 μM	0.29 μM
72 h	0.16 μM	0.062 μM	0.28 μM

Table S3. The IC_{50} values of free CPT, Gd(DTPA-CPT) nanoparticles and Gd(DTPA)/CPT mixture to HepG2 cell line after 48 h incubation.

Incubation time	CPT	Gd(DTPA-CPT)	Gd(DTPA)/CPT mixture
48 h	0.99 μM	0.53 μM	0.71 μM

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