

Electronic Supplementary Information

A Multiple Gadolinium Complexes Decorated Fullerene as a Highly Sensitive T_1 Contrast Agent

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Experimental details

Materials

All chemicals, unless otherwise stated, were purchased from commercial sources and used without further purification. 1-(5-Amino-3-aza-2-oxpentyl)-4,7,10-tris-(tert-butoxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane (DO3A-t-Bu-NH₂, >94%) was purchased from Macrocyclics (Dallas, TX) and used as received. GdCl₃·6H₂O, EDC and NHS (EDC, 1-Ethyl-3-(3-dimethylaminopropyl) Carbodiimide Hydrochloride; NHS, N-Hydroxysulfosuccinimide) were purchased from Alfa Aesar. C₆₀ (purity 99.98%) was obtained from YongXin Fullerenetechnology CO. Ltd., Puyang; Other supplements/chemicals were purchased from J&K Scientific Ltd., Beijing or Sinopharm Chemical Reagent Co. Ltd. Membranes for dialysis (MWCO 1000 Da, 2000 Da, 3000Da) were purchased from Songon Biotech (Spectra/Pro 6, regenerated cellulose dialysis membrane).

Characterization

The molecular weight of complexes that we synthesized were measured by atmospheric pressure chemical ionization mass spectrometry (APCI-MS, Bruker Daltonics Esquire) and matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS, Micro Flex) with 2, 5-Dihydroxybenzoic acid (DHA) as the matrix. ¹H NMR spectra was recorded on a Bruker plus 400. The element analysis of Gd in the sample was measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES) or inductively coupled plasma mass spectrometry (ICP-MS). The dynamic light scattering (DLS) measurements were performed on Malvern Zetasizer nano ZS instrument. The T₁ relaxation time testing was performed on 0.5 T NMI20-Analyst NMR Analyzing & imaging system (Niumag Corporation, Shanghai, China) and 1.5 T imaging system (Siemens Sonata). *In vivo* MR imaging was carried out on a 3 T imaging system (Philips Medical System, ACHIEVA 3.0 T). Gel-filtration chromatography (GFC) analysis was performed on a Superdex-75 10/300 GL column (GE Healthcare Life Sciences) on HPLC system (UltiMate 3000, DIONEX).

Synthesis of C₆₀[C (COOC₂H₅)₂]₅

In a typical synthesis of C₆₀[C (COOCH₂CH₃)₂]₅ (*J. Am. Chem. Soc.*, 2003, **125**, 5471-5478), a suspension of C₆₀ (300 mg, 0.417 mmol) and NaH (210 mg, 5.25 mmol) was prepared in THF (20 mL) with vigorous stirring (15min). With continued stirring, diethyl bromomalonate (1.255g, 5.25mmol) in THF (1mL) is added dropwise to the mixture. Vigorous bubbling is immediately observed (evolution of H₂ (g)), and a dark-brown solution color develops within minutes. The mixture was stirring (30min) after which the dark-brown soluble derivative was separated from excess alkali hydride and small amounts of unreacted fullerene material by filtration (0.45/μm PTFE filter). The product was isolated by THF removal under reduced pressure, rinsed with hexanes, and dried under reduced pressure.

Conversion of C₆₀[C(COOC₂H₅)₂]₅ to C₆₀[C(COOH)₂]₅

Conversion of C₆₀[C(COOCH₂CH₃)₂]₅ to the water-soluble carboxylate salt C₆₀[C(COOH)₂]₅ was accomplished by reflux in toluene with NaH followed by a methanol quench, according to the method reported by Lamparth and Hirsch for the conversion of C_{2n}[C(COOCH₂CH₃)₂]_x to C_{2n}[C(COOH)₂]_x. The aqueous-soluble product was converted to the free acid by passage over an acid-form ion-exchange chromatography column. Next, the solution pH was adjusted to 7.0 with NaOH, and the product was dried under reduced pressure at room temperature.

The modification of C₆₀[C(COOH)₂]₅ with 2-Aminoethy-DOTA-tris (t-Bu ester) (DO3A-t-Bu-NH₂)

A certain amount of EDC/NHS were added in the solution of C₆₀[C(COOH)₂]₅ (40mg, 0.03mmol) in 10 mL DMF at 0 °C (mol_{C60}:mol_{EDC}:mol_{NHS} =1: 1.2: 1.2). The solution was stirred for 1 h. DO3A-t-Bu-NH₂ (200mg, 0.3mmol) and triethylamine (50μL, 0.35mmol) was added, and the mixture was stirred at room temperature for 6~10h. The solution was evaporated, and purified by silica column chromatography[V_{CH₂Cl₂}:V_{CH₃OH} =1: 10].

The synthesis of C₆₀-(Gd-DOTA)_n (CGDn)

The product of C₆₀-(DOTA-t-BU)_n was dissolved in 0.5 mL CH₂Cl₂, and 3 mL trifluoroacetic acid was added. The reaction was carried out overnight at room temperature. MALDI-TOF-MS and ¹H NMR were used to observe the remove of t-butyl protecting groups; once complete, unreacted TFA was removed by evaporation. The resulting brown oil was redissolved in water 3 x 10 mL and evaporated to remove most of TFA. The resulting brown glassy solid was dissolved in 10 mL DI H₂O and GdCl₃·6H₂O (194.7mg, 0.3mmol) was added. The pH was adjusted to ~6 with 1M NaOH and the reaction was stirred overnight at 45 °C. The solution was dialyzed exhaustively with water using dialysis bag (MWCO 2000Da) and further purified by size-exclusion chromatography on Sephadex LH-20 column (mobile phase H₂O).

Gel Filtration Chromatography (GFC)

We performed the GFC analysis on a Superdex-75 10/300 GL column (GE Healthcare Life Sciences) by high-performance liquid chromatography (UltiMate 3000, Dionex) monitored at the absorbance of 256 nm, using PBS buffer (1x, pH 7.4) as the mobile phase. The flow rate was 0.5 mL/min. The calibration of hydrodynamic diameter was performed by injecting 100 μL of protein standards containing blue dextran (2000 kDa, 29.5 nm HD), thyroglobulin (669 kDa, 18.0 nm HD), alcohol dehydrogenase (150kDa, 10.1 nm), ovalbumin (44 kDa, 6.1 nm HD), and vitamin B12 (1.35 kDa, 1.5 nm), denoted as M1-M5 sequentially. All standards and samples were tested in triplicate with high reliability and repeatability. Because the peak of CGDn is close to M3 (alcohol dehydrogenase, 10.1 nm), the size of CGDn is approximately 10 nm.

Cytotoxicity assay

The cytotoxicity of the CGDn was tested by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. HeLa cells were firstly seeded into a 96-well plate with a density of 1×10^4 cells/well in DMEM, and incubated in the atmosphere of 5% CO₂ at 37 °C for 24 h. The cells were then incubated with CGDn at various Gd concentration (0, 0.41, 0.81, 1.63, 3.25, 6.5, 13, 26, 52, and 104 µg/mL) for another 24 h. Subsequently, the culture medium was removed, each well was added with 100 µL new culture medium containing MTT (0.5 mg/mL) and the plate was incubated for 4 h at 37 °C. Then the medium was discarded and each well was added 200µL DMSO. The OD₄₉₀ value (Abs.) of each well was measured by MultiSkan FC microplate reader immediately. Cell viability was calculated from OD₄₉₀ value of experimental group by subtracting that of blank group.

***T*₁ relaxivity and MRI phantom studies**

The samples for *T*₁ relaxivity measurement were prepared separately. CGDn and Gd-DOTA were prepared with concentrations of 0.2, 0.1, 0.05, 0.025, 0.0125 mM of Gd³⁺ ions in 1% agar-containing solution. The control sample denoted as 0 mM was prepared with purified water containing 1% agar. The longitudinal relaxation times were measured at 0.5 T NMR120-Analyst NMR system (Niumag Corporation, Shanghai, China) and 1.5 T on MR scanners (Siemens Sonata) at room temperature, and used for calculating the relaxation rate of the samples. The *T*₁-weighted MR images were acquired at 1.5 T on MR scanners (Siemens Sonata) with a conventional spin echo acquisition (TR = 70 ms, TE = 5.3 ms, field of view (FOV) = 298 mm × 114 mm, slice thickness = 3 mm). Relaxivity values of *r*₁ were calculated through the curve fitting of 1/relaxation time (s⁻¹) versus the Gd³⁺ ions concentration (mM).

***In vitro* MRI study**

HepG2 cells (10⁶ per plate) were seeded on 75 cm² tissue culture flask 24 h before labeling. Subsequently, CGDn and Gd-DOTA with same Gd(III) concentration (320 nmol/mL) were added to each flask respectively, and cultured in a humidified atmosphere containing 5% CO₂ for 4 h at 37 °C. At the end of the incubation period, the cells were washed three times with PBS, and harvested inside microcentrifuge tube (200 µL capacity). MRI study of these cells was performed at 0.5 T NMR120-Analyst NMR system (Niumag Corporation, Shanghai, China). Spin echo *T*₁-weighted imaging was performed with the following parameters: TR = 100 ms, TE = 5.3 ms

***In vivo* MRI studies**

All studies involving animals were approved by the Animal Care and Use Committee of the Institute. MR imaging was carried out on a 3 T imaging system (Philips Medical System, ACHIEVA 3.0 T) by using a rat coil (Philips) for transmission and

reception of the signal. Sprague-Dawley (SD) Rats (180 – 220 g) were anaesthetized by pentobarbital sodium at the dose of 40 mg/kg body weight. Dynamic T_1 -weighted images of upper part of rat body were obtained before and after intravenous injection (via tail vein) of a total 1.0 mL solution of the complex with the dosages of 0.03 mmol Gd/kg body weight. The MRI signal intensity enhancements were monitored before and after injection of MRI agents up to 12 min, with images taken every 1~6 min. T_1 -weighted imaging were obtained with 3DCEMRA sequence with parameters as follows: T_1 -weighted fast field echo (T1FFE), TR = 7 ms, TE = 3 ms, field of view (FOV) = 100 mm \times 100 mm, slices = 60, slice thickness = 1 mm, Flip angle = 30°. To quantify the contrast enhancement, the signal-to-noise ratio (SNR) was measured by finely analyzing regions of interest (ROIs) of the longitudinal images, and the contrast enhancement was defined as the increase of SNR after the injection.

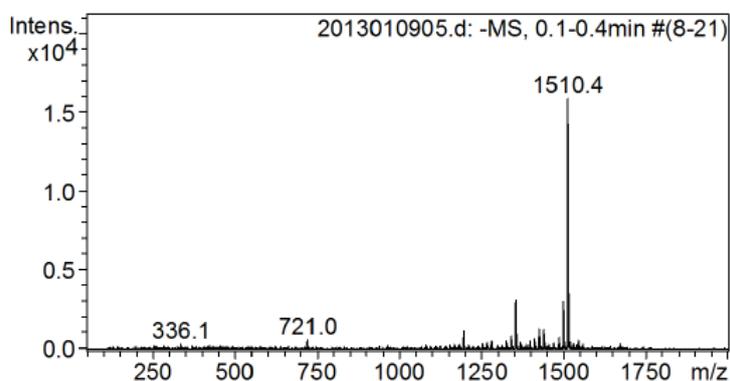


Fig. S1 MS of $C_{60}[C(COOCH_2CH_3)]_5$.

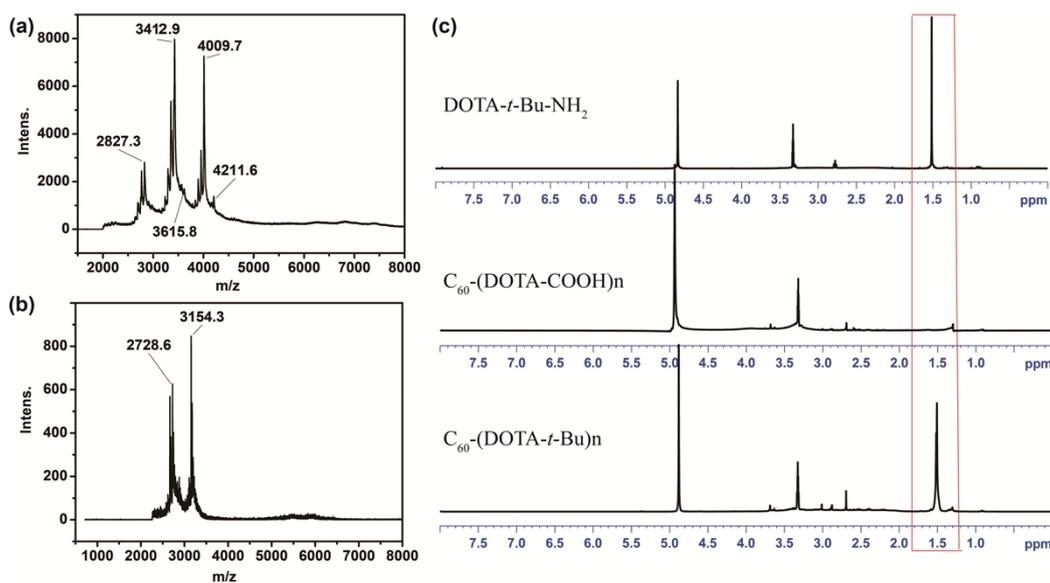


Fig. S2 (a) MALDI-TOF-MS of C_{60} -(DOTA-*t*-Bu)_n. The mass of C_{60} -(DOTA-*t*-Bu)₅ and C_{60} -(DOTA-*t*-Bu)₄ are 4211.6 and 3615.8 Da $[M+H]^+$, which correspond with the theoretical values. 4009.7 and 3412.9 Da would be caused by aborting two tert-butoxyls and CO_2 , leading to high degree of ionization. The little peak of 2827.3 Da is the mass peak of C_{60} -(DOTA-*t*-Bu)₃ aborting several tert-butoxyls.

$$C_{60}\text{-(DOTA-}t\text{-Bu)}_5: 614 \cdot 5 - 18 \cdot 5 + 1230 = 4210$$

$$C_{60}\text{-(DOTA-}t\text{-Bu)}_4: 614 \cdot 4 - 18 \cdot 4 + 1230 = 3614$$

(b) MALDI-TOF mass spectrum of C_{60} -(DOTA-*t*-Bu)_n deprotection.

The mass of C_{60} -(DOTA-COOH)₅ and C_{60} -(DOTA-COOH)₄ was roughly calculated to be 3153.1 and 2727.6 Da, observed: 3154.3 $[M+H]^+$ and 2728.6 Da $[M+H]^+$. The broad peak in the picture was dimerization.

$$C_{60}\text{-(DOTA-COOH)}_5: 4009.6 - 57.1 \cdot 3 \cdot 5 = 3153.1;$$

$$C_{60}\text{-(DOTA-COOH)}_4: 3412.8 - 57.1 \cdot 3 \cdot 4 = 2727.6$$

(c) 1H NMR of DOTA-*t*-Bu-NH₂, C_{60} -(DOTA-COOH)_n, and C_{60} -(DOTA-*t*-Bu)_n. A characteristic singlet of the *t*-butyl protecting groups at δ 1.48 ppm was present on DOTA-*t*-Bu-NH₂ and C_{60} -(DOTA-*t*-Bu)_n, but disappeared on the C_{60} -(DOTA-COOH)_n.

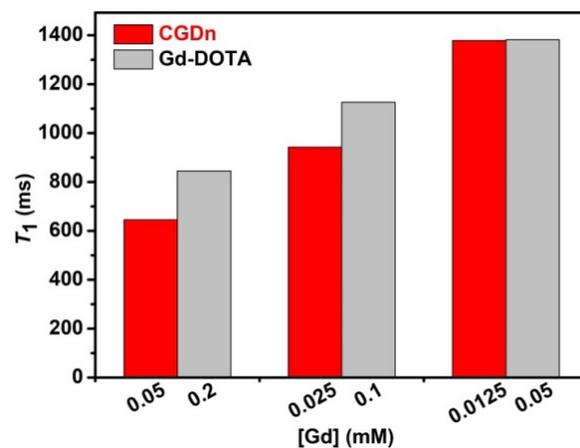


Fig. S3 The proton T_1 relaxation times in CGDn (red) and corresponding Gd-DOTA samples (grey, with four times higher concentration of Gd^{3+} than that in CGDn).

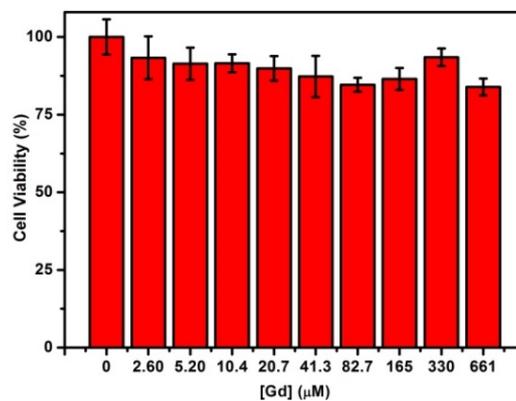


Fig. S4 Viability of HeLa cells after 24 h incubated with CGDn ($n = 5$). The MTT result indicated that CGDn has no obvious cytotoxicity.

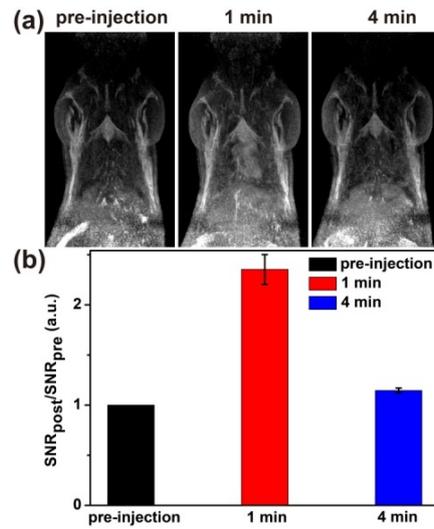


Fig. S5 (a) T_1 -weighted MR images (Philips Medical System, ACHIEVA 3 T, TR= 7 ms, TE = 3 ms) of SD rats at pre-injection, 1 min, 4 min after injection of Gd-DOTA ($0.03 \text{ Gd mmol kg}^{-1}$ body weight). (b) Quantification of signal changes (SNR ratio) in heart at different time points after administration ($n = 3$).

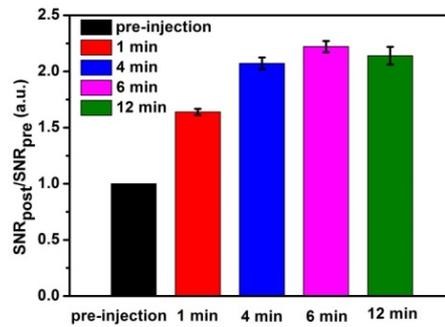


Fig. S6 Quantification of signal changes (SNR ratio) in liver at different time points after administration of CGDn ($n = 3$).