

Supporting Information

Investigation of Cyano-Bridged Coordination Nanoparticles $Gd^{3+}/[Fe(CN)_6]^{3-}/D$ -mannitol as a T_1 -weighted MRI Contrast Agent.

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Experimental Section

Syntheses.

Unless otherwise noted, all manipulations were performed at ambient temperature using reagents and solvents as received. Sodium ferricyanide $Na_3[Fe(CN)_6]$ has been obtained by cation exchange procedure from $K_3[Fe(CN)_6]$ (Acros Organics, 99%) using an acidic DOWEX resin.

Synthesis of $Gd^{3+}/[Fe(CN)_6]^{3-}@D$ -Mannitol: An aqueous solution of $Na_3[Fe(CN)_6]$ (0.2 mmol, 5 ml) containing 1 mmol of D-Mannitol was mixed with a $Gd(NO_3)_3 \cdot 6H_2O$ solution containing the same amount of stabilizer. The mixture is stirred for 2 hours before being centrifugated 10 min at 20000 rpm to remove any precipitate that may form. The supernatant is then filtered on a 0.45 μm filter and the obtained nanoparticles were precipitated with acetone and washed with ethanol. The aqueous solutions for relaxometry measurements have been prepared from precipitated nanoparticles with addition of few drops of the D-Mannitol.

$Gd^{3+}/[Fe(CN)_6]^{3-}@D$ -Mannitol: $\nu_{-OH(H_2O \text{ coordinated})} = 3613 \text{ cm}^{-1}$, $\nu_{-OH(H_2O \text{ non-coordinated})} = 3421 \text{ cm}^{-1}$ and 3260 cm^{-1} , $\nu_{C-H} = 2972 \text{ cm}^{-1}$, 2930 cm^{-1} , $\nu_{C=N} = 2150 \text{ cm}^{-1}$, 2139 cm^{-1} , 2108 cm^{-1} , 2065 cm^{-1} , $\delta_{H-O-H \text{ coordinated}} = 1680 \text{ cm}^{-1}$, 1635 cm^{-1} , 1607 cm^{-1} . El. anal., wt %: Gd, 29.35; Fe, 10.30; N, 15.80; C, 22.13; H, 2.83; O, 19.59. Determined from elemental analysis formula: $Gd(H_2O)_4[Fe(CN)_6]@(D\text{-Mannitol})_{0.38}$.

Physical Characterization.

Infrared spectra were recorded as KBr disks on a Perkin Elmer 1600 spectrometer with a 4 cm^{-1} resolution. Elemental analyses were performed by the Service Central d'Analyses (CNRS, Vernaison, France). The samples were heated at 3000 °C under He. Oxygen was transformed in CO and detected by using an IR detector. Metals were determined with a High resolution ICP-MS using a ThermoFischer element. Transmission Electron Microscopy (TEM) observations were carried out at 100 kV (JEOL 1200 EXII). Samples for TEM measurements were deposited from solutions on copper grids. The NPs size distribution histograms were determined using enlarged TEM micrographs taken at magnification of 100K on a statistical sample of *ca.* 300 NPs. The 1H NMR relaxometry characterizations have been performed by using aqueous solutions of the nanoparticles prepared from powder samples by their redispersion in water with few drops of D-mannitol. The longitudinal (T_1) and transversal (T_2) relaxation times measurements for different concentrations of CAs were performed on a Tecmag Apollo spectrometer operating at 200 MHz. An inversion recovery sequence ($180^\circ - \tau - 90^\circ$) was used to measure the longitudinal relaxation time T_1 . τ was varied from a small value to a large value. For each concentration of the CA, the table of delay (τ) was chosen such that the instantaneous magnetization $M_z(t)$ recovers its equilibrium value M_0 . Thereby each T_1 value was obtained by curve fitting using the relation: $M_z(t) = M_0 \times [1 + (\cos\theta - 1) \times \exp(-t/T_1)]$ (1), where $M_z(t)$ represent the longitudinal magnetization at time t following the inversion pulse $\theta = \pi$. The Carr-Purcell Meiboom Gill pulse sequence ($90^\circ - (180^\circ)_n -$) was applied to achieve the transverse relaxation time, T_2 , measurement. These pulses are equidistantly spaced by an echo time t_e . For each concentration of CA, the number of echos (n) is selected to allow a complete decay of the transverse magnetization. The T_2 value was obtained by fitting the following relation: $M_{x,y}(nt_e) = M_0 \times \exp(-nt_e/T_2)$ (2), where $M_{x,y}(nt_e)$ is instantaneous transverse magnetization. The r_1 and r_2 relaxivities were then calculated from the slope of a linear regression of $R_{1,2} = T^{-1}_{1,2}$

against the concentration of the CA (C), following : $R_{1,2} = R_{1,2}^0 + r_{1,2} \times C$ (3), where $R_{1,2}^0$ are the relaxation rates without CAs.

***In vivo* Magnetic Resonance Image, Processing and Analysis.**

In vivo MRI experiments were performed on mice (6 months, male, 20 g) anesthetized with a gas mixture of isoflurane (1.4%) and medical oxygen (2%). 130 μL of a NaCl solution containing $\text{Gd}^{3+}/[\text{Fe}(\text{CN})_6]^{3-}$ @D-mannitol nanoparticles at a concentration of $2.4 \cdot 10^{-2} \text{ mol.L}^{-1}$ were injected intravenously as a bolus in the tail vein of anesthetised healthy mice (6 months, male) to obtain *in vivo* MRI images.

MRI images were acquired using a 9.4 T horizontal bore Agilent imaging system with a 72-mm-diameter birdcage volume coil. All in-vivo MRI results were obtained after processing the raw “.fid” data that were imported from Agilent’s VnmrJ3.2 software.

Experimental data were classified into two groups: data collected using coronal selection plans and data collected from axial plans. Coronal images were collected using a gems (Gradient Echo Multi Slice) sequence with the following parameters: TR 980ms; 4ms TE; 25° flip angle, FOV 100x60mm², data matrix 256x256, averaging 1, 16 slices with a thickness of 1.5 mm each, no gap was used. Respiration gating was also used. An Inversion Recovery sequence with a 750 ms Inversion Time was used before signal detection for all experiments except anatomic ones. Axial images were collected using a gems (Gradient Echo Multi Slice) sequence with the following parameters: TR 1300ms; 4ms TE; 25° flip angle, FOV 60x60mm², data matrix 256x256, averaging 1, 64 slices with a thickness of 1.5 mm each, no gap was used. Respiration gating was used. An Inversion Recovery sequence with a 750 ms Inversion Time was used before signal detection for all experiments except anatomic ones. Axial anatomy images were collected using gems sequence with a TR of 540 ms.

Toxicity investigations.

Cytotoxicity threshold: The cytotoxicity of $\text{Gd}^{3+}/[\text{Fe}(\text{CN})_6]^{3-}$ @D-mannitol nanoparticles was first evaluated *in vitro*. For this, human colorectal carcinoma cell line (HCT-116) and normal human fibroblasts were purchased from ATCC (American Type Culture Collection, Manassas, VA). HCT-116 cells were cultured in McCoy's 5A-Glut culture medium and fibroblasts in DMEM-Glutamax culture medium, both supplemented with 10% fetal bovine serum and gentamycin 50 $\mu\text{g.mL}^{-1}$. These cells were allowed to grow in humidified atmosphere at 37°C under 5% CO₂. For cytotoxicity assay, HCT-116 cells and fibroblasts were seeded into a 96-well plate, 5,000 cells per well in 150 μL culture medium, and allowed to grow for 24 h. Cells were incubated for 72 h with or without nanoparticles in the concentration range from 0.1 to 50 $\mu\text{g.mL}^{-1}$. Then, a MTT assay was performed to evaluate their toxicity. Cells were incubated for 4 h with 0.5 mg.mL^{-1} of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Promega) in media. The MTT/media solution was then removed and the precipitated crystals were dissolved in ethanol / DMSO (1:1). The solution absorbance was read at 540 nm. Figure S7 shows that there is no obvious decrease in cell viability for HCT-116 cells and cytotoxicity appears in fibroblasts incubated with nanoparticles 20 $\mu\text{g.mL}^{-1}$.

Genotoxicity assay:

In vitro safety evaluation of $\text{Gd}^{3+}/[\text{Fe}(\text{CN})_6]^{3-}$ @D-mannitol nanoparticles was carried out on 3T3 cells. The cytotoxicity analyzed by MTT let us determine the concentration that inhibits 50% of the grown of 3T3 cell culture. The IC₅₀ obtained was 190 $\mu\text{g.mL}^{-1}$. Then, to evaluate the degree of DNA fragmentation in treated cells, a genotoxicity assay was performed by Comet assay. For this, cells were exposed to concentrations of nanoparticles below its IC₅₀, because higher concentrations might alter the integrity of cells. As shown in table 1, non genotoxic damage has been observed in these conditions. The obtained data suggest that nanoparticles have no carcinogenic effect compared to the control group.

Culture cell:

In order to determine the relative toxicity of $\text{Gd}^{3+}/[\text{Fe}(\text{CN})_6]^{3-}$ @D-mannitol nanoparticles, 3T3 cells were used to carry out genotoxicity assay. Cell culture was performed in DMEM media supplemented with 10% FBS, 4 mM Glutamine, 0.5% penicillin/streptomycin (from a stock of 10000 IU/10 mg). Cells were maintained at 37°C in an incubator with 5% CO₂ and humid atmosphere.

Cytotoxicity and Comet assay:

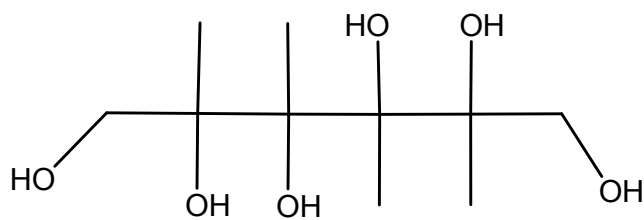
First, cells were seeded at high density on 96 well plates and exposed for 48 h to $Gd^{3+}/[Fe(CN)_6]^{3-}@D$ -mannitol nanoparticles, then MTT and Comet assay were assessed. MTT assay was carried out in order to ensure the survival of the used concentrations.

Genotoxicity studies was conducted as previously described (REF). A preliminary cytotoxicity assay was performed to select a range of no lethal doses suitable for genotoxicity assay. Cells were incubated 48 h with increasing doses of $Gd^{3+}/[Fe(CN)_6]^{3-}@D$ -mannitol nanoparticles (from 63 to 400 $\mu g \cdot mL^{-1}$), and then submitted to a MTT assay.

The Comet assay or Single Cell Gel Electrophoresis (SCGE) is a standard genotoxicity test, to evaluate DNA damages. Sterile water was used as negative control with a 5.5% rate of the total culture volume; 400 mM Methyl Methanesulfonate (MMS) was used as positive control in the comet assay and 0.02% Sodium Dodecyl Sulfate (SDS) was the positive control for the cytotoxicity assay. Cells were exposed to nanoparticles for 48 h, trypsinized from the plate and collected into microtubes. Collected cells were mixed with a suspension of 0.9% low melting point agarose and the mixture was placed on slides previously covered with a first layer of 1% agarose. Once the agarose had solidified, the slides were submerged in lysis liquid for 1 h to release DNA. Later they remained in electrophoresis buffer for 40 min at 4°C to allow DNA unwinding. Electrophoresis was carried out at 25 V and 300 mA for 30 min. then, 3 washes were performed in Tris buffer and slides were dried and kept protected from light until analysis. The slides were stained with DAPI (4,6-Diamidin-2-phenylindol at a concentration of 5 $\mu g/mL$) and they were analyzed by Comet Assay IV software.

In vivo toxicity studies:

Six 8-week-old male C57BL/6J mice were purchased from Harlan (France), and used after acclimatization for a week. During acclimatization and experiment periods, animals were kept under free access to food pellets and tap water. Animals were housed at 22°C in a 12 h light / 12 h dark cycle. Clearances of nanoparticles was evaluated over a period of 16 days after intravenous injection of 200 μL of a solution of physiological serum alone (Control) or added of the $Gd^{3+}/[Fe(CN)_6]^{3-}@D$ -mannitol nanoparticles at a single dose of 0.012 mmol of Gd^{3+} per kg. Gd concentration was determined in the urine and stools of groups of 3 mice injected with nanoparticles or physiological serum by ICP-MS. The body weights of the mice in all groups (control n=3 and treated n=3) were recorded and no symptom and mortality were observed (Fig. S9). Blood was collected before and during the treatment period. Serum was separated by centrifugation (10 min, 4000 rpm). The concentrations of biological markers for toxicity evaluation were measured in control and nanoparticle treated mice. Creatinine activity was determined in urine and serum by Jaffé method. Expression of TNF- α and IL-6 was quantified in serum by specific ELISA immunoassays, and expression of ALT was quantified by enzymatic assay.^[18] After 16 days, animals were sacrificed and the tissues and organs such as the heart, liver, spleen and kidneys were excised and weighted. The coefficients of organs to body weight were calculated as the ratio of organs (weight mg) to body weight (g) (Table 2). The presence of histological tissue abnormalities was studied in hematoxylin- and eosin-stained sections from paraffin-embedded tissues of control and treated mice.



Scheme S1. Schematic representation of D-Mannitol used in this work as a stabilizing agent for the $\text{Gd}^{3+}/[\text{Fe}(\text{CN})_6]^{3-}$ nanoparticles.

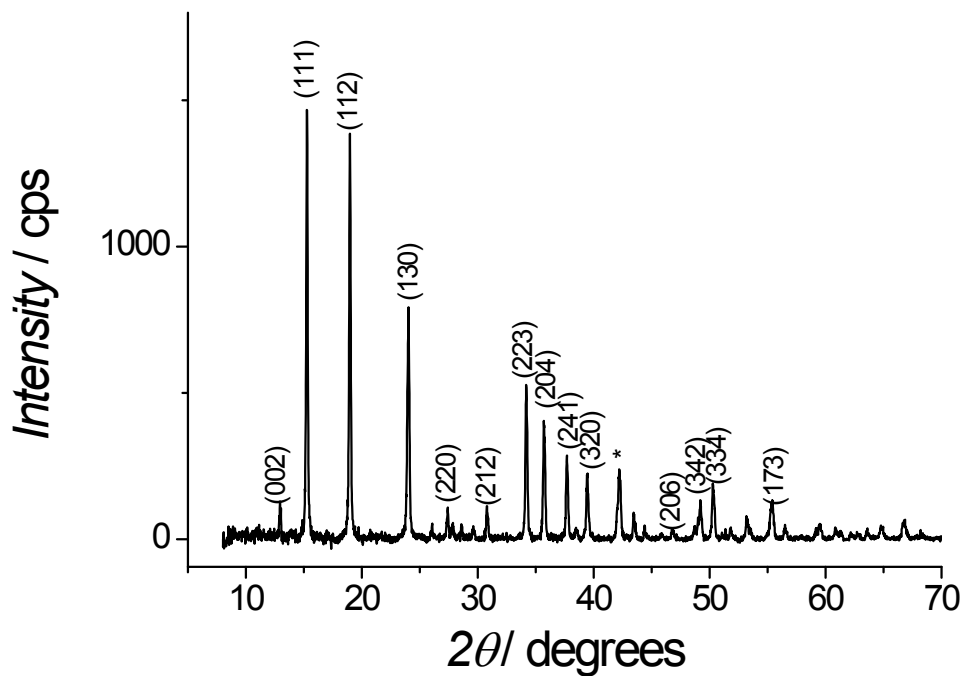


Figure S1. X-Ray diffraction pattern for $\text{Gd}^{3+}/[\text{Fe}(\text{CN})_6]^{3-}@D$ -mannitol nanoparticles.

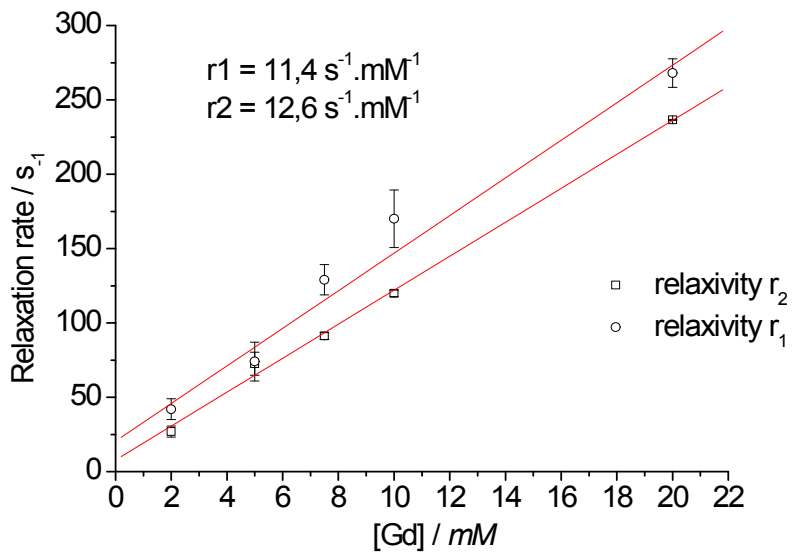


Figure S2. T_1 (\circ) and T_2 (\square) relaxation rate measurements vs concentration of aqueous solutions of the nanoparticles $\text{Gd}^{3+}/[\text{Fe}(\text{CN})_6]^{3-}@D\text{-mannitol}$ performed at an applied magnetic field of 4.7 T.

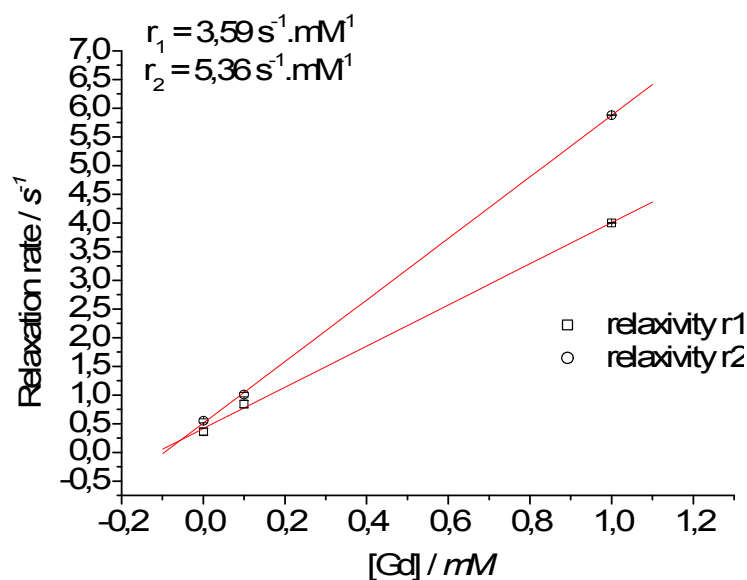


Figure S3. T_1 (\circ) and T_2 (\square) relaxation rate measurements vs concentration of aqueous solutions of the gadoteridol (ProHance[®]) commercial CA performed at an applied magnetic field of 4.7 Tesla.

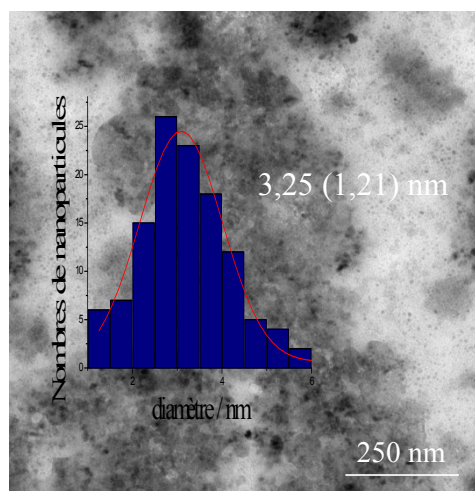


Figure S4. Transmission Electronic Microscopy (TEM) image of $\text{Gd}^{3+}/[\text{Fe}(\text{CN})_6]^{3-}@D\text{-mannitol}$ nanoparticles in 40 % fetal bovine serum (FBS) solution after 1 month. Note that no size and shape evolution has been observed confirming that the nanoparticles aggregation does not occur with time. The relaxivity measurements performed after 1 week also in FBS shows the r_1 and r_2 values of 12.1 ± 0.3 and $12.9 \pm 0.2 \text{ mM}^{-1}\text{s}^{-1}$ indicating that there is no nanoparticles decomposition nor nanoparticles aggregation in time.

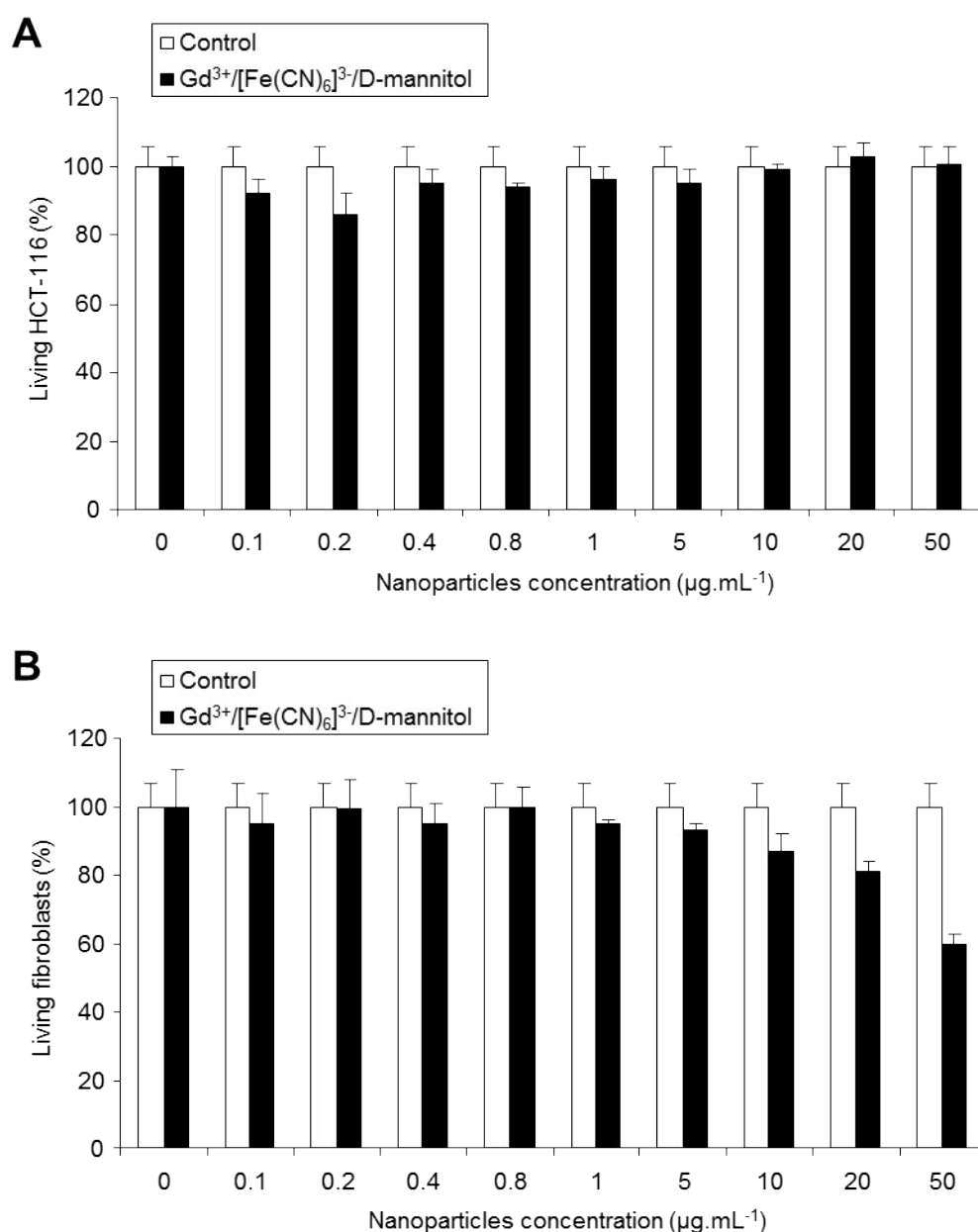
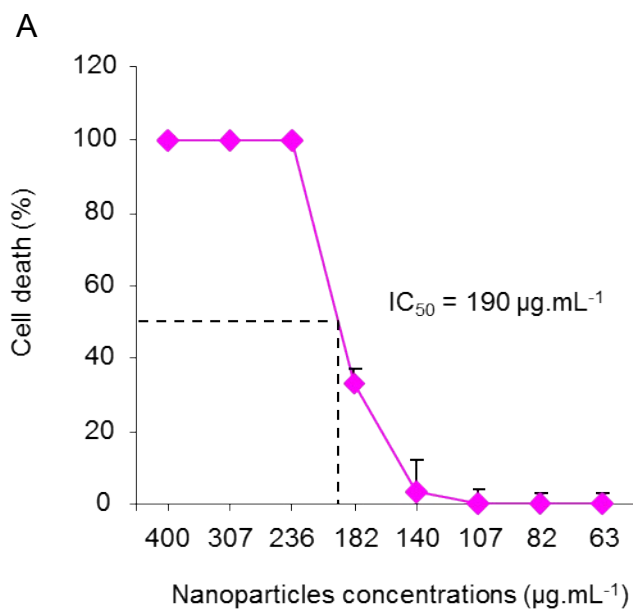


Figure S5. Effect of $\text{Gd}^{3+}/[\text{Fe}(\text{CN})_6]^{3-}/\text{D-mannitol}$ on cell growth showing no evident decrease in cell viability for HCT-116 cells and a start of toxicity in fibroblasts from $20 \mu\text{g.mL}^{-1}$. (A) Human colorectal cancer cells (HCT-116) and (B) human fibroblasts were incubated for 3 days with increasing doses of nanoparticles from 0.1 to $50 \mu\text{g.mL}^{-1}$. Cell proliferation was measured by MTT assay. Values represent the mean \pm standard deviations of triplicates from a typical experiment and were confirmed in two additive experiments. Normal fibroblasts exhibit a slow replication in comparison with HCT-116 cells, which high speed doubling reduces the accumulation of particles by cells.



B

NP (µg.mL ⁻¹)	Mean ± SD
236	2,67 ± 3,48
182	2,63 ± 2,83
140	1,25 ± 1,78
107	1,40 ± 1,85
82	1,0 ± 1,36
63	1,47 ± 2,11
0	1,39
MMS	63,58 ± 20,97

Figure S6. Safety evaluation of Gd³⁺/[Fe(CN)₆]³⁻@D-mannitol nanoparticles. (A) Cell death measurement by MTT assay performed after 48 h incubation with increasing concentrations of nanoparticles. The half maximal inhibitory concentration (IC₅₀) was determined by Probit

function in SPSS ver 18. (B) Results of DNA% in tail obtained for the different concentrations of nanoparticles. Results are mean \pm standard deviation (SD).

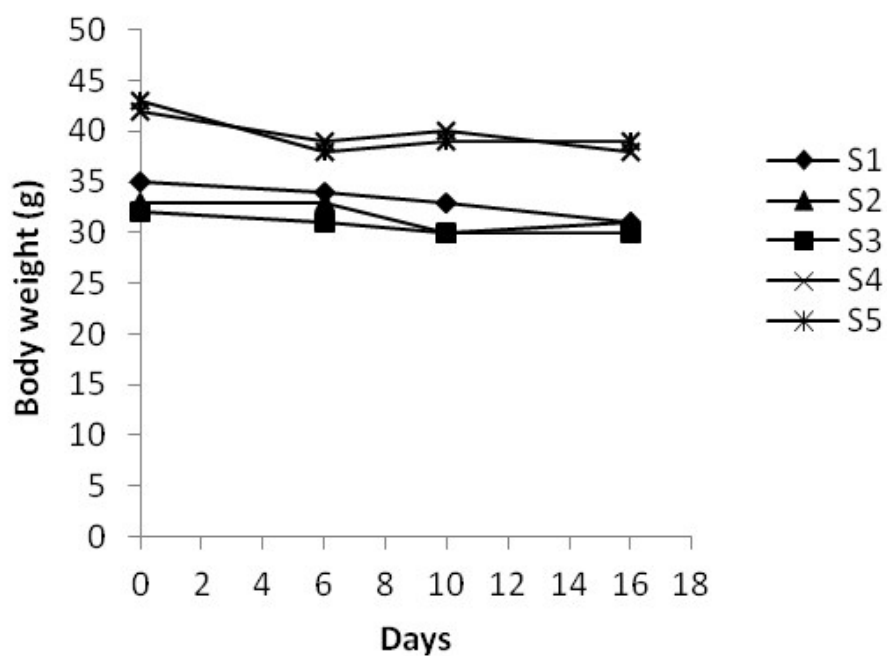


Figure S7. Body weight of control (S1, S2, S3) and $Gd^{3+}/[Fe(CN)_6]^{3-}@D$ -mannitol nanoparticles treated (S4, S5, S6) mice during period exposure.

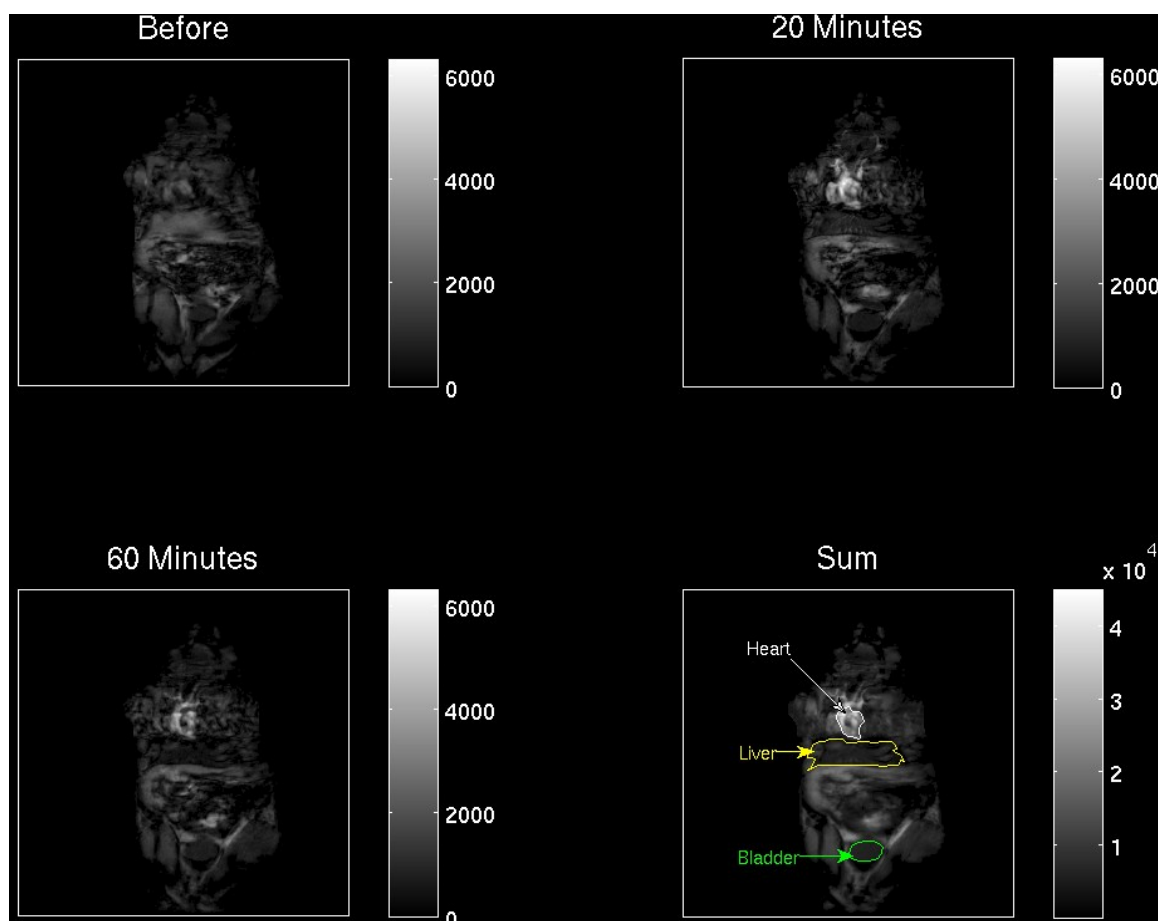


Figure S8. T₁-weighted images of coronal slices of mice acquired before nanoparticles intravenous $\text{Gd}^{3+}/[\text{Fe}(\text{CN})_6]^{3-}/\text{D}$ -mannitol injection (a), 20 min after injection (b), 60 min after injection (c) and the summated images of the organs of interest showing heart, liver and bladder (d).

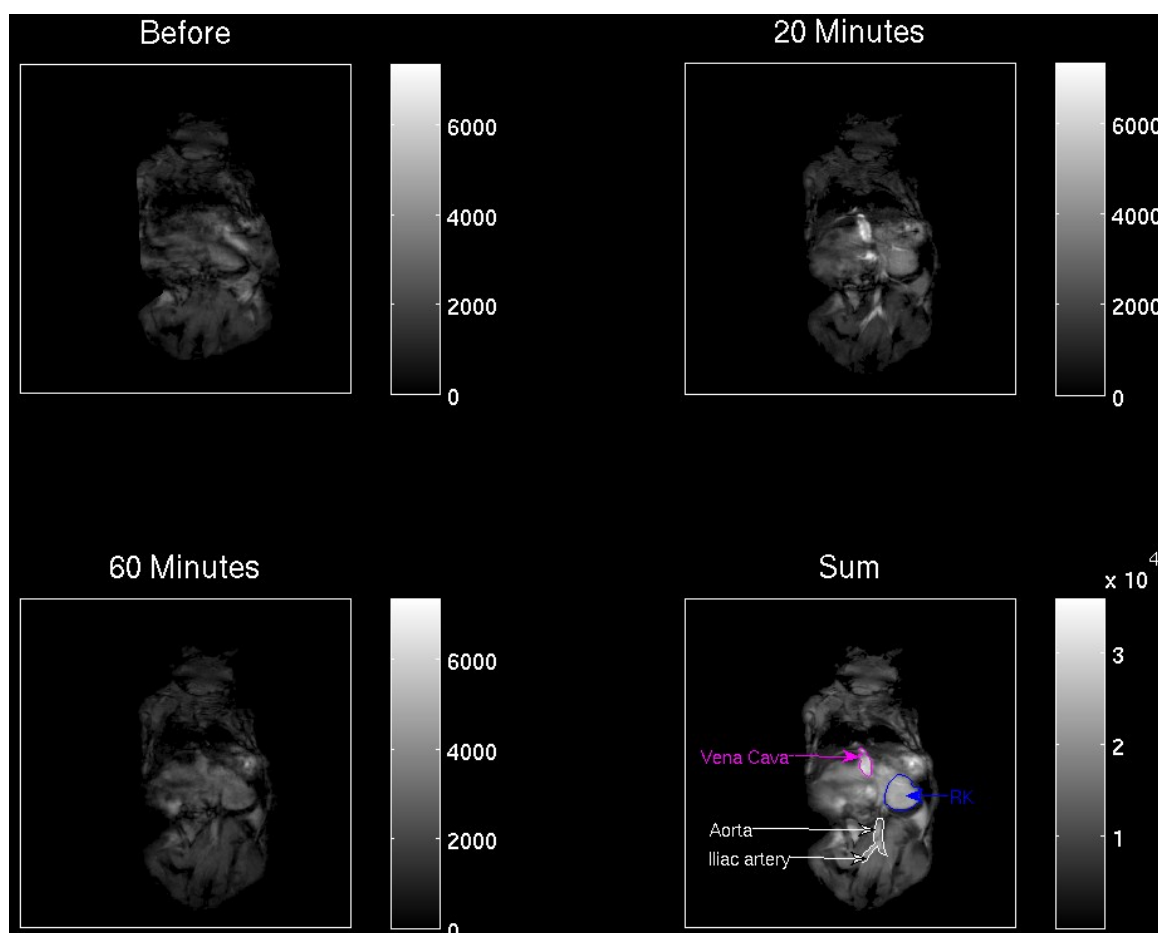


Figure S9. T₁-weighted images of coronal slices of mice acquired before nanoparticles intravenous $\text{Gd}^{3+}/[\text{Fe}(\text{CN})_6]^{3-}/\text{D}$ -mannitol injection (a), 20 min after injection (b), 60 min after injection (c) and the summated images of the organs of interest showing vena cava, aorta, iliac artery and right kidney (d).

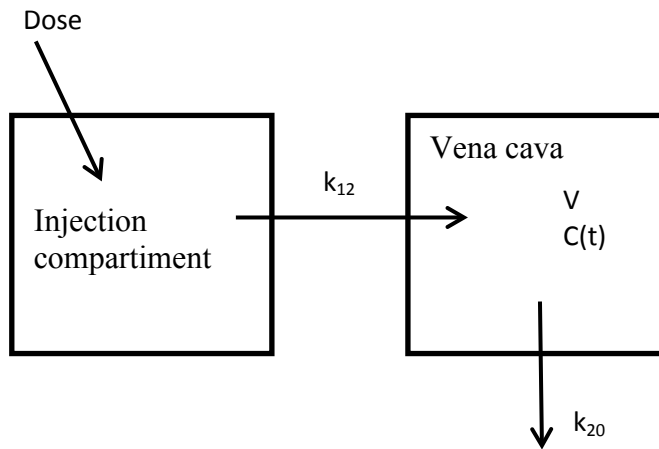


Figure S10. Schematic representation of the bi-compartmental model used for the fitting of the time-dependent evolution of Gd^{3+} concentration in blood showing the passage evolution of the CA from injection to vena cava. Time-dependent evolution of Gd^{3+} concentration in blood, extracted from dynamic acquisitions in a region of interest (ROI) placed upon the vena cava, was fitted with a bi-compartmental model (Fig.3) whose theoretical equation is given as follows :

$[\text{Gd}] = \text{C}(t) = \text{A} \cdot [\exp(-k_{20} \cdot t) - \exp(-k_{12} \cdot t)]$ with $\text{A} = k_{12} \cdot \text{M}_0 / (k_{12} - k_{20})$ and $\text{M}_0 = \text{D}_0 / \text{V}$
 The fitting gave parameters $\text{A} = 7.17$, $k_{12} = 0.099$ and $k_{20} = 0.092$, giving access to $\text{M}_0 = 0,51 \text{ mM/voxel}$. Considering V , the vena cava ROI volume of about 320 voxels of about $9.16 \cdot 10^{-6} \text{ mL}$, this gives access to the "effective injected dose" of contrast medium of about $0.06 \text{ } \mu\text{mol}$ ($6 \cdot 10^{-7} \text{ mol}$).

Table S1. Half maximal inhibitory concentration (IC_{50}) of nanoparticles determined by Probit function in SPSS.

Nanoparticles concentration [$\mu\text{g.mL}^{-1}$]	Mean	\pm	SD
400	99.86	\pm	0.01
307	99.46	\pm	0
236	99.63	\pm	0
182	32.93	\pm	0.04
140	3.31	\pm	0.09
107	-20.86 (0*)	\pm	0.04
82	-22.31 (0*)	\pm	0.03
63	-19.87 (0*)	\pm	0.03

Table S2. Coefficients of collected organs of mice. The mice were intravenously injected with $\text{Gd}^{3+}/[\text{Fe}(\text{CN})_6]^{3-}$ @D-mannitol nanoparticles (treated n=3) or physiological serum (control n=3). Data were expressed as means \pm SD.

	Weight ratio of organ/body (mg.g^{-1})	
	Control (n = 3)	Treated (n = 3)
Liver	45.5 \pm 3.3	48.7 \pm 2.1
Spleen	3.0 \pm 0.7	3.2 \pm 0.1
Heart	12.1 \pm 0.8	12.3 \pm 0.7
Kidneys	5.3 \pm 0.2	5.1 \pm 0.1