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Figure S1. TEM size distribution of samples NP1-4.



Figure S2. TEM images of samples A) S1, B) S2, C) S3 and D) S4 (all images are taken at the same magnification).



Figure S3. XRD pattern of S3 (dry powder). The XRD patterns of samples 1–4 could be unambiguously attributed to the cubic spinel structure of magnetite. The average crystallite size was estimated from the x-ray diffraction line broadening measurement by using the Scherrer formula. The analysis of the (3 1 1) and (5 1 1) peaks gave a mean diameter of ca. 8 nm, in good agreement with TEM observations.



Figure S4. Metabolic activity after 24 hours of A) A3 and B) A4 as a function of the iron concentration.



Figure S5. R_2 relaxation rates obtained at clinical fields of 1.5 and 3T for S3 and S4 are reported as a function of NPs concentration in H_2O solutions; A linear dependence of the relaxation rates on NPs concentration was found for all the different magnetic field strengths used in the study.



Full Experimental Details

Samples preparation

In the preparations, iron pentacarbonyl (Fe(CO)5, 99.5%), oleic acid (OA), dioctyl ether, trimethylamine N-oxide, toluene, ethanol, anhydrous dimethylsolfoxide (DMSO), anhydrous N,N-dimethylformamide (DMF), GdCl₃·6H₂O, Atto 633, PAMAM generation 4 and PAMAM-C12 generation 4 (methanol solution) were purchased from Sigma-Aldrich. Samples were prepared according to the reported method: NP-OAs (13 mg, 83 μ mol Fe, 30 μ mol OA) were added to a solution of 0.25 mL of PAMAM-C12 generation 4 (methanol solution, 20 mg, 1 μ mol) in 10 mL of water. After 16 hours stirring solvents were removed under vacuum and replaced by addition of water. 30 minutes of sonication facilitated the dispersion of the powder in water solution, which became orange. Recovery with a magnet allowed removing the unreacted dendrimers and other residues of the reaction.

For the synthesis of gadolinium or Atto 633 labelled NPs, PAMAM C12 was previously conjugated with N-hydroxysuccinimide (NHS) derivatives of DOTA (and then reacted with an excess of $GdCl_3 \cdot 6H_2O$ in water) and Atto 633. APTES coated samples were synthesized following literature methods.

Structural and Magnetic Characterization

The measurement of iron and gadolinium content in the samples was performed by an Inductively Coupled Plasma-Mass Spectrometry technique (ICP-MS, Agilent Technologies Inc., 7700 series, USA). Dynamic Light Scattering measurements were performed on a Malvern Nano ZS90 Zetasizer instrument at 298 K. Conventional TEM micrographs and electron diffraction patterns were obtained with a Zeiss Libra 120 TEM operating at 120 kV and equipped with in column Omega filter for energy loss electron spectroscopy (EELS). The NP size distribution histogram was determined using enlarged TEM micrographs taken at magnification of 100 K on a statistical sample of ca. 200 NPs. Magnetization data were collected on dry powder with a Quantum Design Ltd. SQUID magnetometer working in the temperature range of 1.8-350 K and the magnetic field range of 0-5 T. Data were corrected for the diamagnetic contribution, separately measured.

Relaxometry

FFC neasurements Water proton relaxation measurements at fixed frequency were obtained on a StelarSpinMaster Spectrometer (Stelar S.n.c., Mede (PV), Italy) operating in the range 20-70MHz. Water proton T₁ data were obtained by means of the Inversion-Recovery technique (16 experiments, 2 scans). Magnetization values were obtained by averaging the first 128 data points of the Free Induction Decay (FID). A phase cycle was applied on the 90° observation pulse to cut off the y-scale receiver offset. A typical 90° pulse width was 3.5 μ s at 20 MHZ. In the 16th experiment the FID is acquired after a single 90° pulse, to get the M_∞ value ³⁵. The reproducibility in T₁ measurements was ± 0.5%. Water proton T₂ data were obtained by means of the CPMG sequence with the following parameters: 8 scans, 2048 data points, delay time equal to 5 times T₁, applying a phase cycle on the pulses. Data were fitted with a monoexponential function. ¹H-NMRD (Nuclear Magnetic Relaxation Dispersion) profiles were recorded at 37°C on a StelarSpinmaster-FFC field-cycling relaxometer (StelarS.n.c., Mede (PV), Italy) by measuring water proton longitudinal relaxation rates at magnetic field strengths in the range from 2.4·10⁻⁴ to 0.47 T (corresponding to 0.01 to 20 MHz proton Larmor frequencies). The relaxometer was able to

switch the magnetic field strength in the millisecond time scale, working under complete computer control with an absolute uncertainty in $1/T_1$ of $\pm 1\%$. The temperature was controlled by a Stelar VTC-91 airflow heater (StelarS.n.c., Mede (PV), Italy), equipped with a copper-constantan thermocouple; the actual temperature in the probe head was set at 37°C and measured with a Fluke 52 k/j digital thermometer (Fluke AG, Zürich, Switzerland), with an uncertainty of ± 0.3 °C.

Phantom MRI studies Phantoms consisting of H_2O and 1% agar gel solutions of NPs at different concentrations (in the range 0.004 – 0.3 mM, [Fe] concentration) were prepared for the evaluation of the relaxation profile. The measurements were conducted using clinical scanners at 1.5 T (Signa Excite, GE Healthcare, USA) and 3.0 T (Signa HDxT, GE Healthcare, USA), equipped with a clinical head coil for MR signal detection. ³⁶ The longitudinal (T₁) relaxation time of the solutions was measured using a T₁-weighted MRI sequence based on a standard Inversion Recovery (IR) with the following parameters: TR = 9000, TE = 7.6 ms, TI = 100 – 2100 ms in steps of 200 ms, FOV = 18x18 cm for 3 T and 23x23 cm at 1.5 T, matrix 192x192 pixels, slice thickness = 3 mm.

The transverse (T₂) relaxation time was measured with a T₂-weighted Fast Spin Echo sequence using the following parameters: TR = 4500 ms; TE = 6.7 - 87.2 ms, 16 scans, FOV = 16x16 cm at 3 T and 23x23 cm at 1.5 T, matrix 192x192 pixels, slice thickness = 3 mm.

The longitudinal (R_1) and transverse (R_2) relaxation rates were calculated as the inverse of the relaxation times T_1 and T_2 respectively and reported as a function of the NPs concentration for the estimation of longitudinal and transverse relaxivity (r_1 and r_2 respectively, mM⁻¹ s⁻¹ [Fe] concentration). r_1 and r_2 were assessed from the slope of the linear fit of R_2 versus concentration T_2 -relaxivities at 0.32 T and 0.5 T were obtained with an open magnet (Paramed Medical System), using a dedicated head multi-array coil: 2-dimensional acquisitions were obtained: proton density (PD)–weighted oblique coronal and oblique sagittal planes, T_2 -weighted fast spin echo (FSE) oblique coronal and oblique axial planes, gradient echo (GRE) oblique axial plane, and short tau inversion recovery (STIR) oblique coronal plane.

Cell viability

Chemicals: All cell culture media and supplements, fetal bovine serum (FBS), phosphate-buffered saline (PBS) pH 7.4, 0,25% Trypsin/EDTA, and LysoTracker-red D99 were purchased from Life Technologies (Carlsbad, CA, USA). WST-8 cell counting kit, dimethyl sulfoxide (DMSO), 3,3'-Dihexyloxacarbocyanine iodide (DiOC6) and Hoechst 33258 nuclei staining solution were purchased from Sigma-Aldrich (St Louis, MO, USA).

Cell cultures Immortalized mouse endothelial cells, bEN5, from Sigma-Aldrich (St Louis, MO, USA) were cultured in high glucose DMEM with 10% FBS, 2 mM L-glutamine, 1000 U/ml penicillin and 1 mg/ml streptomycin. Cells (passage 2–20) were maintained in a humidified cell culture incubator at 37°C and 5% CO₂.

WST-8 cytotoxicity assay: The metabolic activity of bEND5 cell line was determined 24 h following exposure to NP (0.001–1 μ g/ml), using a standard WST-8 assay in 96 multi-well microplates. Cells were seeded at a density of 5000 cells/well and cultured for 24 h in a humidified atmosphere at 37°C and 5% CO₂, before being treated. Overall, 5% DMSO was used as positive control and culture medium as negative. A total of 10 $\mu\mu$ L of Cell Counting Reagent WST-8 were added to each well. Following 3 h incubation, the orange WST-8 formazan product was measured by using a Synergy-HT (BioTEK) microplates reader at a wavelength of 460 nm. To express the cytotoxicity, the average absorbance of the wells containing cell culture medium without cells was subtracted from the average absorbance of the solvent control, 5% DMSO or NP-treated cells. The percentage of cell viability was calculated using the following equation:

 $\frac{(Absorbance_{treated} - Absorbance_{culture medium})}{(Absorbance_{control} - Absorbance_{culture medium})} \times 100$

Data are expressed as mean \pm SE of six replicates from at least three independent experiments. Statistical significance versus controls was calculated using one-way ANOVA with Dunnett's post hoc test (p value < 0.05). At this concentration range, NP did not create any significant interference with the reagents of the WST assay (data not shown). *Flow Cytometry* 24 h after being plated in a 12 well-multiwell plate, bEND5 cells were incubated with NP-1-Atto633 at final concentrations ranging from 1 pg/ml to 10 ng/ml, both with or without Fetal Bovine Serum in complete cell culture medium. After 2 h in humidified atmosphere at 37°C and 5% CO₂, cells were detached with 0,25% Trypsin/EDTA and analysed by flow cytometry using a MACSQuant analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany). 20,000 events per point were analyzed in triplicate.

Confocal microscopy: Uptake of NP-1 conjugated with Atto-633 in cells was tracked by Olympus FV100 confocal microscope. To image cell morphology, bEND3 cells were grown on WillCo-dish for 24 hours. Cells were then treated for 2 hours with 1ng/ml of NS3PAMAM, washed with PBS pH 7.4, fixed in buffered 4% paraformaldehyde for 15 min and Incubated with 50 nM DiOC6 for 30 minutes. Hoechst staining solution was subsequently added for 15 min and each sample was mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA). Lysosome-PAMAM co-localisation was obtained with 50 nM LysoTracker-red D99 3 h after NP-1 exposure (1 ng/ml). Then cell was washed with PBS pH 7.4 and fixed in buffered 4% paraformaldehyde. Hoechst staining solution was subsequently added for 15 min on fixed cells, and each sample, mounted with Vectashield, was visualized within 1 hour.

TEM microscopy: We tested the effect of S4 NPs on U2OS cells (human osteosarcoma cell line) and treated them 24 hours with different concentration of nanoparticles solution. After 24h cells were washed and fixed, then treated with a conventional embedding protocol (as previously shown in Ciofani et al., 2013). Briefly U2OS cells were fixed as monolayer (with a solution of 2% glutaraldehyde in cacodylate buffer) then scraped and centrifuged to obtain a stable pellet. Pellets were deeper fixed in the same fixative solution overnight. Subsequently cells were washed in cacodylate buffer, postfixed with osmium tetroxide 2% in the same buffer, then dehydrated with ethanol growing series and embedded in epoxy resin. After resin polymerization (48 hours, 60°C) samples were cut in thin sections (80-90 nm). Sections were collected over 300 mesh copper grids and were analyzed using a Zeiss Libra 120 Plus transmission electron microscope operating at 120 kV and equipped with an in-column omega filter and 16-bit CCD camera 2k x 2k bottom mounted.

In vivo MRI

MRI *in vivo* studies were performed at 3 T using a clinical scanner (Signa HDxT, GE Healthcare, USA) equipped with a dedicated birdcage coil (Rapid Biomedical, Rimpar, Germany). Nanoparticles sample **S4** (mean diameter = 14.1 nm) were diluted in saline solution to obtain a final injectable concentration of approximately 0.5 mg/mL; afterwards 600 μ L of NPs in solution were manually injected in bolus in Wistar rats (300 g body weight), from the rat tail vein. Rats were anesthetized using Zoletil® + xylazine (50 and 3 mg/kg respectively). Fast Spin Echo sequence for T₂ mapping was applied for the acquisition with the following parameters: TR = 1000 ms; Echo times = 8; FOV = 17x17 cm, matrix = 224x224 pixels, total acquisition time = 24 sec. Images in axial plane were acquired in sequence for the first 5 minutes after injection; subsequently, the measurement was repeated at increasing time intervals up to 60 minutes from the injection. A ROI was selected on the anatomical district of interest on the image and the corresponding T₂ was measured for each acquisition; the transverse relaxation rate R₂ was then estimated as the inverse of T₂. All the experimental protocols conformed to the "Guiding Principles for Research Involving Animals and Human Beings" approved by the Council of the American Physiological Society and were authorized by the local Ethical Committee after approval from Ministry of Health.