ELECTRONIC SUPPORTING INFORMATION (ESI) FOR:

Iron oxide-filled micelles as ligands for *fac*-$[M(CO)_3]^+$ (M = ^{99m}Tc, Re)

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Materials. All reagents and chemicals were obtained from commercial sources and used without further purification: Iron(III) acetylacetonate (99%; Strem Chemicals), 1,2 hexadecanediol: (>98%; Tokyo Chemical Industry Co. Ltd), oleic acid (90%; Alfa Aesar), oleylamine (70%; Sigma Aldrich) and dibenzylether (>98%; Alfa Aesar). 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol)-2000] (ammonium salt) (PEG-COOH), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (ammonium salt) (PEG-NH_2) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) (PEG-OMe) and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (DPPE-Rho) were purchased from Avanti Polar Lipids. A Beckman Optima MAX-XP tabletop ultracentrifuge was used for the purification and isolation of the MNP-filled micelles.

MNP synthesis.[1] $Fe(acac)_3$ (0.7064 g), 1,2-hexadecanediol (2.6371 g), oleic acid (2.123 mL), oleylamine (2.82 mL), and benzyl ether (20 mL) were mixed and magnetically stirred under a flow of nitrogen. The mixture was first heated to 210 °C for 2 h and then to 300 °C for 1 h under nitrogen. The black-colored mixture was cooled to room temperature by removing the heat source. Under ambient conditions, ethanol (40 mL) was added to the mixture, and a black material was precipitated and separated via centrifugation. The black product was dissolved in hexane (10 mL) containing oleic acid (0.05 mL) and oleylamine (0.05 mL). Centrifugation (3,000 g, 10 min) was applied to remove any undispersed residue. The product, 7 nm Fe_3O_4 nanoparticles, was then precipitated with ethanol, and isolated by centrifugation (3000 g, 10 min). The dried powder was redispersed in hexane or chloroform for TEM characterisation or micelle formation.

Micelle formation.

Micelles with individual or small number of MNPs. PEG-phospholipid(s) (2 mg) and hydrophobic Fe_3O_4 NPs (1 mg) were dissolved in chloroform (500 μL). The solvent was allowed to evaporate overnight in a 3 mL round bottom flask at RT. Any remaining solvent was removed under vacuum for 1 h. The flask was placed in a water bath at 80 °C for 30 s, after which 1 mL of nanopure water was added. The solution was transfered to an Eppendorf tube and centrifuged at 9700 g for 5 min. The pellet was discarded and the supernatant was passed through a 0.45 μm filter. This solution was ultracentrifuged (369000 g, 1 h, 3 cycles) to remove the empty micelles. Finally the pellet was dissolved in 400 μL of nanopure water or phosphate buffered saline, PBS (10 mM). The MNP-filled micelles were stored at 5 °C.

Micelles with large number of MNPs: PEG-phospholipid(s) (1 mg) and hydrophobic Fe_3O_4 NPs (1 mg) were dispersed in chloroform (500 μL). The solvent was allowed to evaporate overnight in a 3 mL round bottom flask at RT. Any remaining solvent was removed under vacuum for 1 h. The flask was placed in a water bath at 80 °C for 30 s, after which 1 mL of nanopure water was added. The solution was transfered to an Eppendorf tube and centrifuged at 9700 g for 5 min. The supernatant solution was discarded. The pellet was resuspended in 300 μL of nanopure water and centrifuged (1070 g, 2 min). The supernatant contains the micelles with large number of MNPs.

Fluorescent micelles: Micelles were prepared as above with the modification that DPPE-Rho (4.5 μL of a 2 mg/mL solution in chloroform) was also added to the chloroform solutions of PEG-phospholipid(s) and Fe_3O_4 nanoparticle and that the round bottom flask was left overnight protected from light for the chloroform to evaporate.

Labeling of micelles with *fac*-$[^{99m}Tc(OH_2)_3(CO)_3]^+$.

^{99m}Tc pertechnetate ($Na[^{99m}TcO_4]$) was eluted with saline from a Drytec generator (GE Healthcare, Amersham, UK) at the Radiopharmacy at Guy's and St Thomas' Hospital NHS Trust, London, U.K. and converted to *fac*-$[^{99m}Tc(OH_2)_3(CO)_3]^+$ using the lyophilised kit *Isolink*® (Covidien, Petten, The Netherlands). The synthesis and quality control of tricarbonyltechnetium (^{99m}Tc-TC) were carried out following the manufacturer's instructions. Briefly, 1 mL of $Na[^{99m}TcO_4]$ (1.0 GBq) was injected into the kit and the mixture was then heated at 100 °C for 30 minutes. The vial was allowed to cool to room temperature, and its contents were neutralised with 1M HCl. The reaction was monitored by thin layer chromatography (TLC) using silica gel TLC strips (Merck) with methanol/HCl 99/1 as mobile phase. ^{99m}Tc-TC has an Rf of about 0.3-0.4, while $[^{99m}TcO_4]^-$ moves with the solvent front. TLC strips were

scanned using a Mini-scan radio TLC scanner with a FC3600 Flow-Count detector of γ photons (LabLogic, Sheffield, UK).

400 μL of *fac*-$[^{99m}Tc(OH_2)_3(CO)_3]^+$ was added to 400 μL of an aqueous micelle solution in an Eppendorf tube and the mixture was heated at 90 °C for 25 min. The Eppendorf was then allowed to cool down to RT and the contents were transferred to a Nanosep 100k molecular weight cutoff ultrafiltration centrifugal device (Pall Life Sciences), which was centrifuged at 12000 rpm for 10 min. This allowed separating the radiolabelled micelles, which remained in the retentate, from the unbound ^{99m}Tc-TC, which was in the filtrate. The filtrate was discarded, and water for injection (500 μl) was added to the retentate. Once more, the filter was centrifuged at 12000 rpm for 10 min. This process was repeated twice. The total radioactivity in the filtrates and retentates was measured in a CRC-25R dose calibrator (Capintec, USA) in order to determine the radiolabeling yield (%). The radiolabelled micelles were recovered from the filter by the addition of saline (100 μL) to the retentate.

^{99m}Tc-MNP labeling can also be carried out by directly adding the aqueous solution of *fac*-$[^{99m}Tc(OH_2)_3(CO)_3]^+$ to the solid obtained after the evaporation of chloroform solutions of phospholipids and hydrophobic Fe_3O_4 NPs and the purified/isolated as described above.

Labeling of micelles with *fac*-$[Re(OH_2)_3(CO)_3]^+$.

In a typical experiment a solution of MNP-filled micelles in water (at concentrations ranging from 6 mM to 16 mM Fe, 250 μL) was added to aqueous solutions of *fac*-$[M(OH_2)_3(CO)_3]^+$ (400 μL, 2.8 mM). The mixture was vortexed for 30 s and then heated at 90 °C for 20 min. The reaction mixture was allowed to cool down to RT and then centrifuged at 1000 g for 3 min. The supernatant was transferred to a NanoSep 100k centrifugal device and centrifuged at 8200 g for 10 min. The retentate was washed with water twice and after the final centrifugation the MNP-filled micelles were recovered from the membrane surface by adding and rinsing with a pipette tip (with a total volume of around 500 μL). This purification process was repeated using a new NanoSep 100k centrifugal device. Retentates and filtrates were lyophilised and analyzed by FT-IR.

1 S. Sun, H. Zeng, D. B. Robinson, S. Raoux, P. M. Rice, S. X. Wang, and G. Li, *J. Am. Chem. Soc*,. **2004**, 126, 273-279.

Characterisation.

Transmission electron microscopy (TEM) studies were conducted on a JEOL JEM-2011 electron microscope operating at 200 kV. The samples were prepared by depositing a drop of a solution of nanocrystals onto a copper specimen grid coated with a holey carbon film and allowing it to dry. For MNP size determination from TEM, a minimum of 200 particles were measured using the *Image J* software. Particle size analysis in solution was measured with a NanoSizer (Malvern Nano-Zs, UK). UV/Vis absorption spectra were recorded on a Varian Spectrophotometer (Cary-5000). Fluorescence measurements were made with a HORIBA Jobin-Yvon fluorimeter (Fl-1065) equipped with a Xe 450 W arc lamp. Temperature was maintained at 25 °C. FT-IR spectra were acquired in a Thermo Nicolet FT-IR spectrometer. Magnetic measurements were done using the Vibrating Sample Magnetometry (VSM) technique at SGIker of the Universidad del Pais Vasco (UPV-EHU). The hysteresis loops at RT, with very good low field accuracy (better than 10^{-5} T) were performed in a home-made VSM equipped with an electromagnet up to a maximum field of 1.8 T. Another VSM fitted to a Cryogenic Free 14 T magnet system (Cryogenic Ltd) was used for the measurements below RT from -8 T to +8 T. The thermogravimetric analysis (TGA) were performed on a TGA/SDTA 851 Mettler Toledo thermogravimetric analyzer under nitrogen atmosphere at a heating rate of 10 K/min. The samples were analysed for Fe by ICP-OES using a Perkin Elmer Optima 5300 DV, employing an RF forward power of 1400 W, with argon gas flows of 15, 0.2 and 0.75 L min^{-1} for plasma, auxiliary, and nebuliser flows, respectively. Using a peristaltic pump, sample solutions were taken up into a Gem Tip cross-Flow nebuliser and Scotts spray chamber at a rate of 1.50 mL min^{-1}. The instrument was operated in axial mode. The selected wavelengths (238.024, 239.562, 259.939 nm) were analysed in fully quant mode (three points per unit wavelength). A range of calibration standards were prepared using single element 1000 mg l^{-1} stock solutions (Fisher Scientific UK LTD) and a Merk multi element standard (ICP Multi element standard solution VI CertiPUR®) was employed as a reference standard. Relaxivities were measured at 37 °C on a Bruker Minispec mq60 instrument operating at 1.47 T. The MRI phantom experiments were carried out on a Bruker Biospec 11.7 T with a 9 cm gradient capable of delivering 740 mT/m using a 40 mm volume coil. T2 maps were acquired by using Bruker's MSME (Multi slice Spin echo) sequence. The echo time (TE) values were varied in 128 steps ranging from 10 ms to 1280 ms and a repetition time (TR) of 15 s. T1 maps were obtained by using a spin echo sequence. Images were acquired at ten different TR values 150, 500, 100, 1500, 2200, 3000, 4000, 5200, 7.600, 17500 ms). All data were acquired with: 256 X 256 points and a Field of View of 3 cm X 3 cm, slice thickness of 1.5 mm, no gap between slices and one average. T2 weighted images in Fig. S6 correspond to TE = 36 ms and TR = 15 s.

The T1 and T2 map images were calculated using the Bruker's Paravision 5.1 software via the Levenberg-Margardt method. The relaxivity values, r_1 and r_2, were calculated through linear least-squares fitting of 1/relaxation time (s^{-1}) versus the iron concentration ([Fe] mM).

In vitro studies.

Cytotoxicity studies.

J774.1 cells were obtained from the ATCC and cultured in DMEM supplemented with 5 % FBS, 1 % PS (all Invitrogen, Madrid). Cells were passaged at ~70 % confluence and a low passage number was maintained using cryopreserved stocks stored in FBS supplemented with 10 % DMSO (Sigma Aldrich). The effect of nanoparticle formulations on cell viability was measured using the MTT assay (Roche).

Cells were seeded at 2 X 10^5 cells/ml in 96-well plates (100 µL/well) and allowed to adhere for 24 hrs. Phenol-free media was used when conducting experiments. Media was removed from each well prior to adding 70 µL nanoparticle formulations, diluted accordingly in media, per well in triplicate. Cells were incubated for 24 hrs at 37 °C.

To determine cell viability, media was removed from the wells and 100 µL of MTT reagent diluted in phenol-free media (1/10) was added/well after the 24 hr incubation period. Cells were incubated at 37°C for >4 hrs, after which the liquid was removed and 200 µL DMSO was added/well to lyse cells. The absorbance of samples was measured at 550 nm and converted into cell viability using the average values of control wells.

Cell imaging.

Cells were plated in microslides (Ibili, Germany) at a concentration of 1 X 10^6 cells/mL and left to adhere overnight. Cell nuclei were stained with DAPI (final concentration 0.3 mM in phenol red-free media) for 10 mins at RT, followed by 2 washes with cold PBS (10 mM). Rhodamine-containing MNP-filled micelles were added to cells in phenol red-free media to a final volume of 120 µL ([Fe] = 172 µM measured by ICP-OES). After 30 mins cells were viewed using a Zeiss Axio Observer microscope.

For co-staining with Prussian blue, J774 cells were plated in 24 well plates (2 X 10^5 cells/well/mL) containing glass coverslips (Ø10mm; cleaned in EtOH, then H_2O and UV irradiated) and allowed to adhere overnight. Media was removed from all wells and 400 µL of Rhodamine-containing MNP-filled micelles suspended in phenol-free media was added/well (final [Fe] = 10 mg/L). Samples were added to cells for 24 h. After 24 h, supernatants were removed and wells containing coverslips were washed twice with ice-cold PBS (10 mM) and then fixed in 400 µL/well 2 % formaldehyde made in 10 mM

PBS for 15 min at RT. Wells were washed again twice with ice-cold PBS and subsequently 400 µL/well 1 % H_2O_2 in PBS was added for 15 min at RT. Wells were washed again with ice-cold PBS for at least 5 min, following which a 1:1 mixture of HCl (6 %) and $K_4[Fe(CN)_6]$ (2 %) was added (400 µL/well) and incubated at 65 °C for 45 min. After the incubation period wells were washed in ice-cold H_2O for at least 10 min to remove excess Prussian blue stain. Coverslips were carefully removed from wells and mounted on EtOH cleaned glass slides using DAKI mounting medium. Samples were viewed the following day using a Leica microscope equipped with colour and b/w cameras. Rhodamine-containing NPs were viewed through a 'N2.1' filter (Exc., 515/560; dichromatic mirror, 580; Em., LP590) whilst Prussian blue staining was viewed using either brightfield or polarised light contrast methods.

In vivo studies

Animal studies were carried out in accordance with UK Research Councils' and Medical Research Charities' guidelines on Responsibility in the Use of Animals in Bioscience Research, under a UK Home Office licence. Female BALB/c mice (aged 6–9 weeks, 20.5 ± 3.4 g) were purchased from Harlan Laboratories, UK.

Mice received an intravenous (i.v.) tail vein injection of 24 MBq and 92MBq ^{99m}Tc-TC each. With the mouse under isofluorane anaesthesia in a Minerve imaging chamber, SPECT/CT scans were acquired either from 30 min to 4 h post-injection (p.i.) or at 24 h p.i. respectively, using a NanoSPECT/CT scanner (Bioscan, Paris, France) with SPECT acquisition time 1800 s, obtained in 30 projections using a 4-head scanner with 4 X 9 (1 mm) pinhole collimators in helical scanning mode and CT images with a 45 kVP X-ray source, 500 ms exposure time in 180 projections over 9 min. Images were reconstructed in a 256 _ 256 matrix using the HiSPECT (Scivis GmbH) reconstruction software package, and fused using proprietary Bioscan InVivoScope (IVS) software. Quantification was performed by selecting the desired organs as regions of interest (ROI) using the quantification tool of the IVS software. At the end of the scanning procedure, animals were culled and tissues explanted, blotted dry, weighed and counted on a 1282 CompuGamma gamma counter (LKB Wallac, Finland). The carcass activity was measured with an ionisation chamber cross-calibrated with the gamma counter.

The same procedure was carried out for the *in vivo* imaging of micelles. In this case, mice received an i.v. injection in the tail vail (50 μL of radiolabelled micelles in saline (FreseniusKabi), 20 MBq with [Fe] = 8 mM for large MNP-Tc(CO)$_3$ micelles and 57 MBq with [Fe] = 16 mM for single/small MNP-Tc(CO)$_3$ micelles).

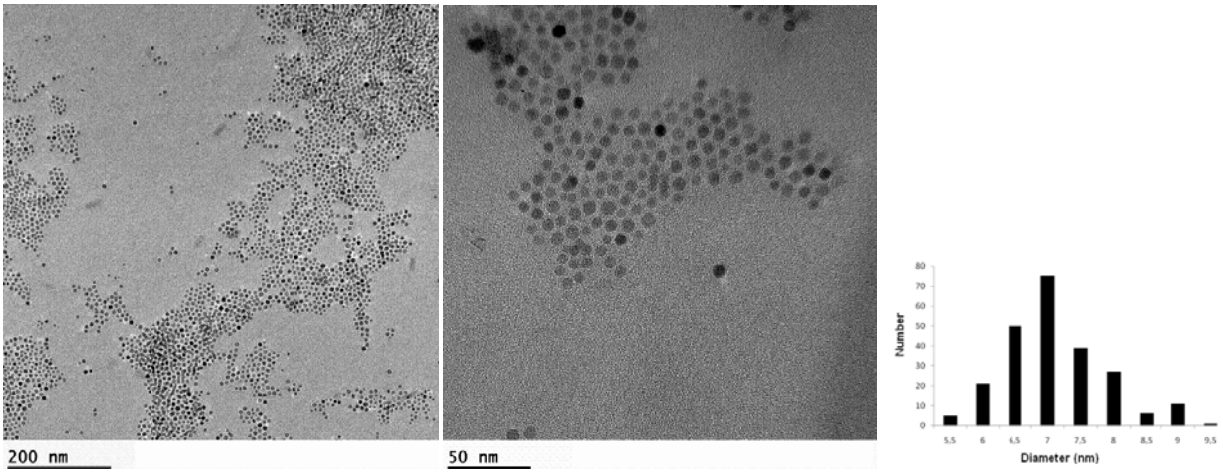


Fig S1 Transmission electron microscopy (TEM) images and size distribution of the hydrophobic MNPs.

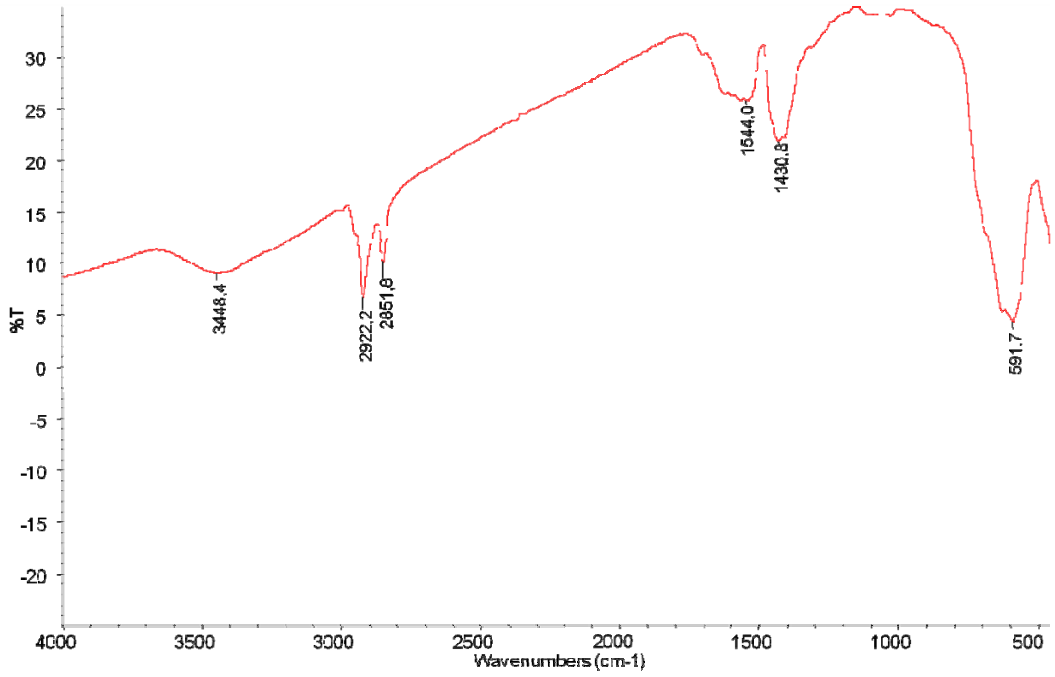


Fig. S2. FT-IR spectrum of the hydrophobic MNPs.

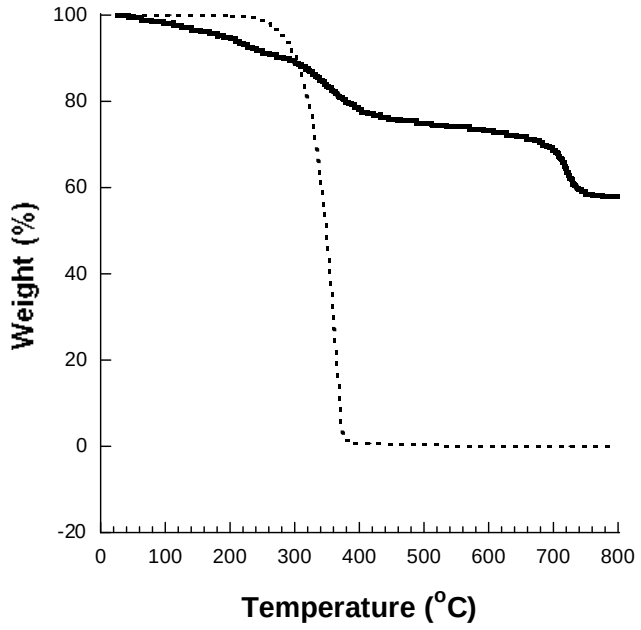


Fig S3. [1]H Termo-gravimetric analysis (TGA) curves for 7 nm iron oxide nanoparticles (solid line) and oleic acid (dotted line)

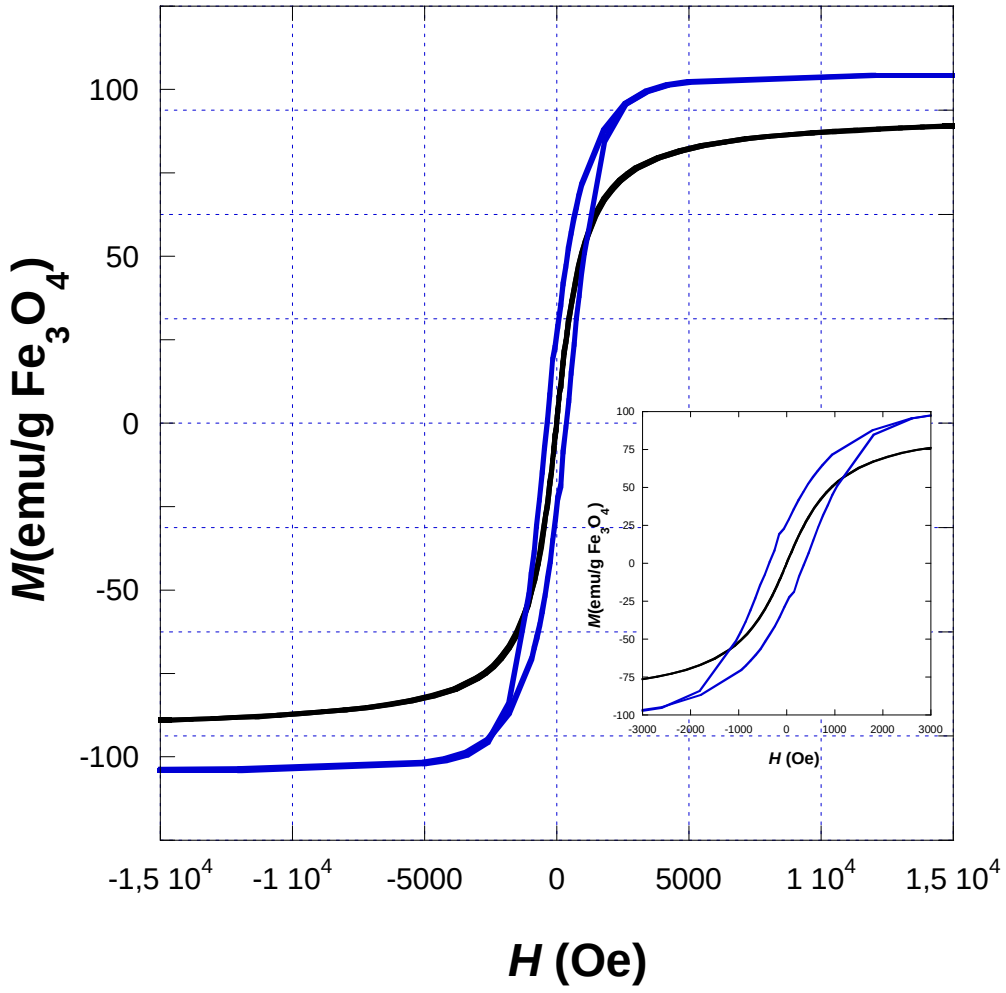


Fig S4. Magnetisation (*M*) loops for the 7 nm MNPs measured at 2 K (blue) and 293 K (black). Inset: expanded view.

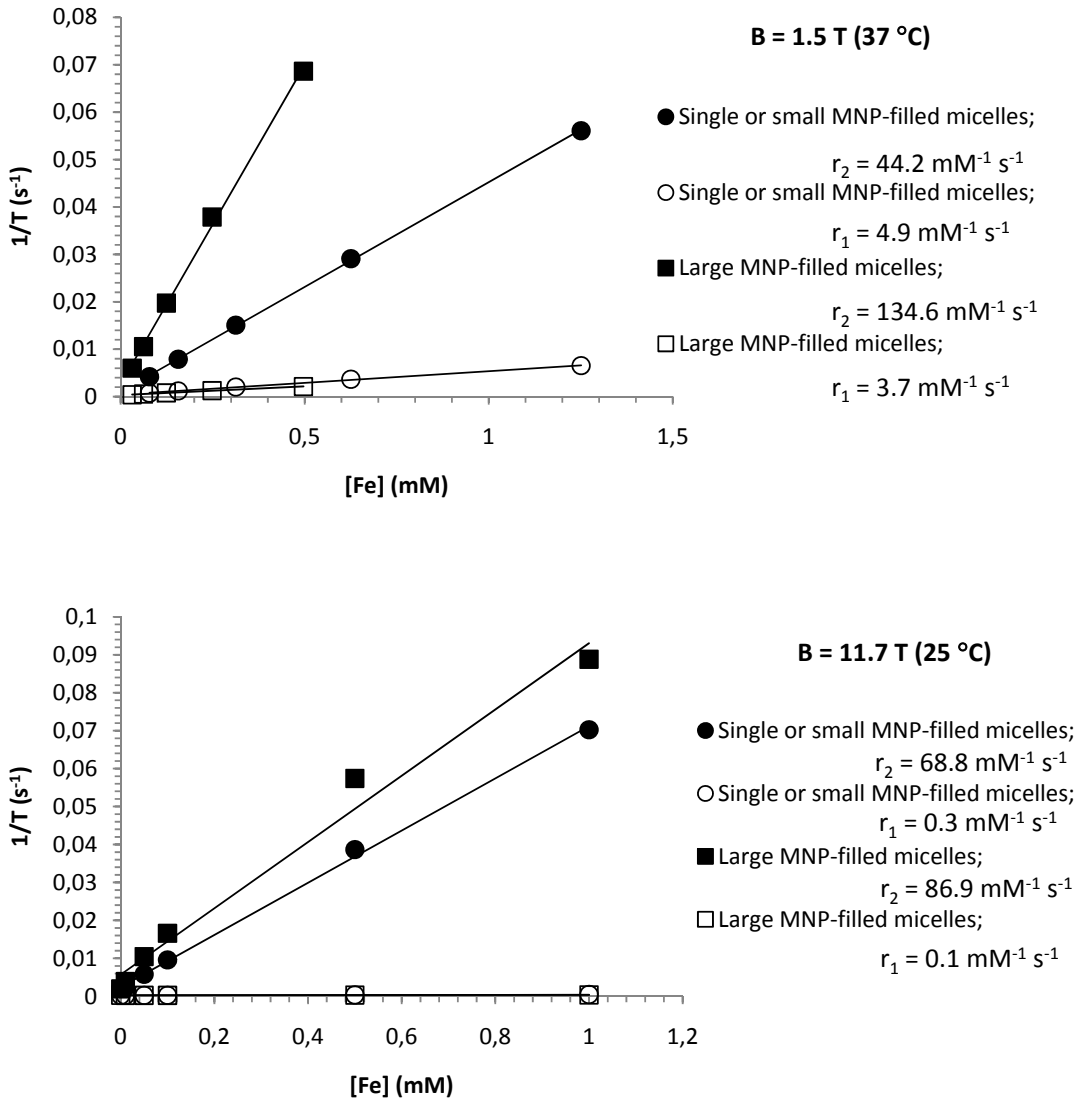


Fig. S5. Plots of $1/T_1$ and $1/T_2$ *vs* Fe concentration of micelles filled with a single/small MNP cluster (main population, D_H = 25 nm) and with large MNP clusters (main population, D_H = 285 nm). Relaxation rates measured at 1.5 T (*top*) and 11.7 T (*bottom*).

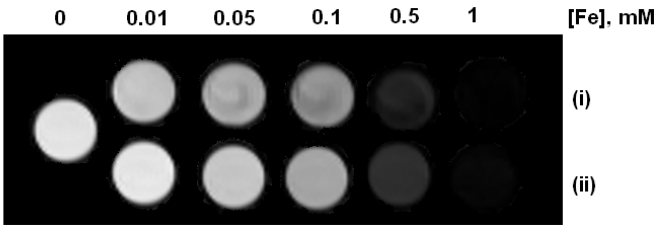


Fig. S6 T_2-weighed MR images of aqueous solutions of micelles with large MNP clusters (i) and single/small MNP clusters (ii) (11.7 T, 25 °C).

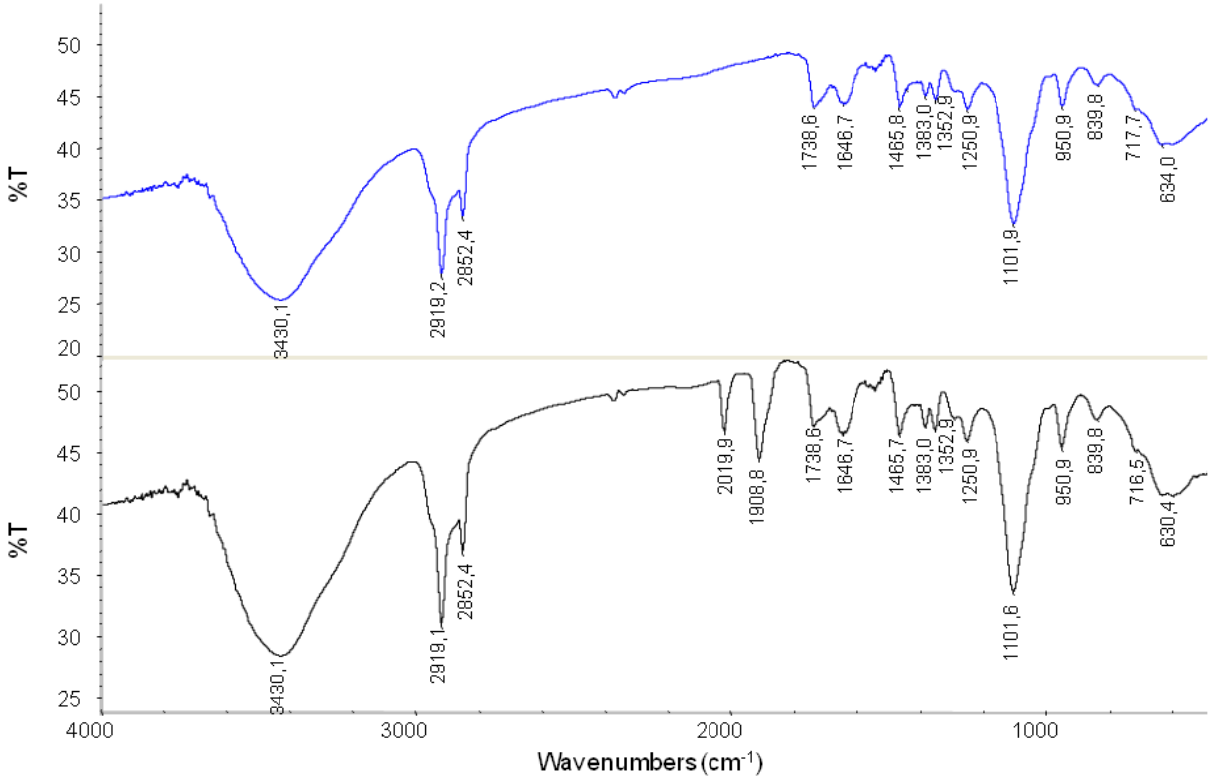
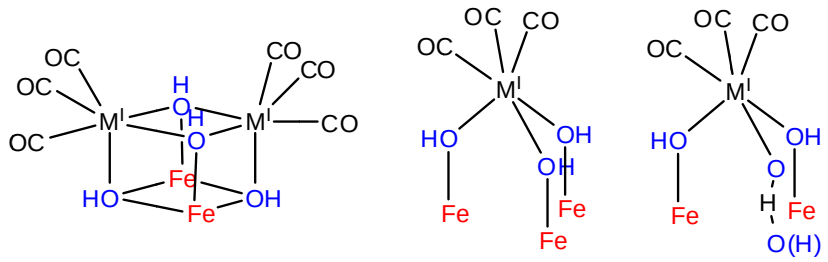


Fig S7. FT-IR spectrum of MNP-filled micelles before (blue) and after (black) reaction with *fac*-$[Re(OH_2)_3(CO)_3]^+$.



Scheme S1. Some plausible modes of attachment of *fac*-$[M(OH_2)_3(CO)_3]^+$ (M = ^{99m}Tc, Re) to the MNP surface.

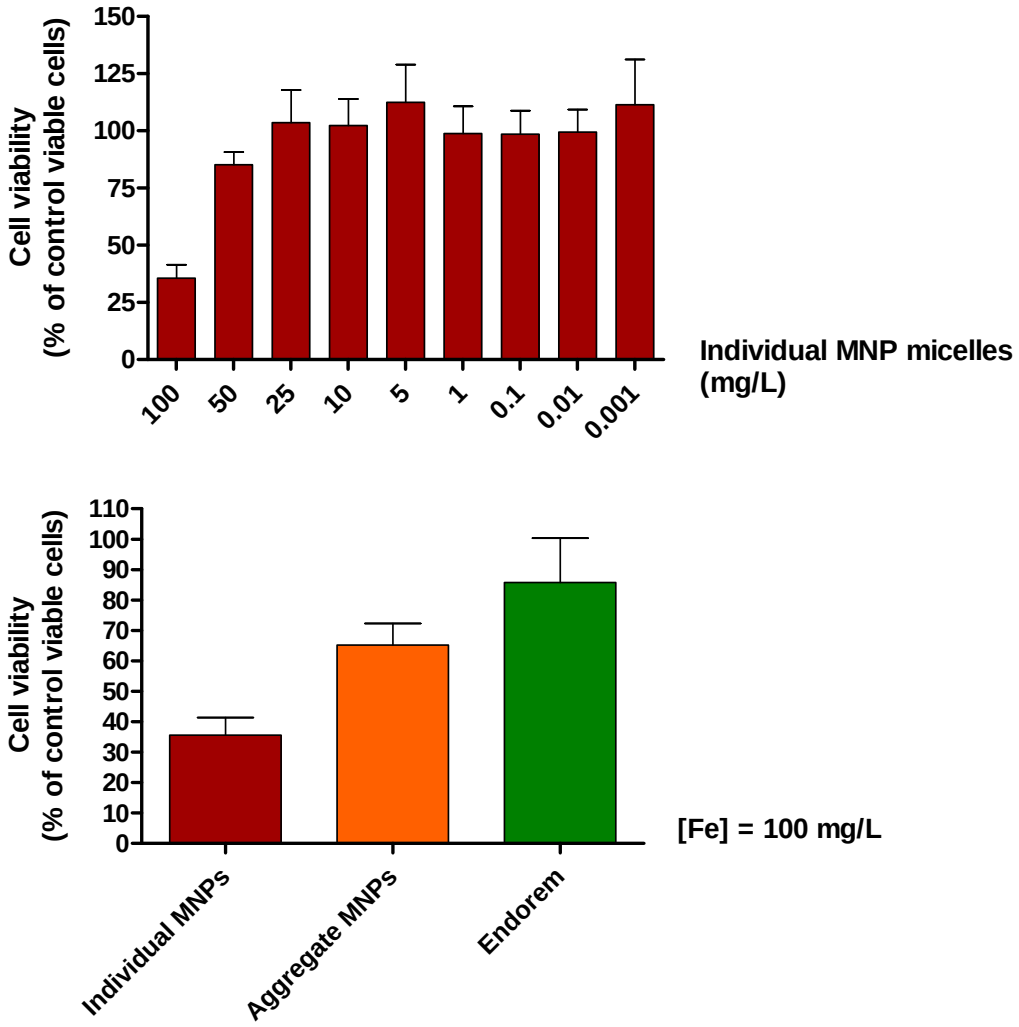


Fig S8. Cell viability of J774 macrophages after 24 h incubation with the MNP micelles.

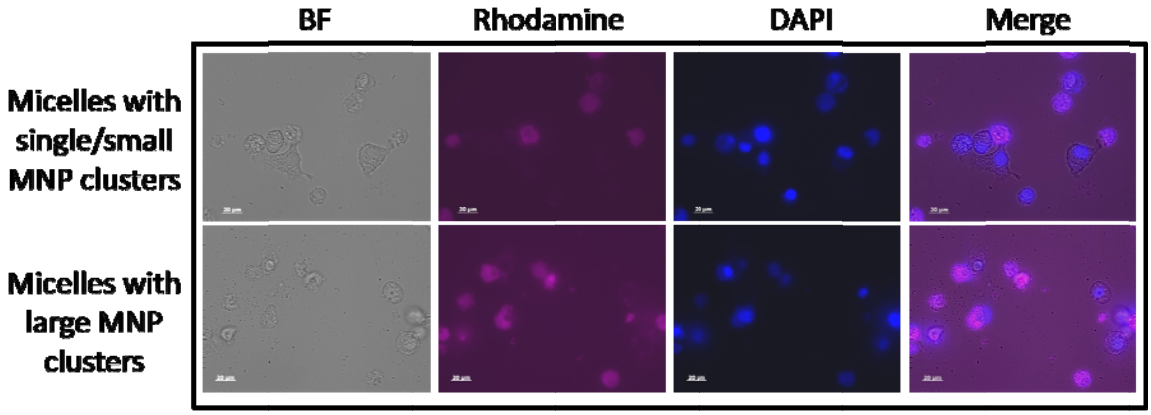


Fig. S9. Representative fluorescence microscope images of DAPI stained J774 macrophages after incubation with Rhodamine-labelled MNP-filled micelles for 30 minutes.

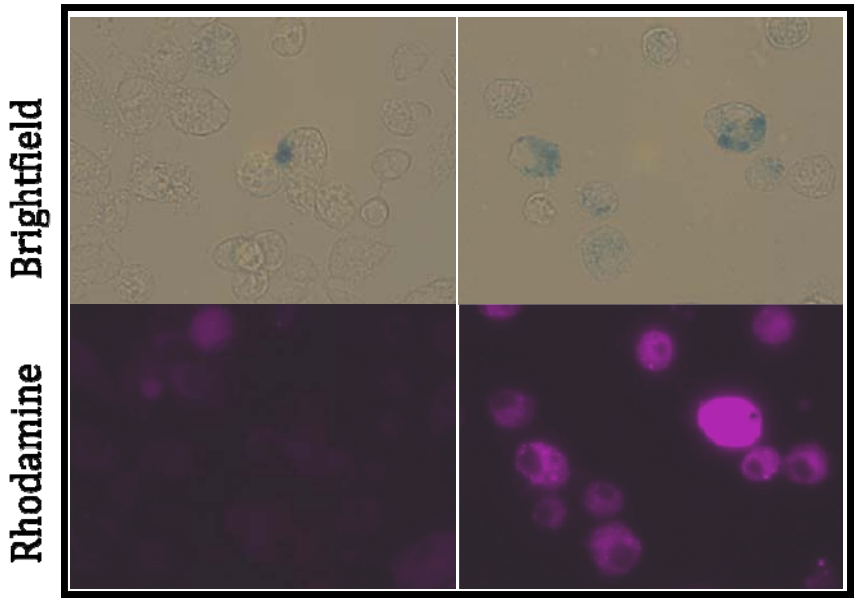


Fig. S10. Representative fluorescence microscope images of J774 macrophages after incubation with Rhodamine-labelled MNP-filled micelles for 24 h and Prussian blue staining.

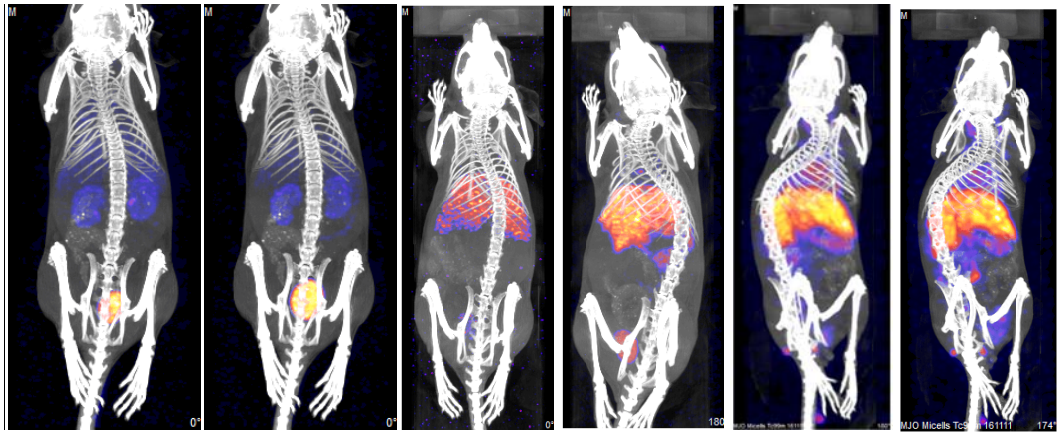


Fig. S11. *In vivo* SPECT/CT images of mice injected with $[^{99m}Tc(OH_2)_3(CO)_3]^+$ and MNP-$^{99m}Tc(CO)_3$ micelles at different times post-injection.

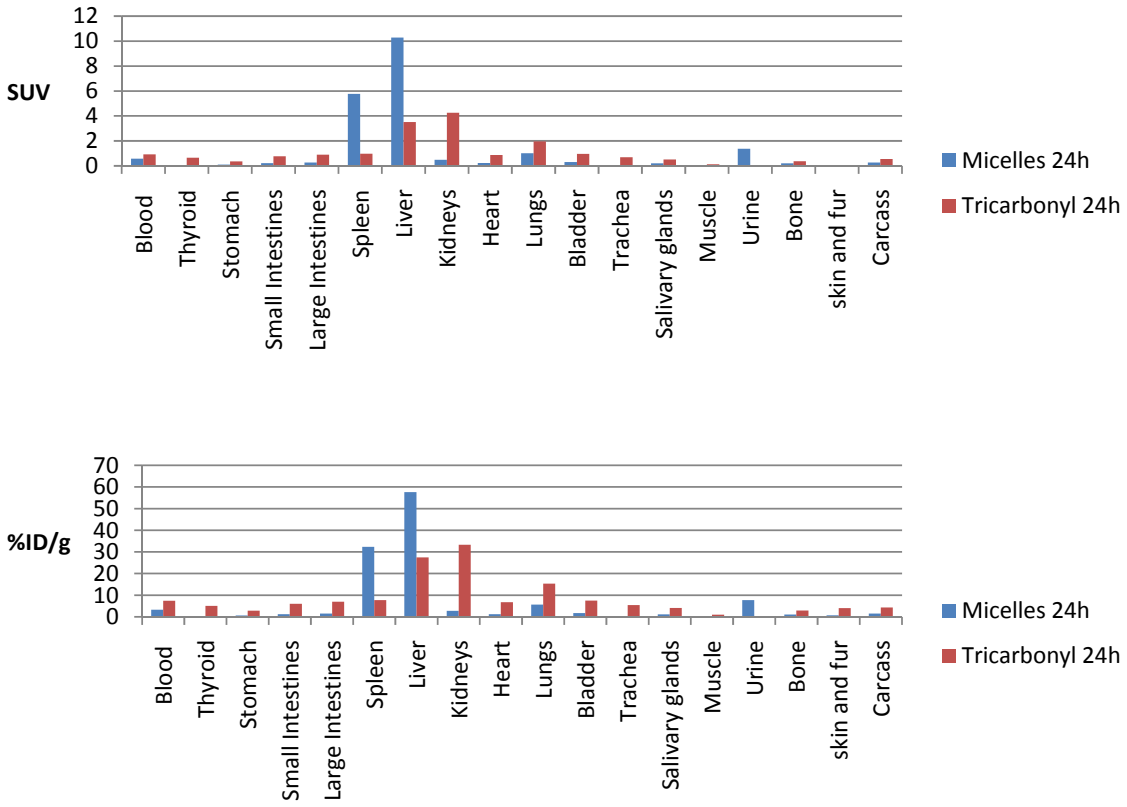


Fig. S12. Ex-vivo biodistribution of *fac*-$[^{99m}Tc(OH_2)_3(CO)_3]^+$ and small/single MNP-$^{99m}Tc(CO)_3$ micelles. Percentage of the injected dose remaining after 24 h: 30 % for *fac*-$[^{99m}Tc(OH_2)_3(CO)_3]^+$ and 46 % for small/single MNP-$^{99m}Tc(CO)_3$ micelles.