Electronic Supporting Information

Combination of Bioresponsive Chelates and Perfluorinated Lipid Nanoparticles Enables *in vivo* MRI Probe Quantification

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Nanoparticles size and structure characterization

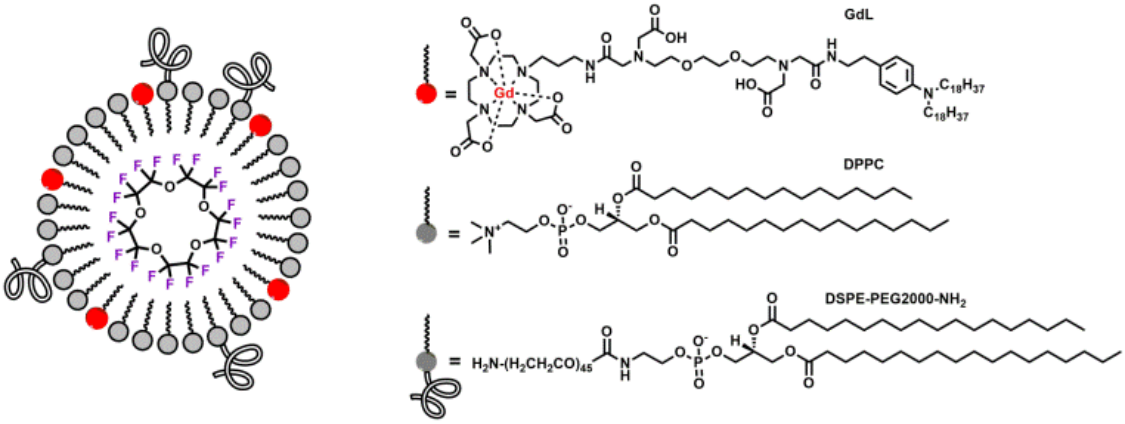


Figure S1. Schematic representation of the structure of **GdPFLNPs**.

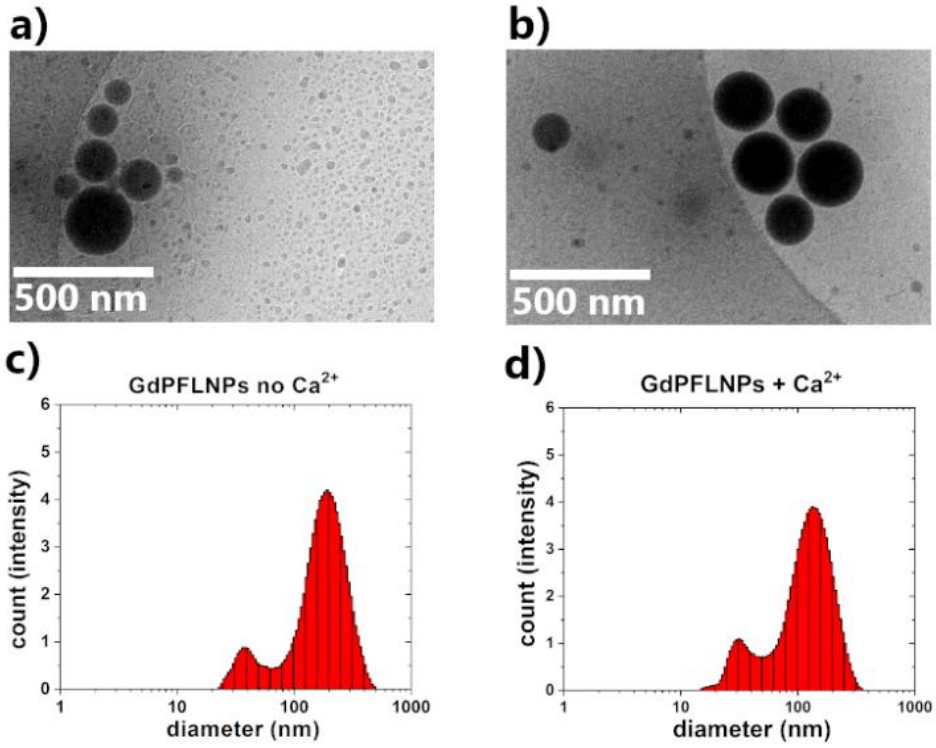
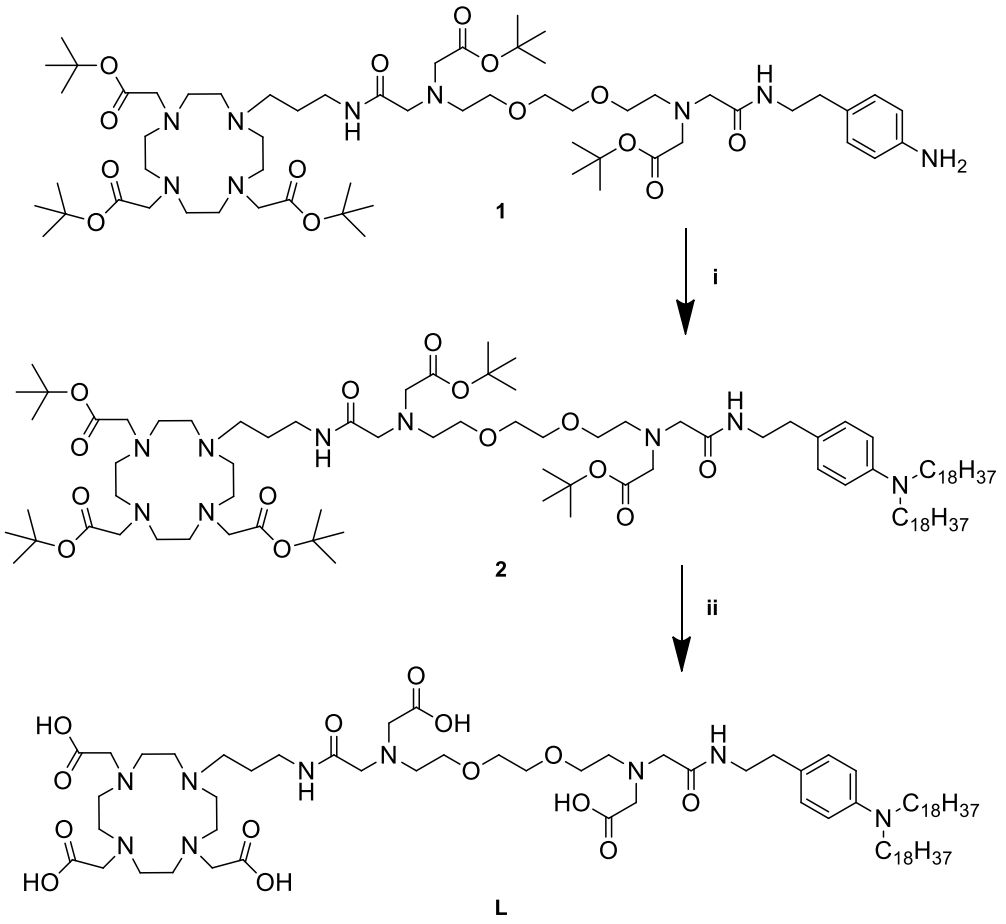


Figure S2. Cryo-TEM images and DLS size distribution of **GdPFLNPs**: Cryo-TEM images (**a**) without and (**b**) with Ca^{2+}; DLS size distribution (**c**) without and (**d**) with Ca^{2+}.

Experimental Section



Scheme S1. Synthesis of the ligand **L**: i) $BrC_{18}H_{37}$, K_2CO_3 in MeCN at 70 °C, 18 h; ii) formic acid, 55 °C, 18 h.

General remarks. All the chemicals commercially available and all the solvents were used as received, without further purification. DPPC was purchased by Sigma-Aldrich. DSPE-PEG2000-amine was purchased by Avanti Polar Lipids (Inc. 700 Industrial Park Drive Alabaster, Alabama 35007-9105). Perfluoro-15-crown-5-ether was purchased by Fluorochem Ltd (14 Graphite Way, Hadfield, Glossop SK13 1QH, UK). Compound **1** was synthesized as previously reported.[27] Low resolution mass spectra were recorded on an ion trap SL 1100 system Agilent with an electrospray ionization source. High resolution mass spectra were recorded on a Bruker Daltonics APEX II

(FT-ICR-MS) with an electrospray ionization source. ^{1}H, ^{13}C and ^{19}F NMR spectra and relaxometric experiments were performed on a Bruker Avance III 300 MHz spectrometer at 25 °C. The NMR spectra were obtained either in $CDCl_3$ or D_2O, using the deuterium lock frequency. Processing was performed using TopSpin 2.1 (Bruker GmbH) and ACD/SpecManager 9.0 (Advanced Chemistry Development, Inc.). The concentration of Gd^{3+} in analyzed solutions was determined using the bulk magnetic susceptibility shift (BMS).[33] The extrusion of nanoparticles was performed on a 1.5 mL Lipex® Thermobarrel Extruder (Evonik Nutrition and Care GmbH). All DLS measurements were performed on a Malvern-Nano-ZS (Zetasizer, software ver. 6.2) instrument. MRI measurements were performed on Bruker BioSpec 70/30 USR magnet (software version Paravision 5.1).

Synthesis of 2 and L. In a 25 mL round bottom flask, **1** (260 mg, 0.22 mmol) was dissolved in 10 mL of acetonitrile. Potassium carbonate (247 mg, 1.79 mmol) and 1-bromooctadecane (1.04 g, 3.13 mmol) were added to the stirring mixture, and the reaction was then heated to 70 °C and stirred for 5 days. The insoluble salts were removed by filtration and the solvent was evaporated *in vacuo*. The residue was purified with liquid chromatography (SiO_2, DCM/MeOH 1-10% gradient), obtaining pure **2** as a brown thick oil (153 mg, 41% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.86-0.90 (t, J = 6 Hz, 6H, CH_3), 1.26 (b, 62H, CH_2), 1.43-1.49 (b, 45H, CH_3tBu), 1.45 (b, 2H, CH_2), 2.31-2.44 (b, 14H, CH_2N), 2.69-2.85 (b, 12H, CH_2), 3.06 (b, 4H, CH_2), 3.18-3.20 (b, 8H, CH_2N), 3.30 (b, 4H, CH_2O), 3.45 (b, 14H, CH_2N), 3.54 (b, 4H, CH_2O), 6.54-6.56 (b, 2H, CH_{Ar}), 7.02-7.04 (b, 2H, CH_{Ar}); ^{13}C NMR (75 MHz, $CDCl_3$): δ 14.1 (CH_3), 22.6 (CH_2), 27.8 (CH_2), 28.0 (CH_{3tBu}), 29.7 (CH_{2chain}), 31.9 (CH_2), 34.7 (CH_2NH), 37.4 (CH_2NH), 50.3 (CH_2N), 51.1 (CH_2N), 51.8 (CH_2N), 53.5

(CH_2N), 55.7 (CH_2N), 56.6 (CH_2N), 57.0 (CH_2N), 58.7 (CH_2N), 59.2 (CH_2N), 68.8 (CH_2N), 69.5 (CH_2O), 70.2 (CH_2O), 81.4 (C_q), 82.4 (C_q), 82.7 (C_q), 82.8 (C_q), 111.8 (CH_{Ar}), 112.8 (CH_{Ar}), 129.4-129.5 (CH_{Ar}), 146.7 (CN_{Ar}), 169.7 (C=O), 170.6 (C=O), 171.1 (C=O), 171.3 (C=O), 171.5 (C=O), 172.4 (C=O), 172.6 (C=O), 173.5 (C=O); HRMS: m/z calculated for $C_{95}H_{178}N_9O_{14}Na^{2+}$ ($[M+H+Na]^{2+}$): 846.1690, found: 846.1682.

Formic acid (5 mL) was used to dissolve **2** (153 mg, 0.092 mmol) in a 10 mL flask and the mixture was heated at 55 °C for 18 hours. Solvent was removed *in vacuo*, obtaining **L** as a white flaky solid (120 mg, 95 % yield). ^1H and ^{13}C NMR signal assignation could not be performed because of the self-aggregation of **L** in $CDCl_3$ and D_2O (see spectra in Supporting Information). HRMS: m/z calculated for $C_{75}H_{137}CaN_9O_{14}Na^{2+}$ ($[M+Ca]^{2+}$): 713.9950, found: 713.9937.

Nanoparticles preparation. A 90 mg of a mixture of DSPE-PEG2000-amine/DPPC/**L** in a 5:75:20 ratio was dissolved in 3 mL of chloroform. The solvent was removed by gentle evaporation *in vacuo*. The obtained lipid film was rehydrated with 2 mL of a 300 mM suspension of PFCE in isotonic (NaCl 150 mM) HEPES buffer (50 mM) at pH 7.4. The resulting colloidal suspension was sonicated for 3 minutes at 55 °C and then extruded 10 times through polycarbonate filters (200 nm) at a pressure of 20-30 bar with an extruder at 55 °C. The obtained suspension was dialyzed overnight against isotonic HEPES/NaCl buffer. Subsequently, the **PFLNPs** emulsion was treated with $GdCl_3$, in order to form **GdL** complexes only on the external surface of the lipid membrane. The excess of lanthanide was then complexated with EDTA and removed by dialysis and ultracentrifugation, obtaining the final **GdPFLNPs** emulsion [Gd^{3+}] = 2.6 mM.

NMR relaxometry. ^{1}H and ^{19}F NMR T_1 and T_2 measurements were performed at 7 T and 25 °C, on a [Gd^{3+}] = 1.0 mM solution of **GdPFLNPs** in isotonic (NaCl 150 mM) HEPES buffer (50 mM) at pH 7.4. Titration profiles were obtained measuring T_1 and T_2 (using inversion recovery and Carr-Purcell-Meiboom-Gill experiments, respectively) of the solution after each addition of Ca^{2+} solution and plotting the values against [Ca^{2+}] expressed in equivalents ([Ca^{2+}]/[Gd^{3+}]).

DLS measurements. Two sets of experiments were measured for **GdPFLNPs** (0.02 mM [Gd^{3+}], 25 mM HEPES, pH 7.4) with and without the addition of 2 equivalents of Ca^{2+}. Sample solutions were filtered before measuring. Each DLS measurement consisted in 5 repetitions of 15 scans (1 scan = 12 s, refractive index 1.345, absorption 1%), without delays in between the scans and with a temperature equilibration time of 30 s prior to recording. The reported size was determined from the analysis of the obtained distributions.

Cryo-TEM. For electron cryo-microscopy of **GdPFLNPs**, samples were vitrified in liquid ethane with a EM GP plunge freezer (Leica) on glow discharged grids (Quantifoil 300 mesh copper grids R2/2) before and after the addition of Ca^{2+}. 3 µL of sample (10 mg/mL) was applied onto the grid and transferred into the plunge freezer with the chamber set to 21°C and 80% humidity, 3 seconds preblot time and 2 seconds blotting. The samples were transferred with a Gatan 626 cryotransfer holder and imaged in a Tecnai Spirit (ThermoFisher Scientific) equipped with a F416 CMOS camera (TVIPS).

MRI on tube phantoms. T_1w ^{1}H and ^{19}F MR images where acquired using Fast Low Angle Single Shot (FLASH), T_2w with Multi-Spin-Multi-Echo (MSME) and Rapid Acquisition with Relaxation Enhancement (RARE), lastly T_2/T_1w using balanced Steady-State Free Precession (trueFISP) pulse sequences. The phantom consisted of 6 x 400 uL vials containing 1.0 and 0.5 mM **GdPFLNPs**, without and with 1 eq. of Ca^{2+}. Additionally, 2 vials containing TFA in concentrations of 100 and 16 mM (300 and 48 mM fluorine, respectively) were used as references. MR images were acquired using a Bruker volume coil (RF RES 300 1H/19F 075/040 LIN/LIN TR). ^{1}H MRI general parameters were: Field of view (FOV) = 32 x 26 mm, matrix size (MTX) = 160 x 130, spatial resolution 200 x 200 μm, slice thickness 1 mm. Sequence specific parameters are listed below. $\boldsymbol{T_1}$**w:** flip angle (FA) = 90^0, repetition time (TR) = 171 ms, echo time (TE) = 2.75 ms, number of averages (NA) = 10, acquisition time (TA) = 3 minutes 42 seconds. $\boldsymbol{T_2/T_1}$**w**: FA = 50^0, TR = 2.3 ms, TE = 1.15 ms, NA = 10, TA = 14 seconds. $\boldsymbol{T_2}$**w**: TR = 1300 ms, TE = 15 ms, NA = 10, TA = 28 minutes 10 seconds. ^{19}F MRI parameters were: FOV= 32 x 26 mm, MTX= 64 x 52, spatial resolution 500 x 500 μm, slice thickness 5 mm, FA = 90^0, TR = 89 ms, TE = 2.05 ms, NA = 790, TA = 60 minutes 56 seconds.

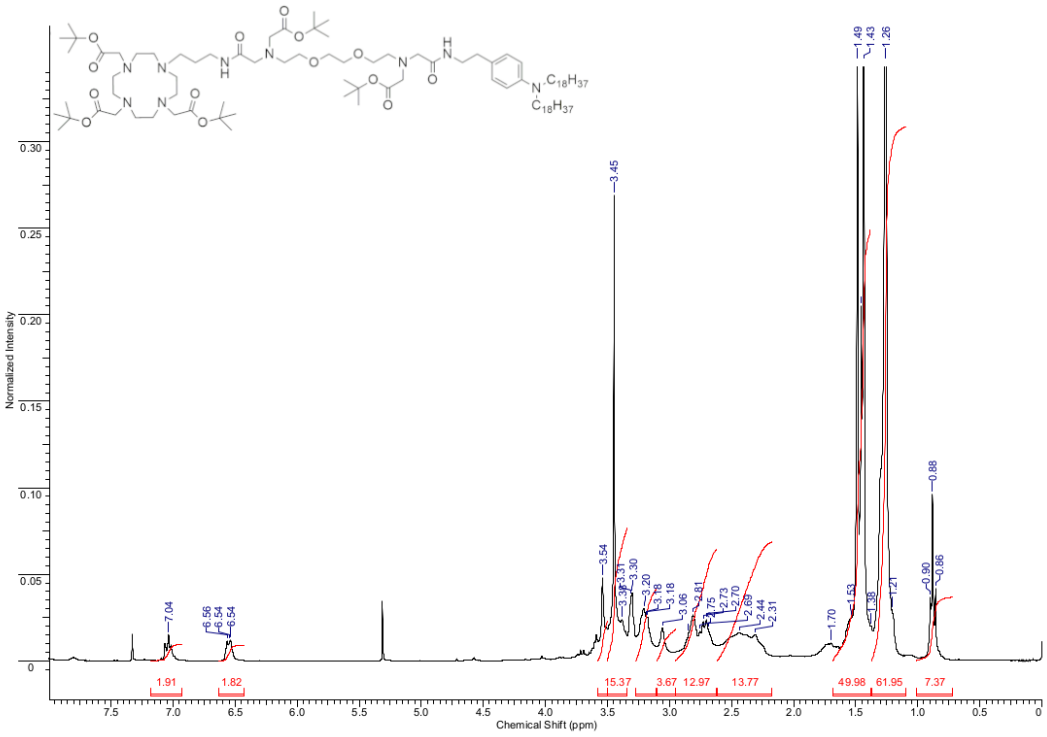
In vivo MRI. Experiments were conducted on male Sprague Dawley rats (300 – 340 g, Charles River Laboratories). Animals were housed and maintained in controlled environmental conditions with 12:12 h light-dark cycle at least for 7 days before the experiment, with food and water provided *ad libitum*. All experiments with animals were approved by the local authorities (Regierungspräsidium Tübingen). After the animal induction with 2.5 % isoflurane in O_2 (Forene, Abbott, Wiesbaden, Germany), urethane was injected i.p. (1.2 g/ kg), giving half the dose on each lateral side of the

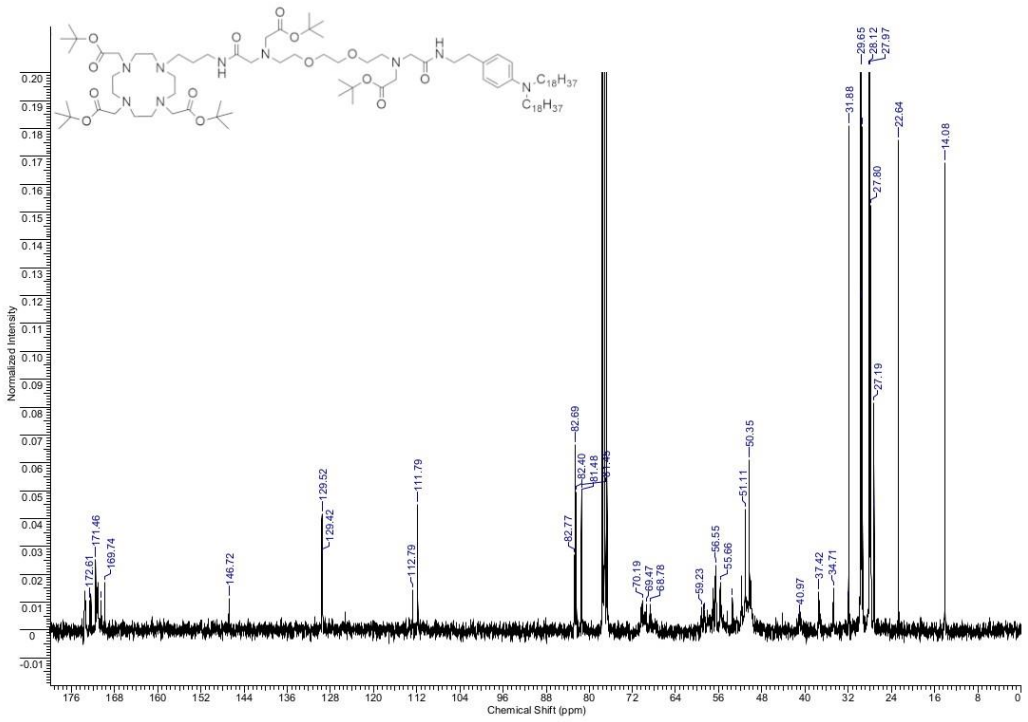
animal. Following 60 minutes animal was monitored closely, and if needed additional (1/10) dose of urethane was given. Once deep anaesthesia was confirmed by absence of pedal withdrawal, stereotaxic (Stoelting Co., IL, US) surgical procedure was initiated, using inhalation mixture of oxygen and nitrous oxide (1:2) to ensure analgesia. Craniotomy was made using manual drill (ML = 2.4, AP = 0.2, DV = 2.5, angle 19.5 ^{0}) and dura was removed. Thereafter, probe was delivered intracranially at a rate of 38 nl / min for a total volume of 3.05 µL with a precision pump (70-4507, Harvard Apparatus) using a 5 µl Hamilton syringe. The body temperature of the animal was maintained at 37.0 ± 0.5 ^{o}C by a rectal probe with a feedback controlled heat pad (50-7221-F, Harvard Apparatus, MA, US). After surgery the animal was transferred to the MRI scanner. A reference sample (100 mM TFA) was placed next to animal head. Inhalation mixture was changed to a mixture of air (800 mL / min) and oxygen (50 mL / min). Body temperature was monitored with a rectal probe and kept around 37 ^{0}C using water bath. Oxygen saturation and heart rate were monitored throughout surgery and experiment with a pulse oximeter (MouseOx, Starr Life Sciences).

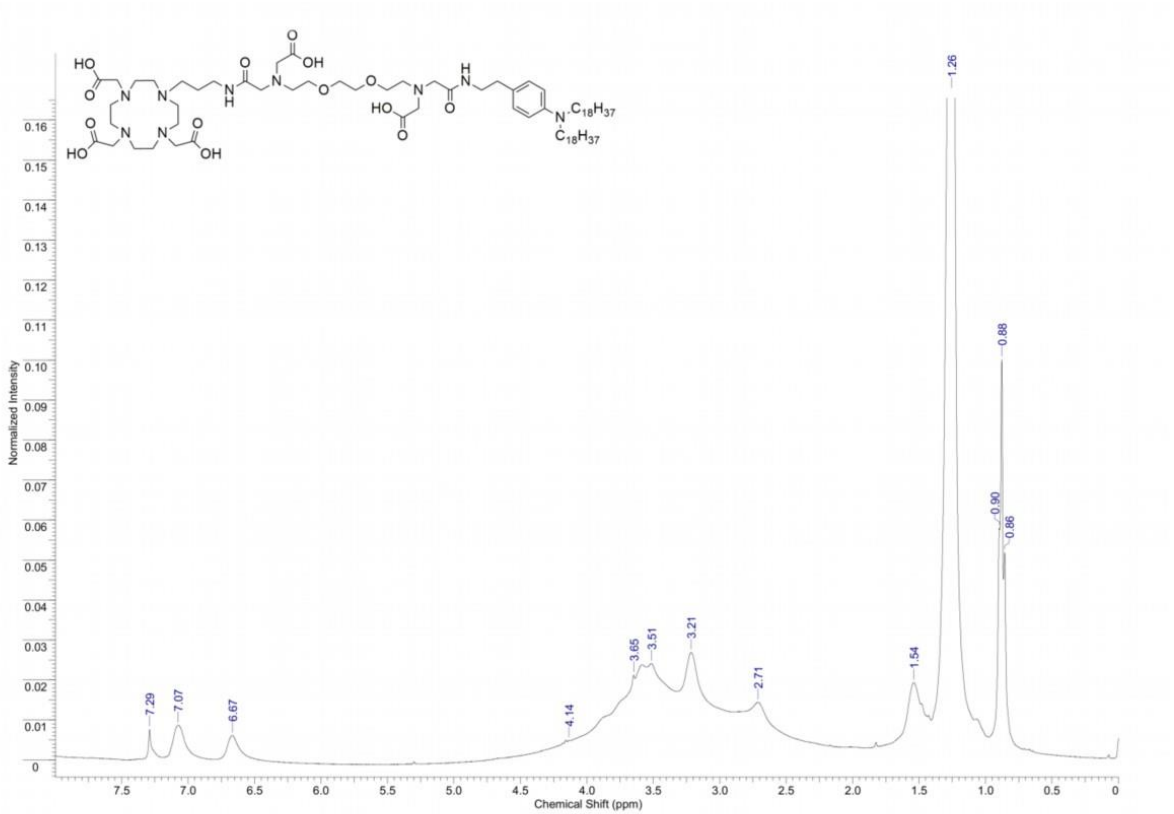
^{1}H MRI *in vivo* was performed using a Bruker volume coil (RF RES 300 1H/19F 075/040 LIN/LIN TR). General acquisition parameters were: FOV = 32 x 32 mm, slice thickness = 2 mm. Other parameters are listed for each imaging protocol. ***T*$_1$w:** MTX = 80 x 80, spatial resolution = 400 x 400 µm, FA = 90^0, TR = 21.58 ms, TE = 2.35 ms, NA = 100, TA = 2 minutes 52 seconds; ***T*$_2$w:** MTX = 160 x 120, spatial resolution = 200 x 267 µm, Rare factor = 8, TR = 2000 ms, TE = 9.73 ms, NA = 30, TA = 15 minutes; ***T*$_2$/*T*$_1$w:** MTX = 160 x 160, spatial resolution = 200 x 200 µm, FA = 50^0, TR = 2.6 ms, TE = 1.3 ms, NA = 100, TA = 4 minutes 43 seconds.

Combined $^{19}F/^{1}H$ MRI measurements were performed using a custom build surface coil for ^{1}H and ^{19}F. General imaging parameters were: FOV = 19.2 x 19.2 mm, MTX = 32 x 32, spatial resolution 600 x 600 μm, slice thickness 3 mm. Other parameters are given below. **^{1}H MRI:** FA = 30^{0}, TR = 94 ms, TE = 1.55 ms, NA = 20, TA = 60 seconds. **^{19}F MRI:** FA = 15^{0}, TR = 5.2 ms, TE = 1.55 ms, NA = 21000, TA = 61 minutes 49 seconds (see Supporting Information S7-8 for details about the images realignment).

^{1}H and ^{13}C NMR spectra of 2 and L



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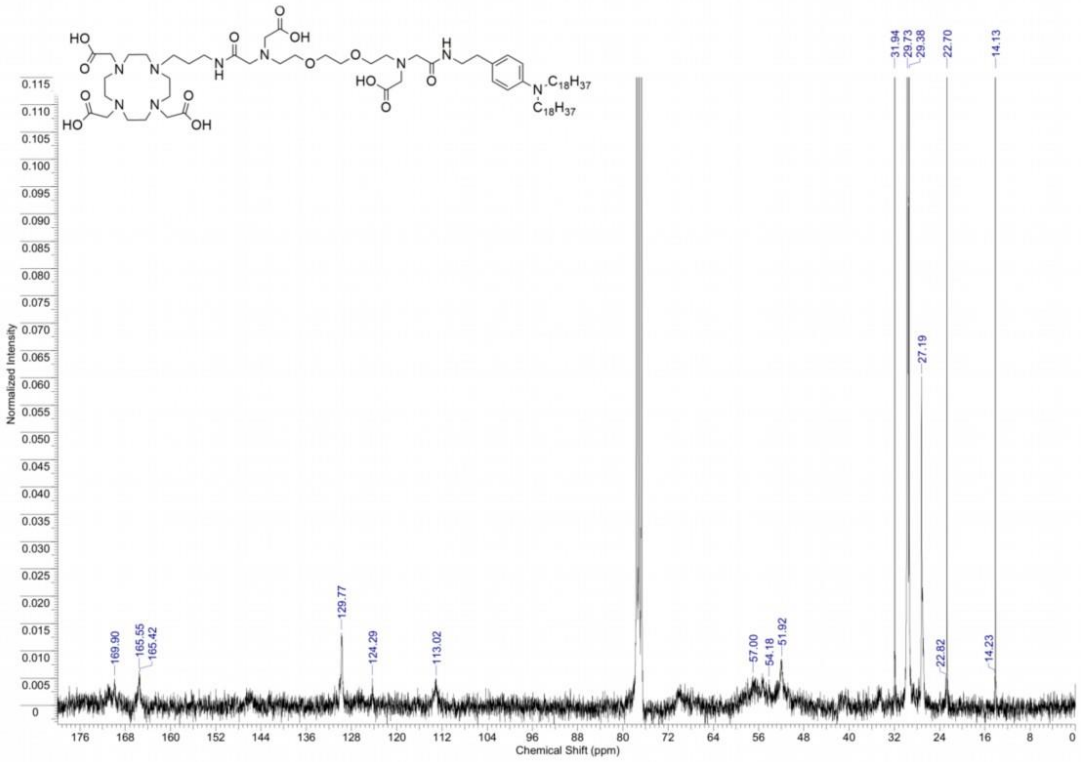
0.86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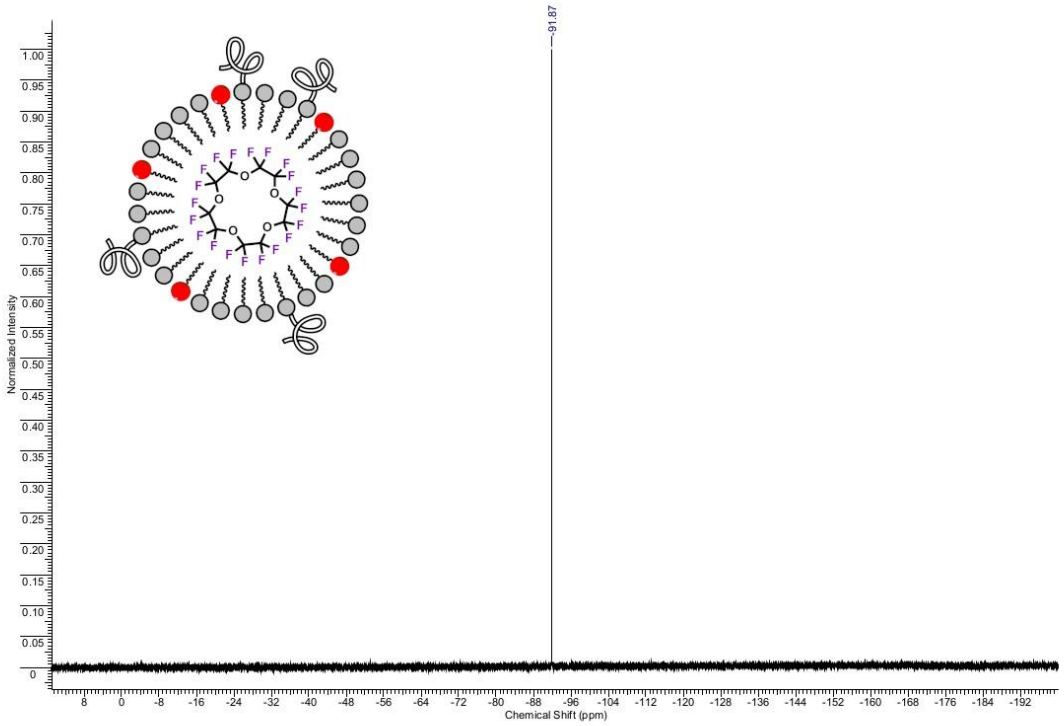
22.70
14.23
14.13

^{19}F NMR of GdPFLNPs



Quantification of fluorine by means of ^{19}F NMR

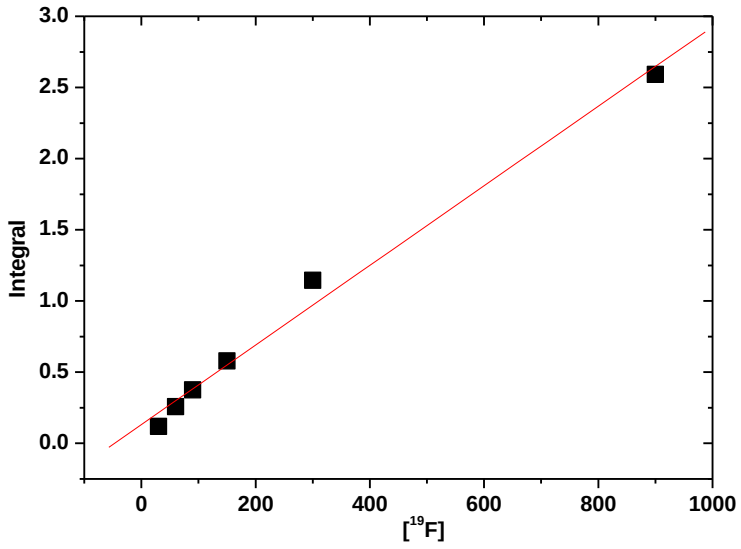


Figure S3. Calibration curve obtained by measuring the area under the ^{19}F NMR peak of TFA for 6 samples at different concentration, using a coaxial capillary containing NaF 104 mM for normalization. A linear fit of the data resulted in a slope of 0.0028 ± 0.0002 and an intercept of 0.1318 (R^2 = 0.99).

For a [Gd^{3+}] = 1.0 mM, a solution of **GdPFLNPs** the provided value of the normalized integral I=1.6572. Using the Eq. S1, the obtained [^{19}F] value was 554.8 mM.

$$I = 0.0028\ [^{19}F] + 0.1318 \qquad \text{(S1)}$$

^{1}H and ^{19}F MRI on tube phantoms

Table S1. Average (n = 3) SNR values for the ^{1}H MRI tube phantom images.

Sample	T_1w SNR (mean)	T_2w SNR (mean)	T_2/T_1w SNR (mean)
1	121.3 ± 1.7	57.1 ± 2.0	28.2 ± 10.0
2	111.2 ± 1.6	68.1 ± 2.7	42.3 ± 14.7
3	164.5 ± 2.6	40.7 ± 1.1	31.5 ± 10.9
4	161.8 ± 2.5	65.7 ± 2.3	46.6 ± 16.1
5	29.9 ± 0.6	29.7 ± 1.9	35.3 ± 11.3
6	29.3 ± 0.7	28.1 ± 1.7	34.7 ± 11.4

Table S2. SNR values for the ^{19}F MRI tube phantoms images.

Sample	1	2	3	4	5	6
SNR	4.9	4.8	10.3	9.6	6.2	2.5

The contrast enhancement % reported in Figure 4e was calculated using the values from Table S1 and Eq. S2 for each [Gd^{3+}] couple of samples with all the three ^{1}H MRI protocols used.

$$\text{Contrast Enhancement} = \frac{SNR_{+Ca} - SNR_{No\ Ca}}{SNR_{NoCa}}\ x\ 100 \qquad \text{(S2)}$$

Generation of [Gd^{3+}] maps

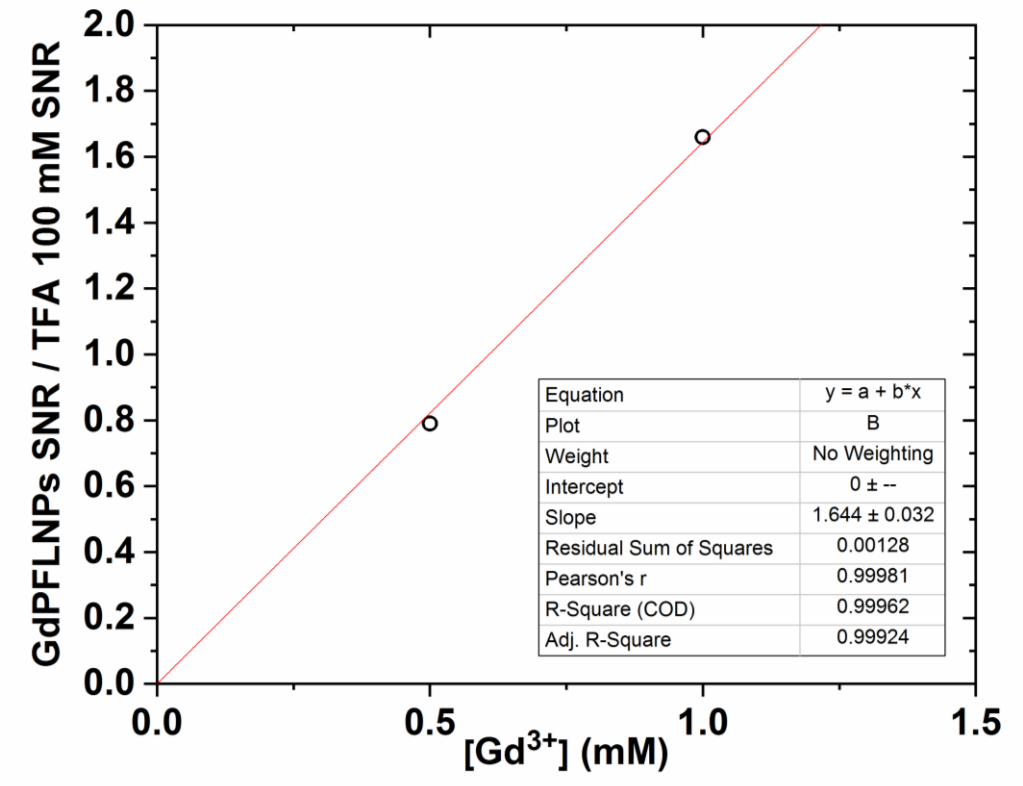


Figure S4. Linear fit of the SNR values obtained with the ^{19}F MRI on tube phantoms.

For generating Gd maps, [Gd] was plotted as a function of the ratio ^{19}F SNR (**GdPFLNPs**) *in vivo* / SNR (TFA) obtained from the MRI experiment on tube phantoms (Figure S2). The acquired linear function was then applied voxel-wise to the SNR (**GdPFLNPs**) / SNR (TFA) ratio map, obtaining the [Gd] distribution map.

SNR (TFA) was obtained as an average signal intensity of the voxels corresponding to the reference sample divided with the standard deviation of the background.

Because the frequency of the TFA sample was used as basic ^{19}F frequency for *in vivo* MRI, the voxels of the area of injection appear to be shifted (chemical shift artifact), compared to the ^{1}H image. This effect could be observed already in the ^{19}F images of the tube phantoms (Figure 4d), which is due to the frequency offset (-17 ppm) of the GdPFLNPs ^{19}F signal from the base frequency used. In particular, the frequency offset generated a shift of ~3 voxels, as

calculated by Eq. S3. For these reasons, the voxel with the highest SI of the ^{19}F image was matched the one of the ^{1}H image.

$$\frac{\Delta_{Hz}}{BW} * MTX = shift\ (voxels) \qquad \text{(S3)}$$

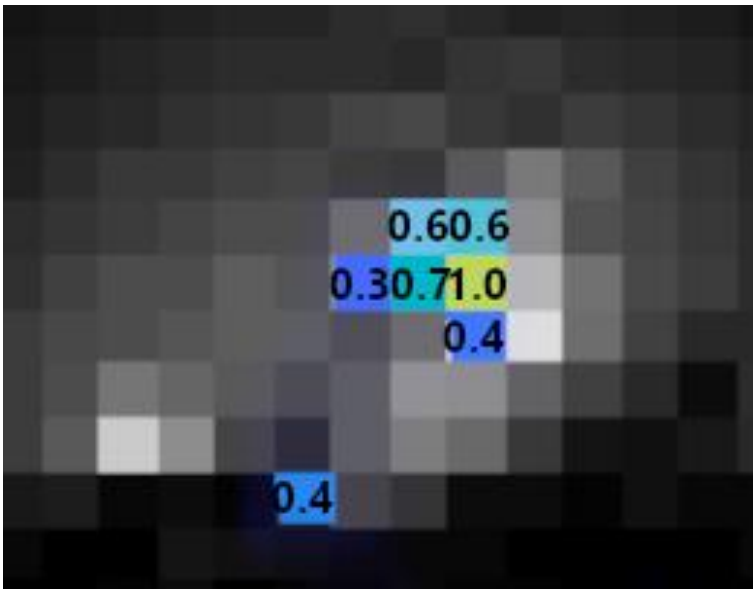


Figure S5. Magnification of the area of injection of Figure 5f. Overimposed are the [Gd^{3+}] values for each voxel (in mM), calculated as described above.