

## Supplementary Information

For

### Gold nanoparticles functionalised with stable, fast exchanging Gd<sup>3+</sup> chelates as high relaxivity Contrast Agents for MRI

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#### Materials and methods

Chemicals were purchased from Sigma-Aldrich and used without further purification. Cyclen was purchased from Chematech, France. Analytical grade solvents were used and, unless specified, not further purified. Reactions were monitored by TLC on silica gel by examination under UV light (250 nm) and staining with iodine vapour. Preparative chromatography was carried on Silica Gel 60 (230-400 mesh). Ion exchange chromatography was performed on Dowex 1X2 OH<sup>-</sup> (50-100 mesh) resin. Size Exclusion Chromatography (SEC) was performed on Sephadex G10 (40-120 μm). Dialysis was performed against water on cellulose membranes with MWCO of 1KDa. UV-VIS spectra were acquired with a Shimadzu UV-2501PC spectrophotometer. The size distribution and zeta potential of the gold nanoparticles were determined with a Malvern Zetasizer, NANO ZS (Malvern Instruments Limited, UK), using a He-Ne laser (wavelength of 633 nm) and a detector angle of 173°. Mass spectrometry was performed at CACTI- Vigo, Spain. <sup>1</sup>H and <sup>13</sup>C NMR spectra were run on Varian Unity Plus 300, Bruker Avance-3 400 Plus and Varian VNMRS 600 NMR spectrometers. Chemical shifts (δ) are given in ppm relative to the CDCl<sub>3</sub> solvent (<sup>1</sup>H, δ 7.27; <sup>13</sup>C 77.36) as internal standard. For <sup>1</sup>H and <sup>13</sup>C NMR spectra recorded in D<sub>2</sub>O, chemical shifts (δ) are given in ppm, respectively, relative to TSP as internal reference (<sup>1</sup>H, δ 0.0) and *tert*-

butanol as external reference ( $^{13}\text{C}$ ,  $\text{CH}_3$   $\delta$  30.29).  $^{13}\text{C}$  NMR spectra were proton broadband decoupled using a GARP-1 modulated decoupling scheme.

The  $^1\text{H}$  Nuclear Magnetic Resonance Dispersion (NMRD) measurements were performed by using a Stelar Spinmaster FFC NMR relaxometer (0.01-20 MHz) equipped with a VTC90 temperature control unit. At higher fields, the  $^1\text{H}$  relaxivity measurements were performed on Bruker Minispecs mq30 (30 MHz), mq40 (40 MHz) and mq60 (60 MHz), as well as Bruker Avance spectrometers connected to 2.35 T, 4.7 T and 9.4 T superconducting magnets. In each case, the temperature was measured by a substitution technique. Variable temperature measurements were performed at 25 and 37 °C. The relaxometric pH dependence and transmetallation studies with  $\text{Zn}^{2+}$  of the nanoparticles were performed on a Bruker Minispec mq20 (20 MHz). The NMRD profiles have been analysed using the Visualiseur/Optimiseur 3.6 program<sup>1</sup> running on a Matlab® 6.5 platform.

## Synthesis and characterization

### Preparation of the DO3A-*N*-( $\alpha$ -cystamido)propionate ligand (4):

**Synthesis of amide (3):** Orthogonally protected cyclen **2**<sup>2</sup> (1.62 g, 2.21 mmol) was dissolved in a mixture of dichloromethane/trifluoroacetic acid (45 cm<sup>3</sup>, 1/3 v/v) and stirred overnight at room temperature. The solvent was evaporated at reduced pressure and the residue was re-dissolved in dichloromethane and evaporated again. This procedure was repeated several times to give a thick light yellow oil.  $^1\text{H}$  NMR spectroscopy ( $\text{CDCl}_3$ ) revealed the disappearance of the signals assigned to the *Boc* groups in precursor compound **2**. The integration ratio between the methine proton  $\text{NCH}_2\text{CH}$  and the methyl or methylene protons (from the ethyl ester groups) indicated that no deprotection of the ethyl ester groups occurred. Amine deprotected compound (**2**) (2.21 mmol, assuming quantitative deprotection) was dissolved in dichloromethane (30 cm<sup>3</sup>). The solution was adjusted to pH 8-9 (pH paper) by dropwise addition of DIPEA (~7.0 cm<sup>3</sup>). To this solution was sequentially added *N*-Boc-*S*-Trityl-*L*-cysteine (1.02 g, 2.21 mmol), Hydroxybenzotriazole (0.557 g, 2.64 mmol) and a solution of DCC (0.752 g, 2.64 mmol) in dichloromethane (5 cm<sup>3</sup>). The reaction mixture was left stirring at room temperature overnight. The DCU byproduct was removed by filtration and the

sample was concentrated under reduced pressure. The residue was re-dissolved in ethyl acetate (100 cm<sup>3</sup>), and the solution was washed sequentially with KHSO<sub>4</sub> (1M, 3 x 30 cm<sup>3</sup>), NaHCO<sub>3</sub> (saturated solution, 3 x 30 cm<sup>3</sup>) and brine (3 x 30 cm<sup>3</sup>). The organic phase was concentrated under reduced pressure and the residue was purified by a flash chromatography (100% CH<sub>2</sub>Cl<sub>2</sub> → CH<sub>2</sub>Cl<sub>2</sub>/EtOH (70:30)) to afford amide (**3**) (0.736 g, 34%). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): δ= 1.26 (m, 9 H, C(O)OCH<sub>2</sub>CH<sub>3</sub>), 1.46 (s, 9H, C(CH<sub>3</sub>)<sub>3</sub>), 2.2-3.60 (broad, overlapped signals with a integration corresponding to 26 H; N(CH<sub>2</sub>)<sub>2</sub>N, NCH<sub>2</sub>CO<sub>2</sub>Et, NHBocCH<sub>a</sub>H<sub>b</sub>CH, NCH<sub>a</sub>H<sub>b</sub>CH), 3.80 (s, 3H, C(O)OCH<sub>3</sub>), 4,15 (m, 6H, OCH<sub>2</sub>CH<sub>3</sub>), 4.77 (m (br), 1H, NHBocCH<sub>a</sub>H<sub>b</sub>CH), 5.87 (m (br), 1H, NCH<sub>a</sub>H<sub>b</sub>CH), 7.1-7.6 (m, 15 H, Trt). HRMS (ESI): m/z: calcd for C<sub>51</sub>H<sub>73</sub>N<sub>6</sub>O<sub>11</sub>S [M+H]<sup>+</sup>: 977.5058, found: 977.5021.

#### **Preparation of fully deprotected DO3A-N-(α-cystamido)propionate metal chelator**

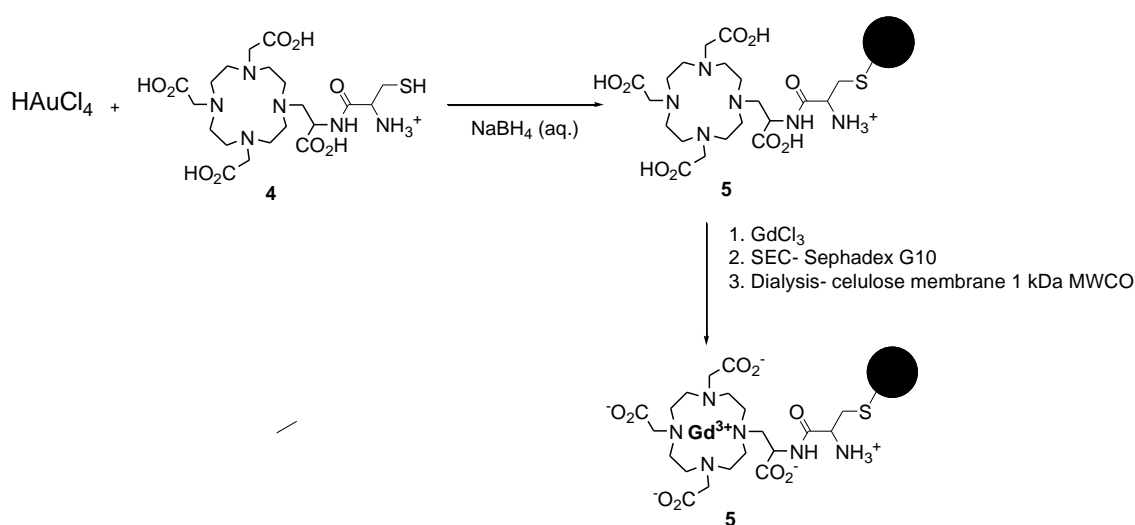
**(4):** Compound **(3)** (0.706 g, 0.722 mmol) was dissolved in hydrochloric acid 3 M (20 cm<sup>3</sup>; water (5 cm<sup>3</sup>)/ethanol (10 cm<sup>3</sup>)/hydrochloric acid 37% (5 cm<sup>3</sup>)). The solution was left stirring at room temperature overnight. The solvent was removed under reduced pressure at room temperature and the residue was taken up into a small volume of water (~ 20 cm<sup>3</sup>) and filtered. The solution was concentrated at reduced pressure, the residue was dissolved in water (~ 15 cm<sup>3</sup>) and the solution was adjusted to pH ~ 10-11 by adding small portions of Dowex 1X2-100-OH<sup>-</sup> resin. The suspension was kept under stirring at room temperature for 2 hours. The wet resin was transferred into a chromatography column, washed with water (~ 50 cm<sup>3</sup>) and eluted with 0.1 M hydrochloric acid. The relevant fractions, identified by TLC (ethanol water 1/1, revelation with iodine vapor) were pooled, concentrated at room temperature and further dried under vacuum to afford the final deprotected compound **(4)** in the hydrochloride form, as a light yellow solid (0.276 g, 71.2%).

<sup>1</sup>H NMR (300 MHz, D<sub>2</sub>O): δ= 2.2-4.40 (broad, overlapped signals with an integration corresponding to, 22 H: N(CH<sub>2</sub>)<sub>2</sub>N and NCH<sub>2</sub>COOH), 3.07 (m, 2H, CH<sub>2</sub>SH), 3.33 (m, 2H, CH<sub>2</sub>CH), 4.22 (m, 1H, CH<sub>2</sub>CH), 4.61 (m, 1H, SHCH<sub>2</sub>CH). <sup>13</sup>C NMR (75.4 MHz, D<sub>2</sub>O): selected signals: 22.14 (CH<sub>2</sub>SH), 46.34 (CH<sub>2</sub>), 47.63 (CH<sub>2</sub>), 48.16 (CH<sub>2</sub>), 48.69 (CH<sub>2</sub>), 49.98 (CH<sub>2</sub>), 51.01 (CH<sub>2</sub>), 53.43 (CH<sub>2</sub>), 53.80 (CH<sub>2</sub>CH), 54.03 (CHCH<sub>2</sub>SH), 54.14 (CH<sub>2</sub>), 54.27 (CH<sub>2</sub>), 55.15 (CHCH<sub>2</sub>), 56.09 (CH<sub>2</sub>), 169.03 (NHC(O)), 169.14

(C(O)), 170.60 (C(O)), 176.61 (CHC(O)), 177.93 (C(O)). HRMS (ESI):  $m/z$ : *calc* for  $C_{20}H_{37}N_6O_9S$   $[M+H]^+$ : 537.2343, *found*: 537.2325.

### Preparation gold nanoparticles functionalized with Gd(DO3A-*N*-( $\alpha$ -cystamido)propionate) chelates.

The strategy envisaged for preparing gold nanoparticles functionalized with Gd(DO3A-*N*-( $\alpha$ -cystamido)propionate) chelates is depicted (as cartoon) in Scheme 1SI).



Scheme 1SI. General strategy (cartoon representation) for the preparation of gold nanoparticles functionalized with Gd(DO3A-*N*-( $\alpha$ -cystamido)propionate) chelates.

An aqueous solution of DO3A-*N*-( $\alpha$ -cystamido)propionate (**4**) ligand (46.13 mM, 4.66  $cm^3$ , 0.099 mmol) was added dropwise, under magnetic stirring, to an aqueous solution of  $H[AuCl_4]$  (58.85 mM, 1.64  $cm^3$ , 0.096 mmol; molar ratio Au/ligand (1/1)). Addition of the ligand to the  $Au^{3+}$  solution generated initially a dark orange color which faded away with further ligand addition. To the stirring reaction mixture was added, in one aliquot, a solution of  $NaBH_4$  (20.40 mM, 0.217  $cm^3$ , 0.094 mmol; molar ratio Au/ligand/ $NaBH_4$  1/1/1). The reaction mixture turned immediately dark brown and was left stirring at room temperature for 24 hours. The nanoparticles preparation was adjusted to pH  $\sim$  7.0 with aqueous  $NaOH$  1 M and filtered through a 0.20  $\mu m$  PTFE syringe filter. A small sample (1.0  $cm^3$ ) was kept for further characterization. To the remaining sample ( $\sim$  6.0

cm<sup>3</sup>) was added a solution of GdCl<sub>3</sub>.6H<sub>2</sub>O (0.0211 g, 0.080 mmol; 1 molar equivalent in relation to the total amount of ligand used in the nanoparticles synthesis) in water (1.0 cm<sup>3</sup>). During the addition of the Gd<sup>3+</sup> ion the solution was kept at pH ~ 5.5 by adding NaOH 0.1 M. The reaction mixture was left stirring at room temperature for 24 hours, adjusted to pH ~7 by with aqueous NaOH (1 M solution) and filtered through a 0.20 μm PTFE syringe filter. The gold nanoparticle preparation was purified by size exclusion chromatography using Sephadex G10 (40-120 μm) eluting with water. During elution a colored broad band separated in the column. No attempt was made to fractionate the sample, the full band was collected as a single fraction (~ 20 cm<sup>3</sup>). The nanoparticles preparation was further purified by dialysis against water using a cellulose membrane with 1 KDa MWCO. The xylenol orange test indicated the absence of free Gd<sup>3+</sup> in the gold nanoparticles preparation. The Gd and Au concentration of the nanoparticle's preparation ([Au]= 3.77 mM; [Gd]= 1.24 mM; [Au]/[Gd]= 2.9) was determined by ICP analysis.

The binding mode of the Gd(DO3A-*N*-(α-cystamido)propionate) chelates to the gold surface, represented in Scheme 1SI through the thiol group of the cysteine linker, is the most likely anchorage mode. Several authors have suggested the simultaneous binding of the thiol and amine groups of cystein to gold (forming a chelate ring) as the (most likely) anchorage mode of cystein containing peptides to gold NPs.<sup>3</sup> The band at 3500 cm<sup>-1</sup> in the IR spectrum (KBr pellets) of our nanoparticles (Figure 1SI) is assigned by several authors to vibrational modes of the -NH<sub>3</sub><sup>+</sup> group in cystein functionalized gold nanoparticles.<sup>4</sup>

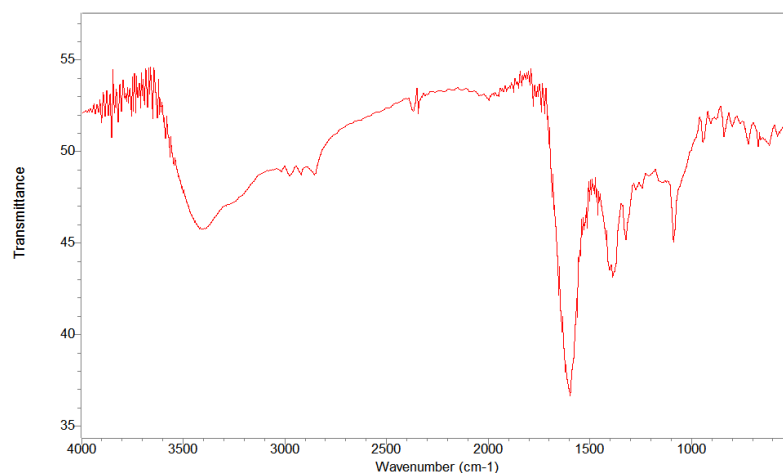


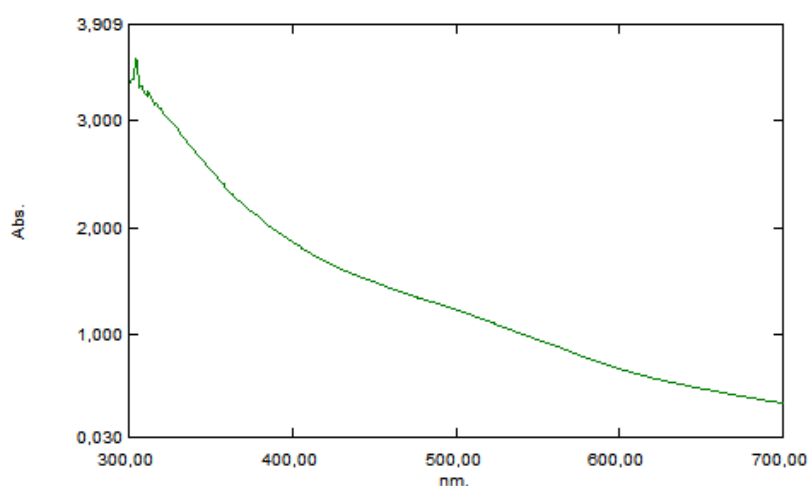
Figure 1SI. IR spectrum (KBr) of gold NPs functionalized with Gd(DO3A-*N*-( $\alpha$ -cystamido)propionate) chelates

The IR spectrum of the functionalized gold NPs suggests that the nanoparticles are linked to the gold core through the thiol group only. The broad band at around  $3500\text{ cm}^{-1}$  has been assigned to vibrational modes of the  $-\text{NH}_3^+$  in cystein functionalized gold NP's.<sup>3,4</sup>

If the amine group is not involved in the binding to gold, then the effect of the solution pH on the overall charge of the immobilized complexes should be apparent as a pH-dependent zeta potential. The negative Zeta potential values measured at pH 7.0 and 10.0 (Figure 3BSI) suggest that the chelates (and whole nanoparticles) bear net negative charges. Moreover, the value and shape of the Zeta potential distribution curves at pH 7.0 and 10.0 suggests a higher extent of ionization of the amine group at pH 10. None the less the putative ionization of the amine group seems not to affect substantially the relaxivity of the NP's in the pH range 2-12 (Figure 7ASI).

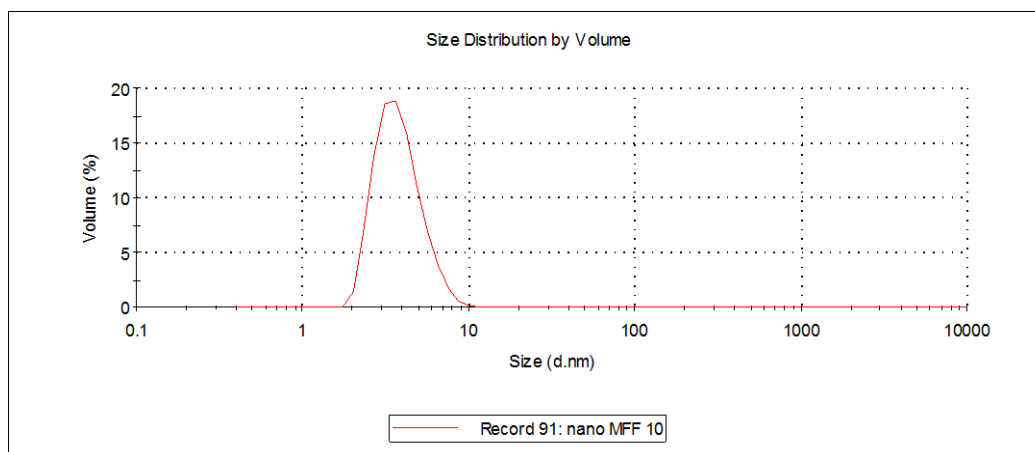
### UV-VIS spectroscopy

A very ill-defined plasmon band is apparent in the UV-VIS spectrum of the gold nanoparticles functionalized with Gd(DO3A-*N*-( $\alpha$ -cystamido)propionate) chelates, suggesting that the nanoparticles display a diameter bellow 6 nm.



**Figure 2SI.** UV-VIS spectrum for the preparation of nanoparticles functionalized with Gd(DO3A-*N*-( $\alpha$ -cystamido)propionate) chelates (diluted by a factor of 3, pH 7.0).

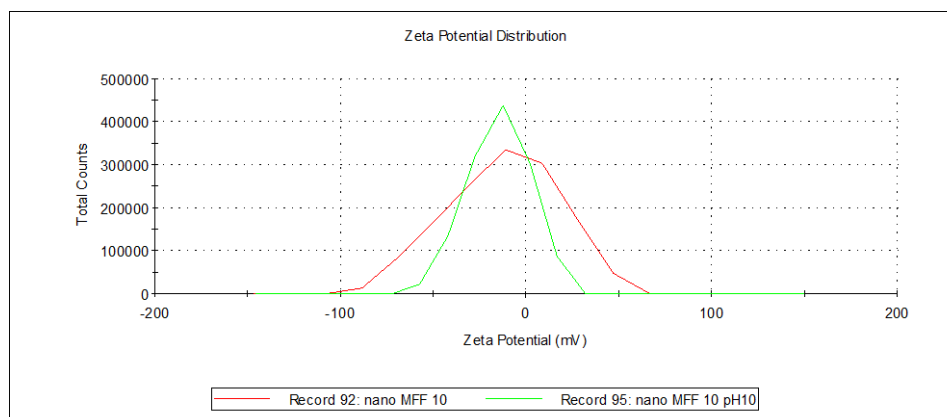
### Size and zeta potential characterization by DLS



Peak	Diam. (nm)	% Volume	Width (nm)
	3.885	100	1.258

**Figure 3ASI.** DLS size distribution (Volume) for the preparation of nanoparticles functionalized with Gd(DO3A-*N*-( $\alpha$ -cystamido)propionate) chelates at pH 7.0.

From the DLS experiments (Figure 3ASI), one can conclude that the main population of nanoparticles in solution displays an average hydrodynamic diameter around 3.9 nm.

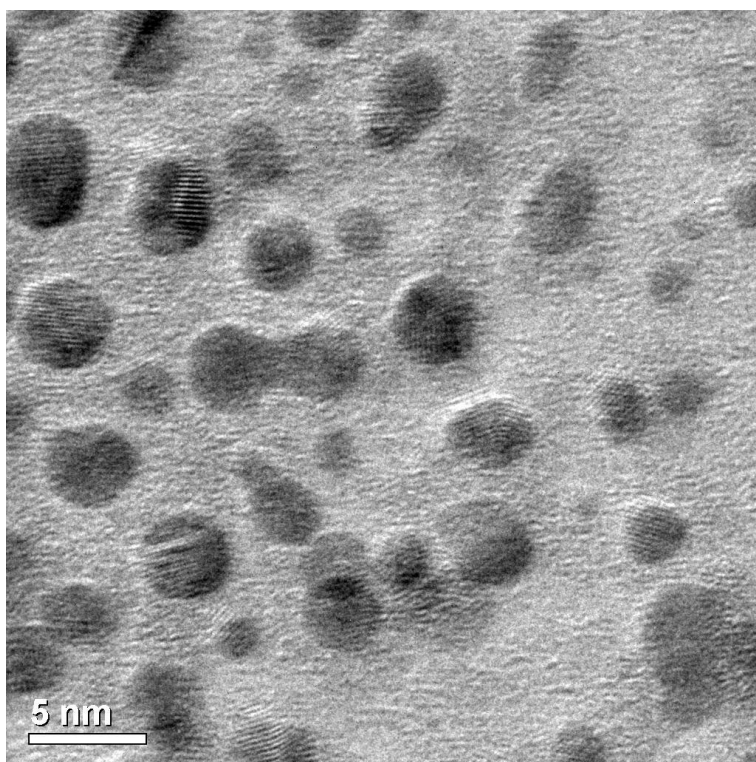




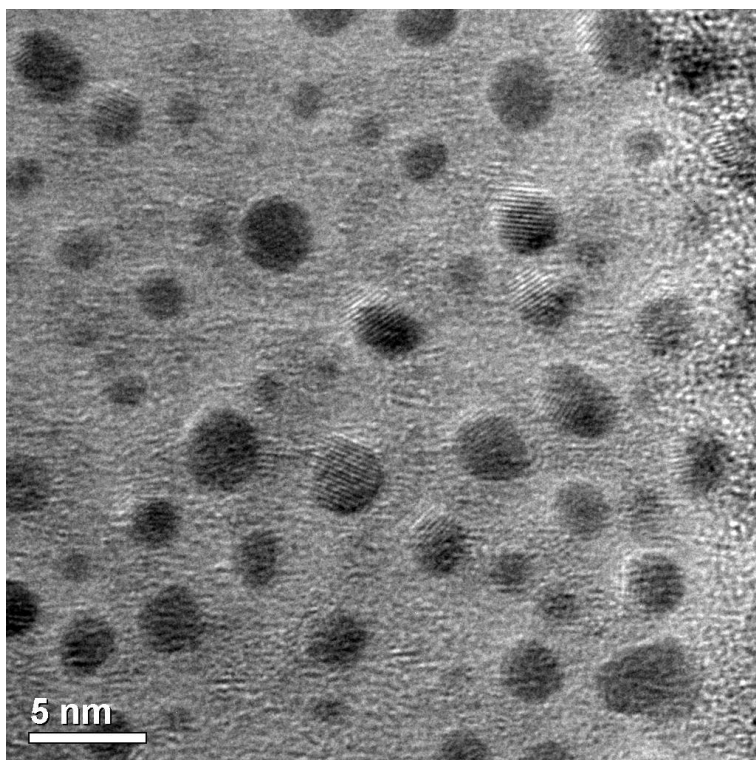
**Figure 3BSI.** Zeta potential distribution at pH 7.0 (red curve) and pH 10.0 (green curve) for the preparation of nanoparticles functionalized with Gd(DO3A-*N*-( $\alpha$ -cystamido)propionate) chelates.

Figure 3BSI shows that the nanoparticles display negative zeta potential both at pH 7.0 and pH 10.0, with a broader distribution at pH 7.0.

### TEM analysis of the nanoparticles





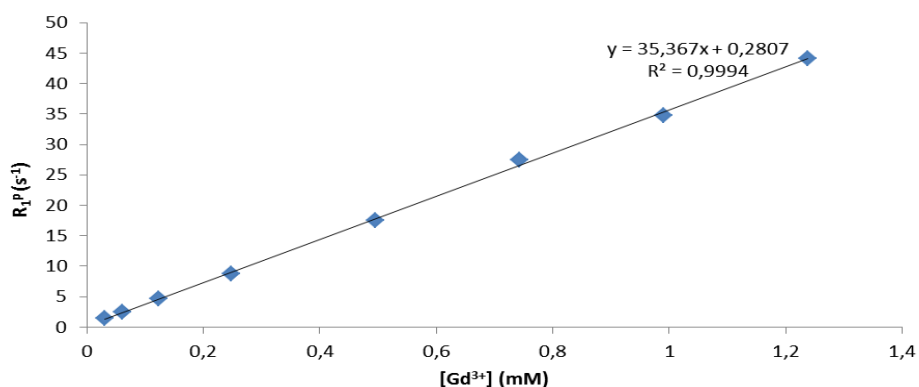


**Figure 4SI.** TEM image of the Au-NPs. The image was measured with a JEOL 2010 UHR transmission electron microscope at the Interdisciplinary Centre for Electron Microscopy (CIME) at EPFL, Lausanne. TEM samples have been prepared by dropping solutions of gold nanoparticles onto carbon-coated-copper grids and evaporating the solvent (water) to dryness.

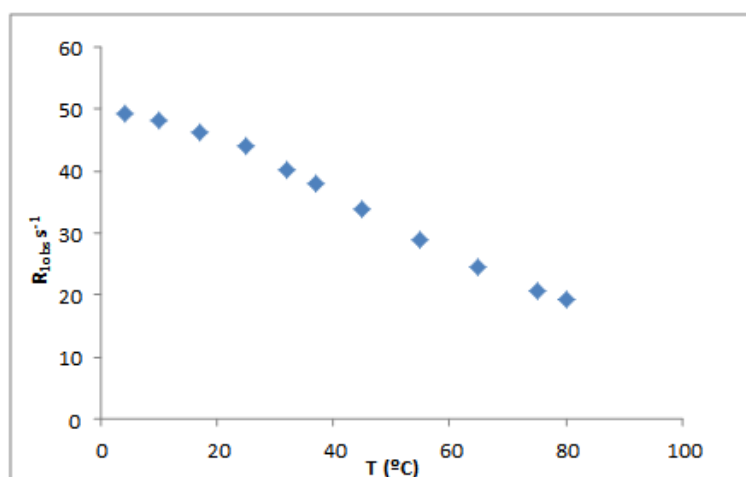
#### **Estimation of the number of Au atoms**

The diameter of the Au-core of the gold nanoparticles has been estimated by choosing the thickness of the organic layer at the surface as 1 nm. This layer should be less thick as in ref. 6 because there are only 4 connecting atoms between the N in the chelate and the S at the Au surface compared to 5 in ref. 6. The Au-core has therefore a diameter of ~1.9 nm. Following ref. 7 the total number of Au atoms can be estimated to be ~210 from  $N_{\text{Au}} = 30.9 \cdot D^3$ . Taking the experimental Au/Gd ratio of 2.9 there are ~70  $\text{Gd}^{3+}$  ions bound to a particle which corresponds roughly to 1  $\text{Gd}^{3+}$  ion per gold atom at the surface.

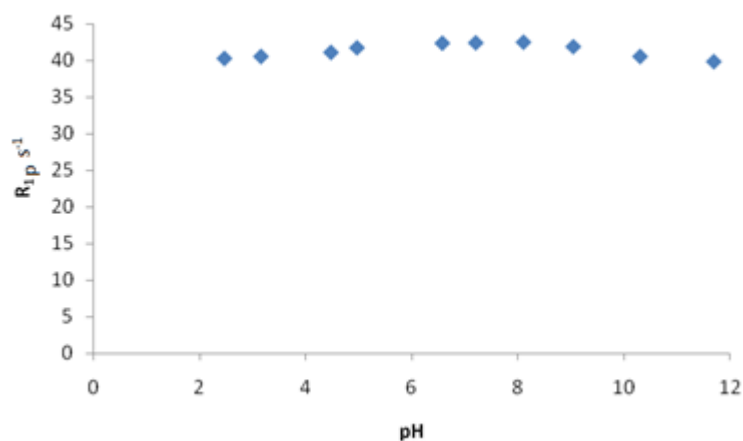
## Relaxometric characterization of the gold nanoparticles



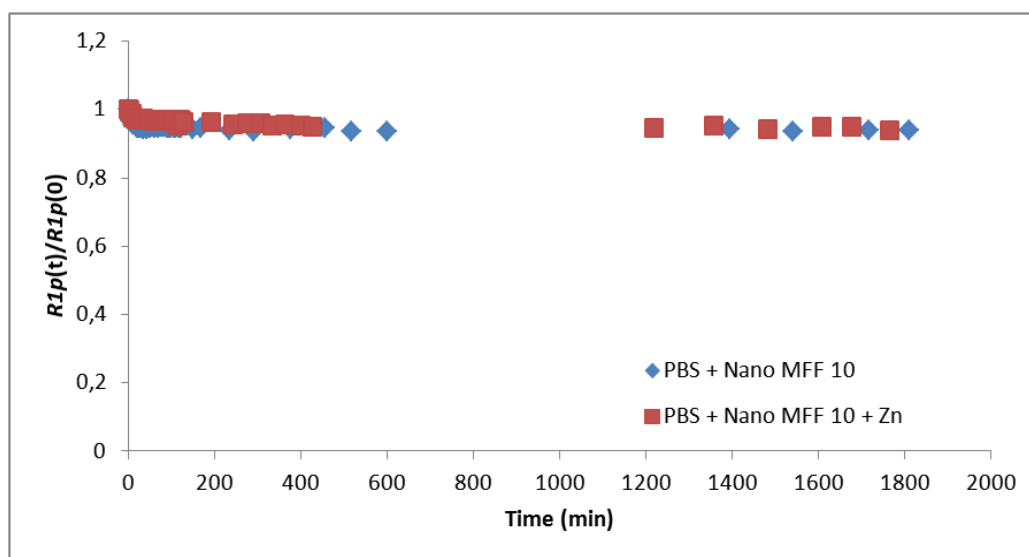
**Figure 5SI.** Dependency of the proton longitudinal paramagnetic relaxation rates ( $R_{1p}$ ) on the  $Gd^{3+}$  concentration for the preparation of nanoparticles functionalized with Gd(DO3A-*N*-( $\alpha$ -cystamido)propionate) chelates (20 MHz, 25 °C, pH 7.5). Preparations with different concentrations  $Gd^{3+}$  were obtained by dilution of the stock solution  $[Gd^{3+}] = 1.238$  mM.



**Figure 6SI.** Dependency of the proton longitudinal paramagnetic relaxation rates ( $R_{1p}$ ) on temperature for the preparation of nanoparticles functionalized with Gd(DO3A-*N*-( $\alpha$ -cystamido)propionate) chelates ( $[Gd^{3+}] = 1.238$  mM, 20 MHz, 25 °C, pH 7.5).



**Figure 7ASI.** Dependency of the proton longitudinal paramagnetic relaxation rates ( $R_{1p}$ ) on the solution's pH for the preparation of nanoparticles functionalized with Gd(DO3A-*N*-( $\alpha$ -cystamido)propionate) chelates ( $[Gd^{3+}] = 1.238$  mM, 20 MHz, 25 °C).



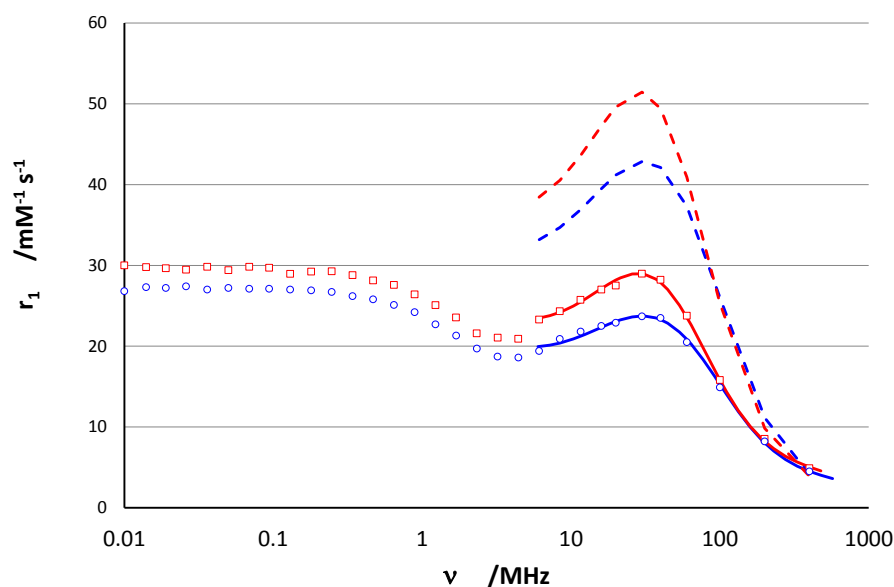
**Figure 7BSI.** Time evolution of the relative water proton paramagnetic relaxation rate  $R_{1p}(t)/R_{1p}(0)$  (20 MHz, 37 °C) for the preparation of nanoparticles functionalized with Gd(DO3A-*N*-( $\alpha$ -cystamido)propionate) chelates ( $[Gd^{3+}] = 0.928$  mM) in PBS 2.5 mM, pH 7.1 in the presence ( $\bullet$ ) and in the absence ( $\blacklozenge$ ) of 0.75 mM  $ZnCl_2$ .

## Fitting of NMRD profiles

**Table 1SI.** Full list of parameters used for the fitting of the high-field part of the NMRD profiles using SBM theory.

Parameters	Value
$q(\text{H}_2\text{O})$	$\frac{1}{2}$
$\Delta H^\ddagger$ [J/mol]	$\frac{17^a}{2}$
$k_{\text{ex}}^{298}$ [ $10^7 \text{ s}^{-1}$ ]	$\frac{5.14^a}{2}$
$E_{\text{R}}$ [kJ/mol] (global)	$18 \pm 1.3$
$\tau_{\text{RH}}^{298}$ [ps] (global)	$2470 \pm 151$
$E_{\text{R}}$ [kJ/mol] (local)	$\frac{18^b}{2}$
$\tau_{\text{RH}}^{298}$ [ps] (local)	$177 \pm 27$
$S^2$	$0.48 \pm 0.02$
$E_{\text{V}}$ [kJ/mol]	1
$\tau_{\text{V}}^{298}$ [ps]	$11 \pm 1$
$\Delta^2$ [ $10^{20} \text{ s}^{-2}$ ]	$0.040 \pm 0.003$
$r_{\text{GdH}}$ [ $\text{\AA}$ ]	$\frac{3.1^c}{2}$
$D_{\text{GdH}}^{298}$ [ $10^{-10} \text{ m}^2 \text{ s}^{-1}$ ]	$\frac{23^c}{2}$
$E_{D\text{GdH}}^{298}$ [kJ/mol]	$\frac{20^c}{2}$
$d$ [ $\text{\AA}$ ]	$\frac{3.6^c}{2}$

Parameters underlined have been fixed: <sup>a</sup> from reference 5; <sup>b</sup> fixed to  $E_{\text{R}}(\text{global})$  after a preliminary fit, <sup>c</sup> fixed to usual parameters



**Figure 8SI.**  $^1\text{H}$  nuclear magnetic relaxation dispersion profiles for gold nanoparticles functionalized with Gd(DO3A-*N*-( $\alpha$ -cystamido)propionate complex (5 mM): 25 °C ( $\square$ ), and 37 °C ( $\circ$ ). The full drawn lines are from the fit with parameters in Table 1SI. The

dashed lines are calculated using the same parameters except  $S^2 = 1$ .

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