Electronic Supplementary Material (ESI) for Journal of Materials Chemistry

Supporting information for

Polymer/Gold Nanohybrids with Potential Use in Bimodal MRI/CT: Enhancing the Relaxometric Properties of Gd(III) Complexes

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Table of Contents

1. Experimental Section

Materials.

Oligo(ethylene glycol) methyl ether acrylate (480 g mol^{-1}, 99%, OEGA), trifluoroacetic acid (99%), gadolinium(III) chloride hexahydrate (99%), gold(III) chloride trihydrate ($HAuCl_4.3H_2O$, >99.9%), trisodium citrate dihydrate (>99%), fluorobenzene and arsenazo III (>99%) were purchased from Aldrich and used as received. Pentafluorophenyl acrylate (PFPA)[1] and 3-(benzylsulfanylthiocarbonyl-sulfanyl)propionic acid (BSPA)[2] were synthesized according to previously reported procedures. 2,2'-Azobisisobutyronitrile (AIBN, Wako Chemicals) was crystallized twice from methanol before use. 1-(5-Amino-3-aza-2-oxpentyl)-4,7,10-tris(*tert*-butoxycarbonyl-methyl)-1,4,7,10-tetraaza-cyclododecane (DO3A-*t*-Bu-NH_2, >94%) and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) were purchased from Macrocyclics (Texas) and used as received. Acetonitrile, triethylamine, dichloromethane, dimethylsulfoxide (DMSO), petroleum ether and diethyl ether were used without further purification. Deuterated solvents, $CDCl_3$-*d* and DMSO-d_6, were obtained from Cambridge Isotope Laboratories, Inc. High purity N_2 (Linde gases) was used for degassing. Membranes for dialysis (MWCO 3500 Da) were purchased from Fisher Biotec (Cellu SepT4, regenerated cellulose tubular membrane). Ultrapure deionized water (17.8 mΩ cm) was obtained using a MilliQ purification system.

Syntheses.

Synthesis of Linear Statistical Copolymers of OEGA and PFPA

Typically, OEGA, PFPA, BSPA and AIBN were dissolved in 3 mL acetonitrile (experimental quantities in Table S1) and introduced into a septum-sealed vial. The mixture was degassed by purging with N_2 for 30 min at 0 °C and then placed in an oil bath at 60°C. The polymerization was conducted for 12h and then interrupted by cooling the mixture in an ice bath and exposing it to air. Monomer conversion was determined by ^1H and ^{19}F NMR and size exclusion chromatography (SEC) analysis was undertaken to assess molecular weight distribution. The polymer was purified by repeated precipitations in a mixture of petroleum ether/diethyl ether (1:1) and then dried under vacuum.

Table 1. Experimental quantities of reactants used for the synthesis of P30 and P50.

Polymer	Reactants	m (g)	n (mmol)
P30	OEGA	1.01	2.1
	PFPA	0.511	2.1
	BSPA	0.042	0.15
	AIBN	2.4×10^{-3}	0.015
P50	OEGA	1.03	2.1
	PFPA	0.214	0.9
	BSPA	0.061	0.22
	AIBN	3.6×10^{-3}	0.02

Chemical modification with DO3A-tBu-NH_2 chelate

A typical procedure for the chemical modification of P(OEGA-*stat*-PFPA) polymers with DO3A-*t*Bu-NH_2 is as follows: P(OEGA-*stat*-PFPA) (250 mg, 0.43 mmol of PFPA moieties) was dissolved in acetonitrile (3 mL). DO3A-*t*Bu-NH_2 (369 mg, 0.53 mmol) and triethylamine (125 µL, 0.9 mmol) were added and the mixture was stirred at 50 °C for 48h. A sample was taken and ^{19}F NMR was performed (Figure S), showing that all PFPA moieties had reacted. Then, the polymer was precipitated repeated times in diethyl ether/petroleum ether (1:1) and the percentage of conjugation was assessed by ^{1}H NMR (Figure S).

Deprotection of t-butyl groups of the chelating agent

Purified P(OEGA-*stat*-(DO3A-*t*Bu-MA) was dissolvend in 3 mL of trifluoroacetic acid (and the mixture was left to react, by stirring overnight at room temperature. Then, unreacted TFA was removed by evaporation and the polymer was further purified by dialysis against water for 4 days. The polymer [P(OEGA-*stat*-DO3A-MA)] was recovered as a yellowish tacky solid after freeze-drying.

Complexation with Gd(III). P(OEGA-*stat*-DO3A-MA) and $GdCl_3.6H_2O$ (1.5 equiv.) were dissolved in 3 mL of water. The pH was adjusted to 6 and the mixture was stirred at 50 °C for 24h. DOTA was then added to the aqueous solution to remove unreacted Gd(III) ions and stirred overnight. An aqueous solution of arsenazo III (0.25 mM) was prepared and 100 µL were added to the previous solution to confirm the absence of free Gd(III) ions (pink color). Then, the polymer was dialysed against water for 5 days and freeze-dried.

Synthesis of Citrate-Stabilized Gold Nanoparticles (12 nm)

Prior to synthesis, the glassware was washed with aqua regia solution, then rinsed with MilliQ water and oven-dried.

First, 450 mL of a 1 mmol L^{-1} aqueous solution of $HAuCl_4$ was prepared and brought to boil on a hot plate with vigorous stirring. Then, 10 mL of an aqueous solution of trisodium citrate (0.38 mol L^{-1}) was rapidly added to the previous solution. The mixture was maintained under reflux for another 30 min. The color progressively changed from yellow to wine red. The solution was allowed to cool to room temperature. The final AuNP solution contained a total Au^0 concentration of 0.22 mg mL^{-1} (1.10 mmol L^{-1}).

Synthesis of P(OEGA-stat-(Gd(III)-DO3A-MA)/Gold Nanohybrids

A "grafting to" approach was employed for the synthesis of nanohybrids. Approximately 30 mg of P30-Gd(III)-DO3A-MA (or P50-Gd(III)-DO3A-MA) was dissolved in 1 mL of ultrapure water. This polymer solution was introduced into a falcon tube, to which 49 mL of the citrate-stabilized AuNPs solution was added. The mixture was then stirred overnight in the absence of light. To remove non-grafted polymer chains and excess citrate, the coated AuNPs were centrifuged for 2h at 13,500 rpm at 20 °C. After removal of the supernatant, the coated AuNPs were redispersed in 10 mL of ultrapure water and this centrifugation-resuspension process was repeated another three times. After the final cycle, the AuNPs were redispersed in a low volume of water to yield solutions with a gold concentration of at least 10 mg mL^{-1} (determined by ICP-OES).

Physical and Analytical Methods.

NMR spectroscopy.

Monomers conversions and polymer compositions were assessed by ^{1}H and ^{19}F NMR using a Bruker AC300F (300 MHz) spectrometer or a Bruker DPX300 (300 MHz) spectrometer. Typically, 500 µL of $CDCl_3$ was added to 100 µL of the final polymerization mixture and the monomer conversion was determined as described in the next section. For determination of polymer composition, a known amount of fluorobenzene was added into the NMR tube to act as a standard for integrals determination (cf. Figure S).

Size exclusion chromatography (SEC)

SEC analyses of polymer samples were performed in *N*,*N*-dimethylacetamide (DMAc with 0.03% w/v LiBr and 0.05% 2,6-di-butyl-4-methylphenol (BHT) using a Shimadzu modular

system comprising a DGU-12A degasser, an SIL-10AD automatic injector, a 5.0 μm bead-size guard column (50×7.8 mm) followed by four 300×7.8 mm linear Phenogel columns (bead size: a 5.0 μm; pore sizes: 10^5, 10^4, 10^3, and 500 Å) and an RID-10A differential refractive-index detector. The temperature of columns was maintained at 50 °C using a CTO-10A oven and the flow rate was kept at 1 mL min^{-1} using a LC-10AT pump. A molecular weight calibration curve was produced using commercial narrow molecular weight distribution polystyrene standards with molecular weights ranging from 500 to 10^6 g mol^{-1}. Polymer solutions at 2-3 mg mL^{-1} were prepared in the eluent and filtered through 0.45 μm filters prior to injection.

UV-Visible Spectrophotometry

UV-Visible spectra were recorded in a Cary 300 spectrophotometer from Bruker at 25 °C.

Dynamic Light Scattering (DLS) and Zeta Potential

DLS and zeta potential measurements were performed using a Malvern Zetasizer Nano Series running DTS software and using a 4 mW He–Ne laser operating at a wavelength of 633 nm and an avalanche photodiode (APD) detector. The scattered light was detected at an angle of 175° for DLS measurements and at 12.8° for zeta potential measurements. The temperature was stabilized to ±0.1 °C of the set temperature. Dilute aqueous suspensions (0.1 wt.%) of AuNPs were prepared in an aqueous phosphate buffer (10 mM, pH=7.4) and the filtered through a 0.45 μm pore size filter to remove dust prior to measurement. Hydrodynamic radii were determined by the non-negative least squares (NNLS) algorithm and the zeta potential was assessed by the Smoluchowski approximation using the manufacturer's software.

Transmission Electron Microscopy (TEM)

The sizes and morphologies of the nanoparticles were observed using a Tecnai G2 20 transmission electron microscope from FEI at an accelerating voltage of 200 kV. Typically, a drop of AuNPs suspension (0.1 mg mL^{-1}) was deposited onto 200 mesh, carbon-coated holey film, copper grid and the water was evaporated under air. The polymer-coated AuNPs were also negatively stained. For that, the grids containing the AuNPs were deposited onto a drop of the an aqueous solution of sodium phosphotungstic acid (1%) for 2 min. The excess

solution was then removed by swabbing with filter paper and then the grid was left to dry. The particles size was assessed by counting more than 250 particles using ImageJ software.

X-ray Photoelectron Spectroscopy (XPS)

A Kratos Axis ULTRA XPS incorporating a 165 mm hemispherical electron energy analyzer was used. The incident radiation was monochromatic A1 X-rays (1486.6 eV) at 225 W (15 kV, 15 ma). Survey (wide) scans were taken at an analyzer pass energy of 160 eV. Survey scans were carried out over 1200-0 eV binding energy range with 1.0 eV steps and a dwell time of 100 ms. Samples were prepared by adding a droplet onto aluminium foil and drying it.

Indutively Coupled Plasma-Optical Emission Spectrometry (ICP-OES)

Gold and gadolinium content of the macromolecular CAs was determined by inductively coupled plasma-optical emission spectrometry (ICP-OES) using a Perkin Elmer OPTIMA 7300 spectrometer. Samples were prepared as follows: 50 µL of the nanohybrids aqueous suspension was added into a falcon tube and digested in 200 µL of aqua regia. Then, water was added up to 10 mL and analysis was carried out.

Relaxivity measurements

^{1}H NMRD curves (Nuclear Magnetic Resonance Dispersion) of the macromolecular CAs were obtained at 310 K on a Stelar fast cycling relaxometer (PV, Mede, Italy) over a range of magnetic fields extending from 0.25 mM to 0.94 T (0.01 – 40 MHz). Measurements of T_1 and T_2 relaxation times were performed at 310 K on Minispec Mq-20 and Mq-60 (Bruker, Karlsruhe, Germany) working at 20 MHz (0.47 T) and 60 MHz (1.4 T) respectively.

Toxicity studies

Cell culture.

MRC5 fibroblast cells were cultured in growth media consisting of Dulbecco's Modified Eagle's Medium: Nutrient Mix F-12 (DMEM) supplemented with 10% (v/v) Foetal Bovine Serum (FBS) in a ventilated tissue culture flask T-75 and passaged every 2-3 days when monolayers at around 80% confluence. The cells were used only when stable cell growth was obtained (approximately 3-4 passages). The cells were incubated at 37°C in a 5% CO2 humidified atmosphere. The cell density was determined by counting the number of viable cells using a trypan blue dye (Sigma-Aldrich) exclusion test. The cells were detached using 0.05% trypsin-EDTA (Invitrogen), stained using trypan blue dye, and loaded on the

haemocytometer. One day prior to the treatment, the cells were seeded at required cell densities on and 96-well plates.

Cell viability.

The cytotoxicity of prepared polymers was tested in-vitro by a standard Alamar Blue assay, which provides a homogeneous, fluorescent method for monitoring cell viability. The assay is based on the ability of living cells to convert a redox dye (blue resazurin) into a fluorescent end product (red resorufin). Nonviable cells rapidly lose metabolic capacity and thus do not generate a fluorescent signal. The cells were seeded in a tissue culture treated 96-well plate in 100 µL medium per well at a density of 5 000 cells/well and incubated for 24 h. The medium was then replaced with fresh medium containing Au@P30 and Au@P50 and incubated for 72h. Alamar Blue assay dye (20 µL) was then added to each well and the cells were incubated for 5 h. After an incubation step, data were recorded using a fluorescence plate reader (570ex/595em). Cell viability was determined as a percentage of untreated control cells.

The amount of fluorescence produced (F) was proportional to the number of metabolically active (viable) cells in the culture. Wells without cells was set up as the negative control for the determination of background fluorescence. Wells without polymer treatment were used as the positive control. The cell viability was calculated by comparing the fluorescence products of treated and non-treated cells according to Eq. as follows:

$$\text{Cell viability (\%)} = \left(\frac{\bar{F}_{sample} - \bar{F}_{negative\ control}}{\bar{F}_{positive\ control} - \bar{F}_{negative\ control}} \right) \times 100\%$$

where, $\bar{F}_{sample}$ is the average fluorescence product in the sample wells (cell treated with prepared polymer), $\bar{F}_{negative\ control}$ is the average fluorescence product in the negative control wells (without cells), and $\bar{F}_{positive\ control}$ is the average fluorescence product in the positive control wells (no treatment with polymers).

2. Synthesis of P(OEGA-*stat*-PFPA) copolymers

Table S2. Conversions and macromolecular characteristics of the polymers synthesized in this work.

Polymer	Conversion (%)		$M_{n,theor}$ [c] (g mol^{-1})	$M_{n,NMR}$ [d] (g mol^{-1})	$M_{n,SEC}$ [e] (g mol^{-1})	M_w/M_n [e]	f_{PFPA} [f]	F_{PFPA} [g]	
	OEGA [a]	PFPA [b]						Before purification [h]	After purification [i]
P30	75	83	4250	5600	12800	1.12	0.3	0.32	0.26
P50	34	49	3900	5700	13800	1.18	0.5	0.59	0.44

[a] Determined by ^{1}H NMR (cf. Figure S1).
[b] Determined by ^{19}F NMR (cf. Figure S1).
[c] Calculated by the following equation:
[d] Determined by a combination of ^{1}H and ^{19}F NMR (cf. Figure S2 and S3).
[e] Assessed by SEC in DMAc (0.03% w/v LiBr, 0.05% BHT) using a conventional calibration curve with narrow PS standards.
[f] Feed molar composition in PFPA.
[g] Cumulative copolymer composition in PFPA.
[h] Determined by:
[i] Determined by a combination of ^{1}H and ^{19}F NMR (cf. Figure S2).

Determination of Conversions

Typically, for the determination of conversions the following equations were used:

$$\text{Conv.OEGA} = \frac{\int_{3.9ppm}^{4.4ppm} - 2\times\int_{5.7ppm}^{6.0ppm}}{\int_{3.9ppm}^{4.4ppm}} \quad \text{(eq. 1)}$$

from the ^{1}H NMR spectrum (Figure S1-A) of the final polymer mixture, where $\int_{3.9ppm}^{4.4ppm}$ corresponds to the C(=O)OCH$_2$ protons of both monomer and polymer and $\int_{5.7ppm}^{6.0ppm}$ is attributed to one vinylic proton of OEGA monomer.

$$\text{Conv.PFPA} = \frac{\int_{-156.0ppm}^{-158.0ppm}}{\int_{-156.0ppm}^{-158.0ppm} + \int_{-158.0ppm}^{-158.5ppm}} \quad \text{(eq. 2)}$$

from the ^{19}F NMR spectrum (Figure S1-B) of the final polymer mixture, where $\int_{-156.0\,ppm}^{-158.0\,ppm}$ and $\int_{-158.0\ ppm}^{-158.5\ ppm}$ corrrespond to the signal of ^{19}F at the *para* position of the monomer and polymer, respectively.

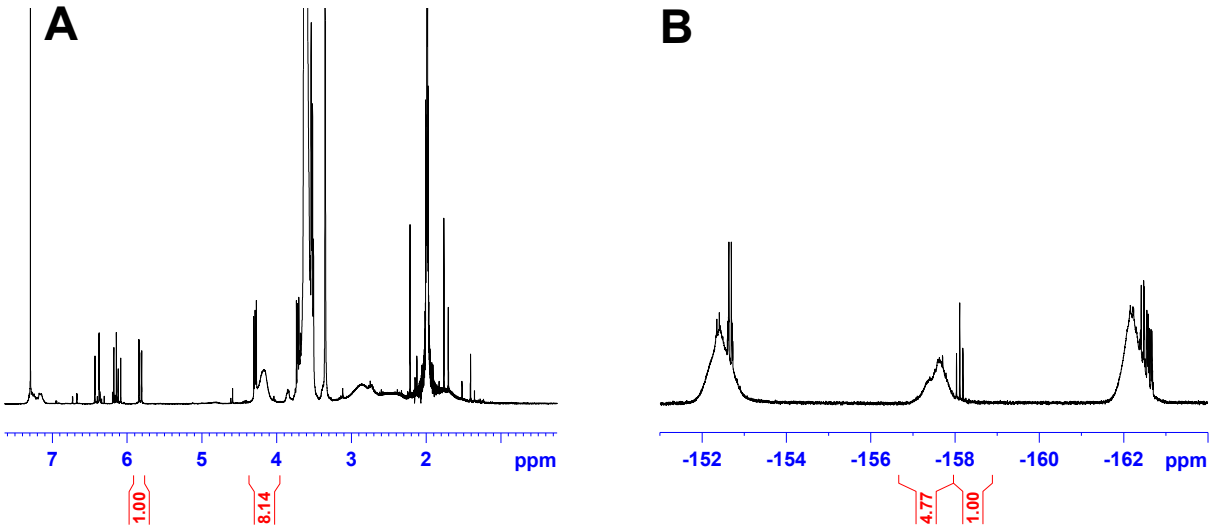

Figure S1. (A) ^{1}H NMR and (B) ^{19}F NMR spectra of P30 final polymerization mixture (300 MHz, 25 °C, $CDCl_3$).

Determination of Polymer Composition

The PFPA content of the final polymer was calculated using fluorobenzene as a reference in ^{1}H NMR and ^{19}F NMR according to the following equations:

$$F_{PFPA} = \frac{B}{A+B} \quad \text{(eq. 3)}$$

with $A = \frac{\int_{3.9ppm}^{4.4\ ppm} / 2}{\int_{6.9\ ppm}^{7.2\ ppm} / 3}$, in the ^{1}H NMR (Figure S2-A), where $\int_{3.9ppm}^{4.4ppm}$ corresponds to the C(=O)OCH$_2$ protons of OEGA and $\int_{6.9\ ppm}^{7.2\ ppm}$ is the integral of the signal attributed to three aromatic protons (*ortho and para*) of fluorobenzene.

and $B = \frac{\int_{-156.0\ ppm}^{-158.0\ ppm}}{\int_{-112.0\ ppm}^{-115.0\ ppm}}$, in the ^{19}F NMR (Figure S2-B), where $\int_{-156.0\ ppm}^{-158.0\ ppm}$ and $\int_{-112.0\ ppm}^{-115.0\ ppm}$ are the integrals from signals attributed to the ^{19}F at the *para* position of PFPA and to the to the ^{19}F of fluorobenzene, respectively.

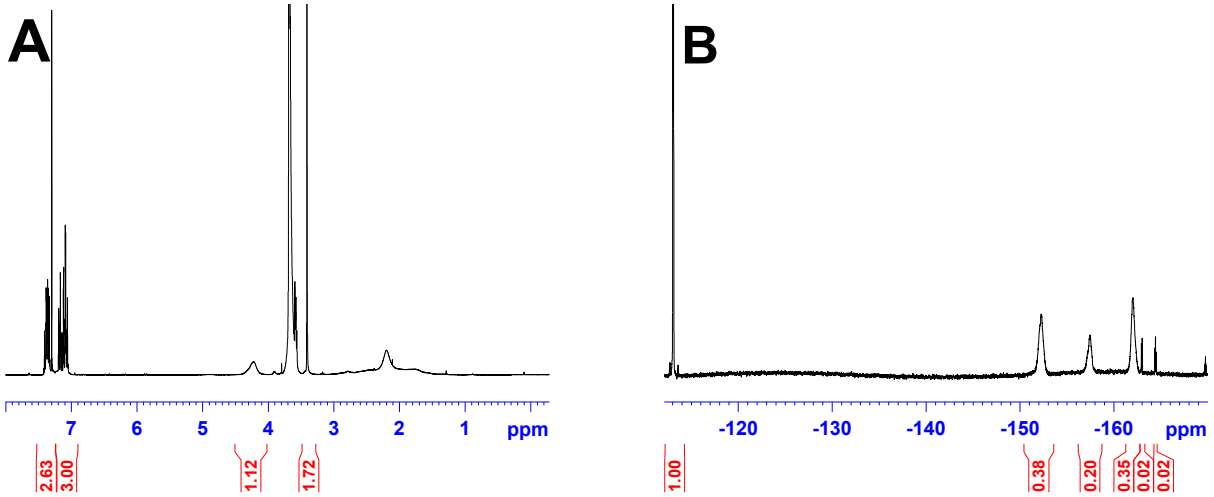

Figure S2. (A) ^{1}H NMR and (B) ^{19}F NMR spectra of P30 after repeated precipitations in diethyl ether/petroleum ether 1:1 (300 MHz, 25 °C, $CDCl_3$).

Curiously, during precipitation some of the PFPA groups were hydrolysed as evidenced by the appearance of signals corresponding to pentafluorophenol (chemical shifts: -162.9 ppm, -164.4 ppm and -169.8 ppm) in the ^{19}F NMR spectrum (Figure S2-B). We noticed that this side reaction was more prone to occur in low molecular weight polymers, such as the ones used in this work (degree of polymerization lower than 15), and with higher content in PFPA (i.e. in polymers with around 10-20% of PFPA almost no hydrolysis occurred).

Determination of Number-Average Molecular Weight by NMR

Since it was not possible to accurately integrate the peak corresponding to the protons of PFPA units, we based our calculation of the number-average molecular weight (M_n) on the NMR spectra from Figure S2 and Figure S3. To better visualize the peaks corresponding to the benzyl group at the polymer α–end, we recorded a ^{1}H NMR spectrum in DMSO-d_6 (Figure S3). Then, using the following equation, we determined the M_n:

$$M_n = MW_{OEGA} \times DP_{OEGA} + MW_{PFPA} \times DP_{PFPA} + MW_{BSPA} \quad \text{(eq.4)}$$

where DP_{OEGA}, DP_{PFPA} are the degrees of polymerization of OEGA and PFPA, and are respectively given by:

$$DP_{OEGA} = \frac{\int_{3.9ppm}^{4.4\ ppm} / 2}{\int_{7.0\ ppm}^{7.5\ ppm} / 5}$$

$$DP_{PFPA} = DP_{OEGA} \times \frac{F_{PFPA}}{F_{OEGA}}$$

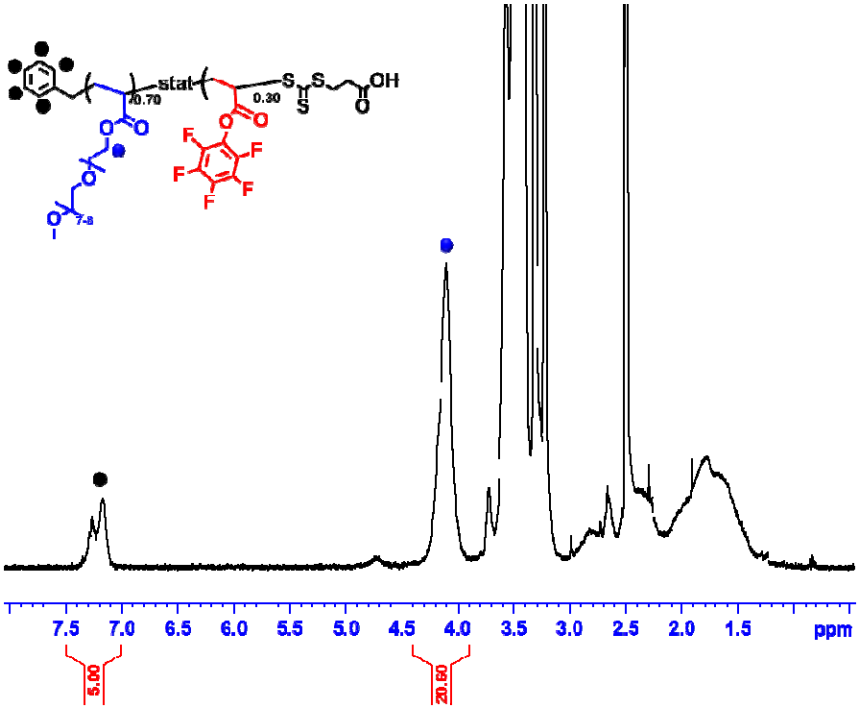

Figure S3. ^{1}H NMR spectrum of pure P30 (300 MHz, 25 °C, DMSO-d_6).

Molecular Weight Distributions by Size Exclusion Chromatography

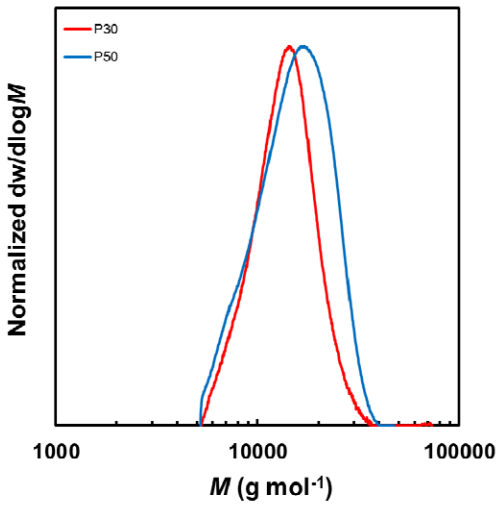

Figure S4. Size exclusion chromatograms of P30 and P50.

Preliminary Kinetic Studies of Copolymerization of OEGA and PFPA

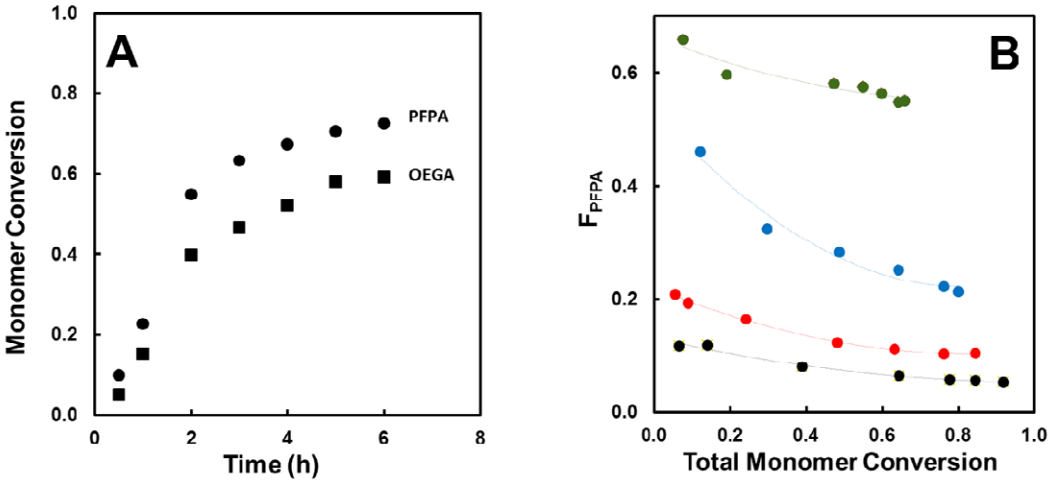

Figure S5. (A) Monomer conversion as a function of time for the RAFT copolymerization of OEGA and PFPA ($[PFPA+OEGA]_0$=1 M; $[PFPA+OEGA]_0$:$[CTA]_0$:$[AIBN]_0$=50:1:0.2; f_{PFPA}=0.20). (B) Dependence of cumulative copolymer composition (F_{PFPA}) with the total monomer conversion for the RAFT copolymerization OEGA and PFPA at various feed monomer compositions: f_{PFPA}=0.05 (●), f_{PFPA}=0.10 (●), f_{PFPA}=0.20 (●) and f_{PFPA}=0.5 (●).

3. Post-polymerization modification of P(OEGA-*stat*-PFPA) copolymers with chelating agents

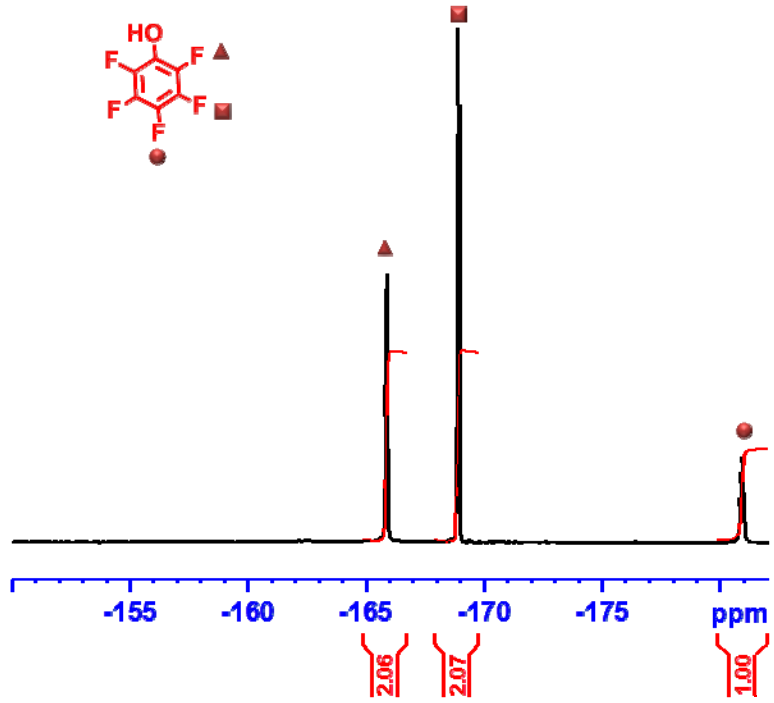

Figure S6. ^{19}F NMR spectrum of crude mixture of P50 after reaction with DO3A-t-Bu-NH_2 (300 MHz, 25 °C, $CDCl_3$). The signals corresponding to the polymer (cf. Figure S2B) are no longer visible

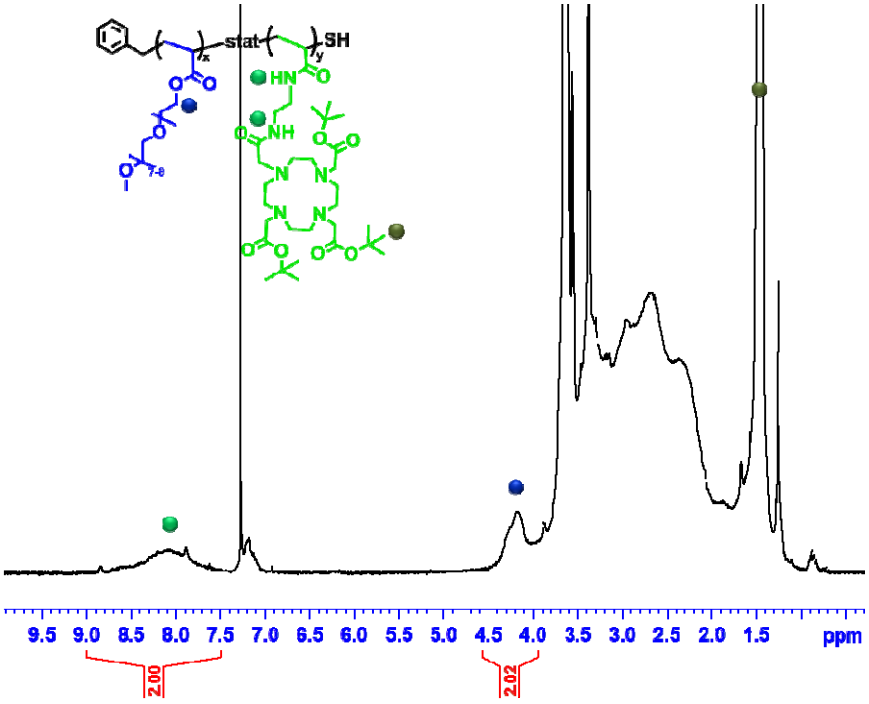

Figure S7. ^{1}H NMR spectrum of pure P50-DO3A-*t*Bu (300 MHz, 25 °C, DMSO-d_6).

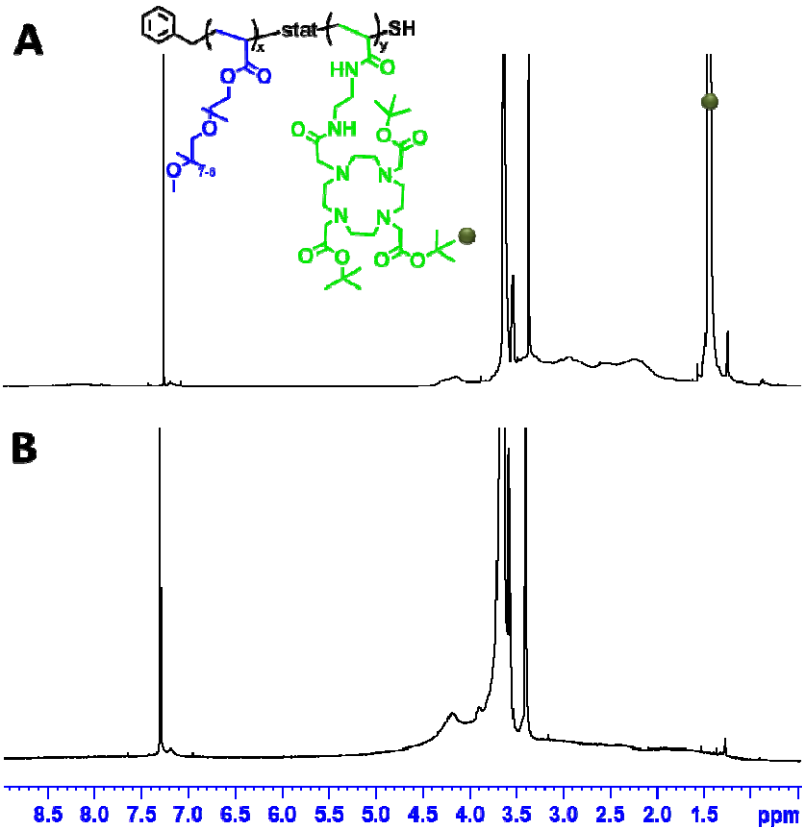

Figure S8. ^{1}H NMR spectra of P30-DO3A-*t*Bu (A) before and (B) after deprotection (300 MHz, 25 °C, $CDCl_3$).

4. Synthesis of citrate-stabilized AuNPs

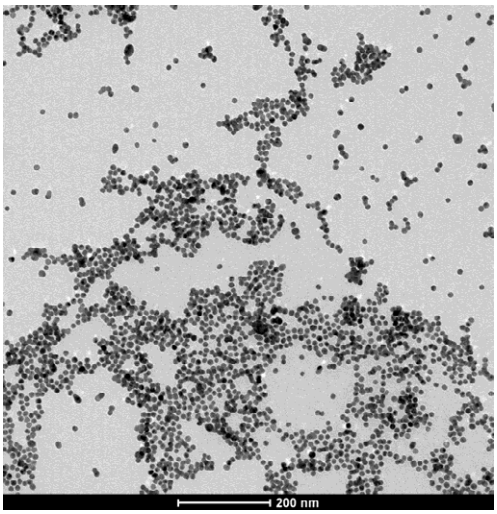

Figure S9. TEM images of citrate-stabilized AuNPs.

5. Grafting of P(OEGA-stat-(Gd(III)-DO3A-MA)) onto as-prepared AuNPs

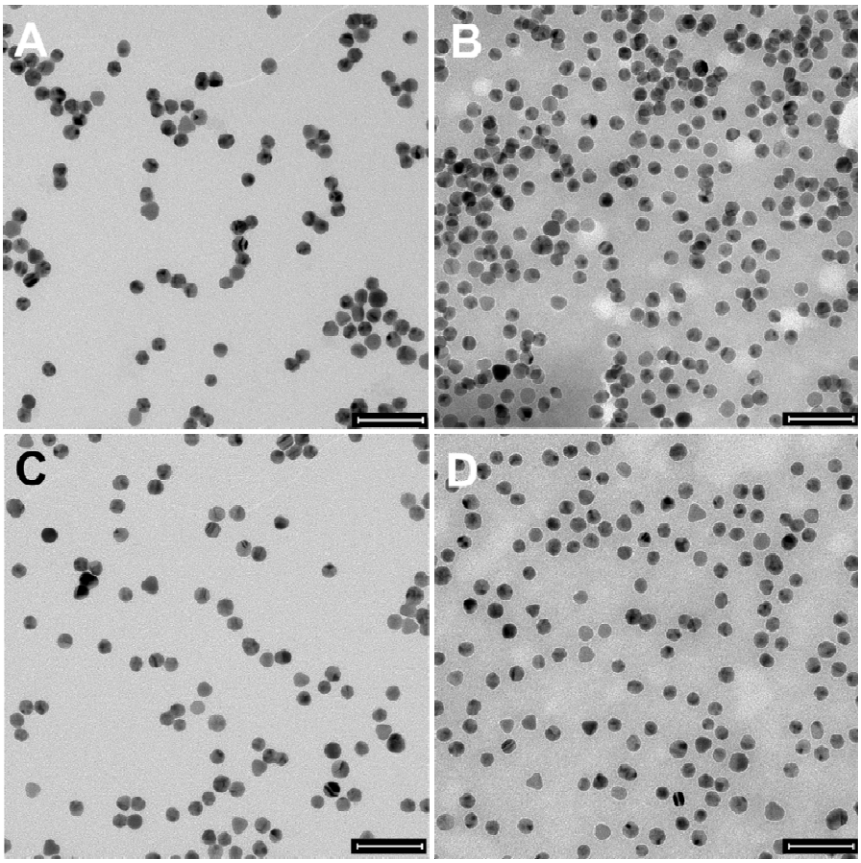

Figure S10. TEM images of (A) Au@P30 – non stained, (B) Au@P30 – stained with phosphotungstic acid, (C) Au@P50 – non stained, (B) Au@P50 – stained with phosphotungstic acid. Scale bar: 50 nm.

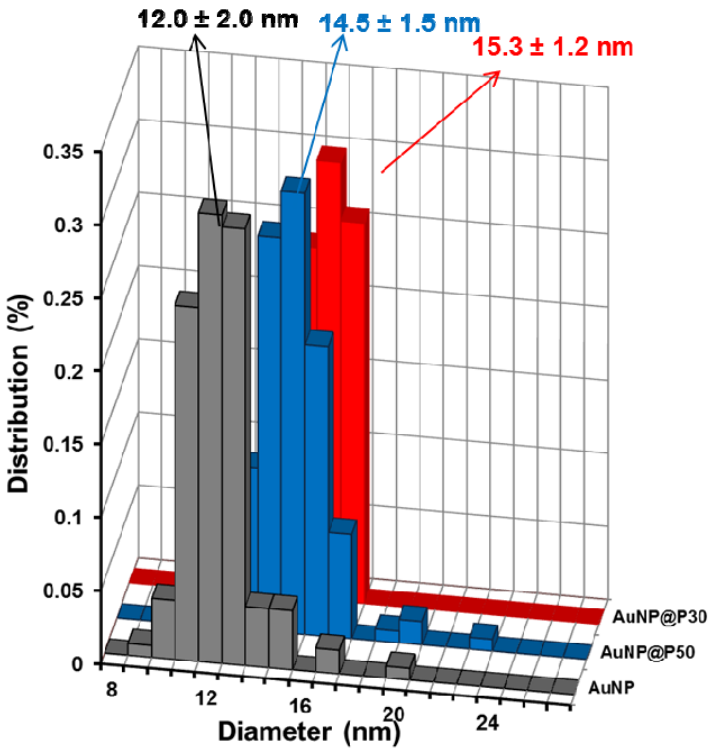

Figure S12. Distributions of sizes assessed by counting 250 particles including the shell observed with staining.

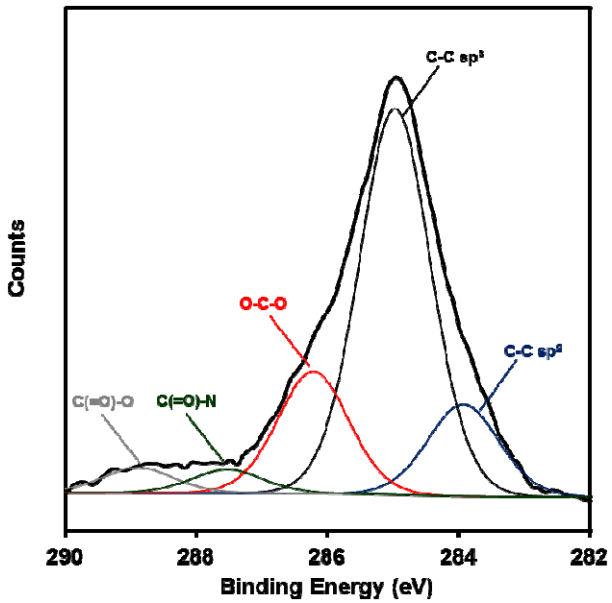

Figure S13. C1s XPS spectrum of Au@P30.

Calculation of Surface Density

The surface density is given by the following equation:

$$\text{Surface density (chains/nm}^2) = \frac{\left(\frac{W_{polymer}}{100 - W_{polymer}}\right) \times \rho \times V_{particle} \times N_A}{M_{polymer} \times S_{particle}} \quad \text{(eq.5)}$$

where W_{polymer} corresponds to the polymer weight loss in TGA (i.e. the weight loss was corrected by taking into account the Gd/Au ratio obtained by ICP/MS), ρ is the density of gold (19.3 g cm^{-3}), M_{polymer} is the polymer molecular weight assessed using NMR (cf. section 2 above) and N_A is the Avogadro Number.

V_{particle} and S_{particle} are respectively the volume and surface of the nanoparticle and since they can be considered as spherical, the following equations were employed:

$$V_{particle} = \frac{4\pi R_{particle}^3}{3} \quad \text{(eq.6)}$$

$$S_{particle} = 4\pi R_{particle}^3 \quad \text{(eq.7)}$$

where R_{particle} is the radius of nanoparticle as obtained by TEM.

Stability in FBS (10%)

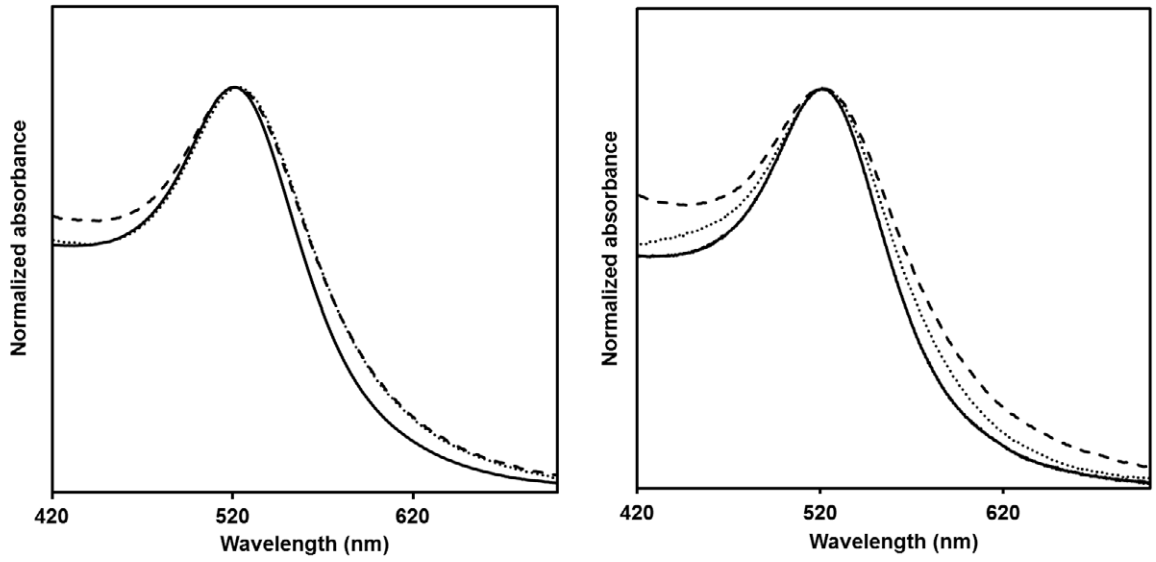

Figure S14. Normalized absorption spectra of Au@P50 (left) and Au@P30 (right) in phosphate buffer pH=7.4 (solid line) and in 10% aqueous FBS immediately after preparation (dotted line) and after 72h (dashed line).

Cytotoxicity studies

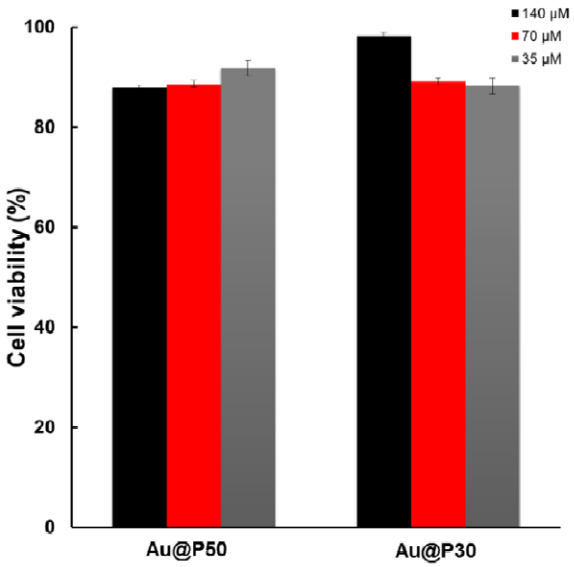

Figure S15. Cytotoxicity studies of Au@P50 and Au@P30 at various Au^0 concentrations (Gd/Au is equal to 0.009 and 0.006 wt.%, respectively). Note: all the data have been repeated in triplicate experiences.

References

(1) Boyer, C.; Liu, J.; Bulmus, V.; Davis, T. P. *Aust. J. Chem.* **2009**, *62*, 830.
(2) Boyer, C.; Bulmus, V.; Davis, T. P. *Macromol. Rapid Commun.* **2009**, *30*, 493.