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Stealth macromolecular platforms for the design of MRI blood pool contrast agents

Mathurin Grogna,^a Rudi Cloots,^b André Luxen,^c Christine Jérôme,^a Catherine Passirani,^d Nolwenn Lautram,^d Jean-F. Desreux,^e Mike Collodoro,^f Marie-Claire De Pauw-Gillet^f and Christophe Detrembleur^{*a}

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Stealth macromolecular platforms bearing alkyne groups and poly(ethylene oxide) brushes were synthesized by reversible addition fragmentation chain transfer (RAFT) polymerization. The anchoring of Gd³⁺-chelates bearing an azide group was then carried out by the Huisgen 1,3-dipolar cycloaddition (“click”) reaction in mild conditions, leading to macrocontrast agents for MRI applications. The gadolinium complex is hidden in the PEO shell that renders the macrocontrast agents free of any cytotoxicity and stealth to proteins of the immune system. Relaxometry measurements have evidenced an improved relaxivity of the macrocontrast agent compared to ungrafted gadolinium chelate. Moreover, this relaxivity is further enhanced when the spacer length between the Gd³⁺-chelate and the polymer backbone is shorter, as the result of its decreased tumbling rate. These novel products are therefore promising candidates for MRI applications.

Introduction

Magnetic resonance imaging (MRI) is a routine diagnostic tool in modern clinical medicine. MRI has many advantages as a diagnostic imaging modality. It is noninvasive, delivers no radiation burden, and has excellent (submillimetre) spatial resolution. Contrast agents (CAs) (chelating Gd³⁺ with suitable organic ligands such as DTPA (diethylene triamine pentaacetic acid) or DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid)), that increase the relaxation rate of water molecules and improve the contrast between tissues of interest, are widely used in experimental and clinical settings. Unfortunately these CAs present two main drawbacks that lead to injection of large gadolinium doses: (i) rapid blood extraction (circulation half lifetime of about 5 min¹) and (ii) poor contrast at high magnetic field.

These two drawbacks can be solved by grafting CAs to biocompatible polymers. Indeed some biocompatible and water-soluble polymers have demonstrated unique pharmacokinetic

properties with long blood circulation time and good tissue retention. Due to its biocompatibility, hydrophilicity, non-toxicity, good steric stabilization effect and its capacity to prevent protein adsorption,² poly(ethylene oxide) (PEO) has emerged as a suitable candidate for that purpose. It enhances solubility of hydrophobic drugs, prolongs circulation time, minimizes non-specific uptake, and allows for specific tumor accumulation through the enhanced permeability and retention effect (EPR effect³). Moreover, according to the SBM theory^{4,5} (Solomon–Bloembergen–Morgan), it is recognized that slowing down molecular tumbling by grafting the contrast agent onto high macromolecular weight structures increases the relaxivity and so the contrast. In this way, recent approaches for high-relaxivity agents have involved the incorporation of Gd(III) chelates into dendrimers,^{6–8} micelles^{9–11} and linear polymers.^{12–16}

In this paper, we aim at designing and characterizing new stealth macromolecular platforms that bind gadolinium based MRI contrast agents under very mild conditions (Scheme 1). This multifunctional platform is composed of (i) poly(ethylene oxide) grafts for ensuring water solubility and prolonged blood circulation, and (ii) alkyne groups for anchoring the gadolinium complexes by click chemistry, a well-known efficient and selective cycloaddition reaction between azide bearing molecules and alkynes.¹⁷ Furthermore, the rigid nature of the triazole linker formed during this click reaction hinders the local rotation of the Gd(III) complex that should consequently enhance the relaxivity compared to a macrocontrast agent bearing a linear and flexible linker¹⁸ (Scheme 2).

The combination of the stealth character of the macrocontrast agent imparted by the PEO chains with its improved relaxivity

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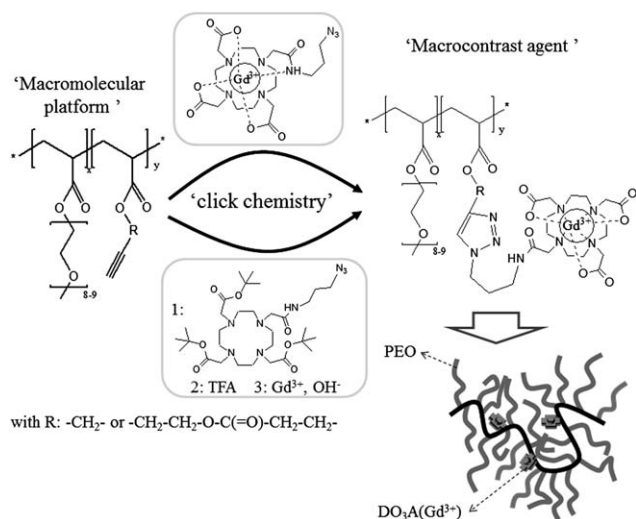
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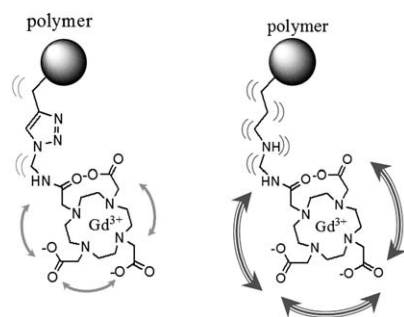
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Scheme 1 General procedure for the synthesis of the macrocontrast agent by grafting $\text{DO}_3\text{A}(\text{Gd}^{3+})-\text{N}_3$ (up pathway) and $\text{DO}_3\text{AtBu}-\text{N}_3$ (bottom pathway) onto alkyne bearing copolymers.



Scheme 2 Local hindering of contrast agent due to triazole ring vs. a linear flexible linker.

promoted by the conjugation of the gadolinium complex to the hindered macromolecule is expected to decrease the doses of injected gadolinium while maintaining satisfactory image acquisition times. Besides the design of those macrocontrast agents, their relaxivity (contrasting efficiency), cytotoxicity, and stealth character will also be evaluated in order to study their potential as MRI blood pool agents.

Experimental procedures

Materials

S-1-Dodecyl-*S'*-(α,α' -dimethyl- α'' -acetic acid)trithiocarbonate (CTA) was synthesized according to Lai *et al.*¹⁹ 4-Pentynoic acid, 2,2'-azobis(isobutyronitrile) (AIBN), propargyl alcohol, triethylamine, acryloyl chloride, ethyl acetate, bromopropylamine sodium azide (NaN_3), dichloromethane (CH_2Cl_2), heptane, dimethylsulfoxide (DMSO), poly(ethylene oxide) methyl ether acrylate (PEOMA $M_n \approx 454 \text{ g mol}^{-1}$, $\text{DP}_{\text{PEG}} = 8-9$, Aldrich), methyl bromoacetate, trifluoroacetic acid (TFA), dimethylformamide were purchased from Sigma-Aldrich. Magnesium sulfate (MgSO_4), sodium hydroxide (NaOH), diethyl ether and potassium carbonate (K_2CO_3) were purchased from VWR. 1,4,7,10-Tetraazacyclododecane-1,4,7-triacetic acid, 1,4,7-tris

(1,1-dimethylethyl) ester (DO_3AtBu) was purchased from Chematech. Bathophenanthroline disulfonic acid disodium salt hydrate was purchased from Alfa Aesar. All components were used as received without further purification.

Synthesis

Synthesis of propargyl acrylate. Propargyl acrylate was synthesized as follows: 5.6 g of propargyl alcohol (100 mmol) and 10.6 g of triethylamine (105 mmol) were added in a glass reactor with 200 ml of dry dichloromethane. The reactor was cooled in an ice bath. Then 10.9 g of acryloyl chloride (120 mmol) was added dropwise under stirring while maintaining the temperature to 0°C . After the complete addition of acryloyl chloride, the solution was stirred for 3 h at 0°C . Then, the solution was washed two times with brine, two times with water and dried with MgSO_4 . Finally, flash chromatography (100% ethyl acetate) was used to purify the product. Solvent was removed under reduced pressure to give colorless liquid (8.8 g, 80% yield). Propargyl acrylate was analyzed by ^1H NMR and ^{13}C DEPT135.

^1H NMR (CDCl_3 , TMS, 250 MHz): $\delta = 2.47$ ppm (s, $-\text{C}-\text{H}$, 1H), $\delta = 4.72$ ppm (s, $-\text{O}-\text{CH}_2-\text{C}$, 2H), $\delta = 5.8$ and 6.5 ppm ($\text{CH}_2-\text{CH}-\text{C}(=\text{O})-$, 3H).

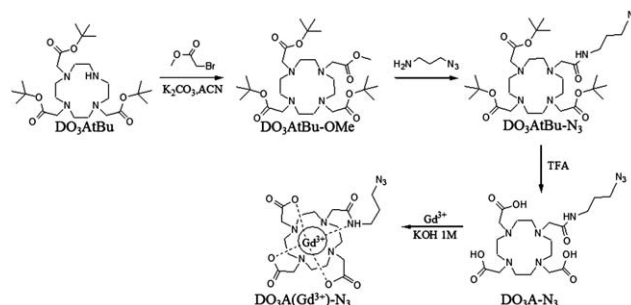
^{13}C DEPT135: $\delta = 51.78$ ppm ($-\text{O}-\text{CH}_2-\text{C}$), $\delta = 75.06$ ppm ($-\text{C}-\text{H}$), $\delta = 77.48$ ppm ($-\text{C}-\text{H}$), $\delta = 127.34$ ppm ($\text{CH}_2-\text{CH}-\text{C}(=\text{O})-$), $\delta = 131.59$ ppm ($\text{CH}_2-\text{CH}-\text{C}(=\text{O})-$).

Synthesis of N_3 -propylamine²⁰(APA). N_3 -Propylamine was synthesized according to an Experimental section reported elsewhere.²¹

Synthesis of $\text{DO}_3\text{AtBu}-\text{N}_3$ and $\text{DO}_3\text{A}(\text{Gd}^{3+})-\text{N}_3$ (Scheme 3)

Synthesis of $\text{DO}_3\text{AtBu}-\text{OMe}$ [1-methyl ester-4,7,10-tris(tert-butoxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane]. 957 mg of DO_3AtBu (1.86 mmol) was dissolved in 15 ml of acetonitrile. 0.6 g of K_2CO_3 and 0.212 ml of methyl bromoacetate (2.42 mmol) were then added. The resulting solution was stirred for 4 hours under reflux. After cooling to room temperature, the solvent was removed under reduced pressure and toluene (30 ml) was added to extract the product from the mixture. The organic phase was washed three times with water and was then dried with MgSO_4 . Solvent was removed under reduced pressure to give a yellow oil (875 mg, 80% yield). Product was analyzed by ^1H NMR and ESI-MS.

^1H NMR (CDCl_3 , TMS, 250 MHz): $\delta = 1.42$ ppm (s, $-\text{C}-\text{CH}_3$, 27H), $\delta = 2.79$ ppm (m, $-\text{N}-\text{CH}_2-\text{CH}_2-\text{N}$ 16H), $\delta = 3.24$ ppm (s,



Scheme 3 Synthetic pathway for $\text{DO}_3\text{AtBu}-\text{N}_3$ and $\text{DO}_3\text{A}(\text{Gd}^{3+})-\text{N}_3$.

N-CH₂-C(=O), 6H), δ = 3.38 ppm (s, N-CH₂-C(=O)-OMe, 2H), δ = 3.64 ppm (s, -O-CH₃, 3H).

ESI-MS: m/z : 587.41 [M + H]⁺; 609.39 [M + Na]⁺.

Synthesis of DO₃AtBu-N₃ [1-N-3-azidopropylamine amide-4,7,10-tris(tert-butoxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane]. 875 mg of DO₃AtBu-OMe (1.49 mmol) was dissolved in 3 g of N₃-propylamine (APA; 30 mmol) and was stirred for 3 days at room temperature. The coupling reaction between DO₃AtBu-OMe and APA was certified by mass spectrometry with the complete disappearance of the molecular peaks of DO₃AtBu (m/z) 586/609 and the appearance of the molecular peak of the DO₃AtBu conjugated APA (DO₃AtBu-N₃) (m/z) 655/677. After complete disappearance of the molecule peak of the DO₃AtBu-OMe, the solvent was removed under reduced pressure. Product was purified by silica gel chromatography (100% CH₂Cl₂) leading to the pure compound (730 mg, 75%) as a white foam. Product was analyzed by ¹H NMR, ¹³C NMR and ESI-MS.

¹H NMR (CDCl₃, TMS, 250 MHz): δ = 1.42 ppm (s, -C-CH₃, 27H), δ = 1.81 ppm (quin, -CH₂-CH₂-N₃), 2.49–3.01 ppm (very broad signals with an integration corresponding to 16H), δ = 3.19–3.35 (m, N-CH₂-C(=O)- and -NH-CH₂-, 10H), δ = 8.72 ppm (t, -C(=O)-NH-CH₂-, 1H).

¹³C NMR (CDCl₃, TMS, 63 MHz): δ = 27.76 ppm (-C-CH₃), δ = 28.77 ppm (-NH-CH₂-CH₂-), δ = 36.18 ppm (-NH-CH₂-CH₂-), δ = 48.81 ppm (-CH₂-N₃), δ = 51.63, 52.05, 53.17, 54.55, 55.84 ppm (-N-CH₂-CH₂-N-), δ = 56.44 ppm (-N-CH₂-C=O-), δ = 57.61 ppm (-N-CH₂-C(=O)-), δ = 80.3 ppm (-C-CH₃), δ = 170.03 ppm (-C(=O)-O-*t*Bu), δ = 171.69 ppm (-C(=O)-NH-).

ESI-MS: m/z : 655.45 [M + H]⁺, 677.43 [M + Na]⁺.

Synthesis of Gd³⁺ complex of [1-N-3-azidopropylamine amide-4,7,10-tris(carbonylmethyl)-1,4,7,10-tetraazacyclododecane] (DO₃A(Gd³⁺)-N₃). Ligand (DO₃AtBu-N₃) (200 mg, 0.3 mmol) was dissolved in trifluoroacetic acid (TFA) (3 ml) and was stirred at room temperature overnight. TFA was evaporated under reduced pressure and the ligand was dissolved in water (5 ml). GdCl₃·6H₂O (120 mg, 0.33 mmol) was added to the solution and the pH was increased to 6 by the slow addition of a KOH solution (1 M). The solution was heated to 40 °C overnight. Finally, the uncomplexed Gd³⁺ ions were removed by eluting the solution through a Chelex 100 (Bio-Rad Laboratories, sodium form) resin. The resulting solution was brought to dryness under reduced pressure and the DO₃A(Gd³⁺)-N₃ complex was recovered as a pale yellow solid in almost quantitative yield (>95%).

ESI-MS: m/z : 642.16 [M + H]⁺.

Synthesis of poly(poly(ethylene oxide) methyl ether acrylate)-*st*-poly(propargyl acrylate) (P[PEOMA-*st*-PA]) statistic copolymer. P[PEOMA-*st*-PA] (DP50; 70% mol PEOMA/30% mol PA) statistic copolymer was synthesized by RAFT copolymerization of poly(ethylene oxide) methyl ether acrylate (PEOMA) with propargyl acrylate (PA) as follows. 5.39 mg of 2,2'-azobis(isobutyronitrile) (AIBN; 0.0329 mmol), 120 mg of *S*-1-dodecyl-*S'*-(α,α' -dimethyl- α'' -acetic acid)trithiocarbonate (CTA; 0.329 mmol), 5.22 g of poly(ethylene oxide) methyl ether acrylate (PEOMA; 11.5 mmol), 0.542 g of propargyl acrylate (PA;

4.93 mmol) and 10 ml of DMF were mixed in a 25 ml Schlenk flask. The mixture was degassed by bubbling nitrogen for 5 minutes. This reaction mixture was heated in an oil bath at 80 °C for 300 min. The resulting polymer was precipitated two times by addition of the solution into a large amount of heptane/diethyl ether (50/50) under vigorous stirring. Molecular weight composition and conversion were determined by ¹H NMR in CDCl₃. Relative molecular weight (M_n , SEC and polydispersity were determined by size exclusion chromatography (SEC) in DMF/LiBr using a polystyrene calibration ¹H NMR (CDCl₃, 250 MHz, TMS): δ = 0.86 ppm (t, -CH₂-CH₃, 3H), δ = 1.16 ppm (CH₃-C-, 6H), δ = 1.24 ppm (-CH₂-(RAFT), 20H), δ = 1.66 and 1.92 ppm (large, -CH₂-CH-, 92H), δ = 2.34 and 2.63 ppm (large, -CH₂-CH-, 46H), δ = 3.36 ppm (s, CH₃-O-PEO, 96H), δ = 3.65 ppm (large, -CH₂-CH₂-O-, 1088H), δ = 4.16 ppm (large, -C(=O)-O-CH₂-PEO, 64H), δ = 4.64 ppm (large, -C(=O)-O-CH₂-alkyne, 28H). M_n , NMR = 16 450 g mol⁻¹, DP_n, NMR = 46, M_n , SEC = 20 000 g mol⁻¹, M_w/M_n = 1.15. Conversion is 70% for both monomers.

The same experimental section is used for synthesizing other P[PEOMA-*st*-PA] with different molecular weights, excepted that the RAFT agent to monomers ratio was accordingly adapted. [PEOMA]/[PA] = 7/3, DP_{th} = 10, [AIBN]/[CTA] = 0.1, ([PEOMA]+[PA])/[CTA] = 10.

M_n , NMR = 3400 g mol⁻¹, DP_n, NMR = 7, M_n , SEC = 7500 g mol⁻¹, M_w/M_n = 1.08. Conversion is 70% for both monomers.

Synthesis of poly(poly(ethylene oxide) methyl ether acrylate)-*st*-poly(2-hydroxyethyl acrylate) (P[PEOMA-*st*-HEA]) statistic copolymer. P[PEOMA-*st*-HEA] (DP50; 70 mol% PEOMA/30 mol% HEA) statistic copolymer was synthesized by RAFT polymerization of PEOMA and 2-hydroxyethyl acrylate (HEA) as follows. 21.6 mg of 2,2'-azobis(isobutyronitrile) (AIBN) (0.132 mmol), 480 mg of *S*-1-dodecyl-*S'*-(α,α' -dimethyl- α'' -acetic acid)trithiocarbonate (CTA; 1.32 mmol), 20.88 g of poly(ethylene oxide) methyl ether acrylate (PEOMA; 46 mmol), 2.29 g of 2-hydroxyethyl acrylate (HEA; 19.7 mmol) and 70 ml of DMF were mixed in a 250 ml Schlenk flask. The mixture was degassed by bubbling nitrogen. This reaction mixture was heated in an oil bath at 80 °C for 4 h. The polymer was precipitated two times by addition of the solution into a large volume of a heptane/diethyl ether mixture (50/50). Molecular weight composition and conversion were determined by ¹H NMR in CDCl₃. Relative molecular weights (M_n , SEC) and polydispersity were measured by size exclusion chromatography (SEC) in DMF/LiBr using a polystyrene calibration. Conversion > 98%.

¹H NMR (CDCl₃, 250 MHz, TMS): δ = 0.86 ppm (t, -CH₂-CH₃, 3H), δ = 1.16 ppm (CH₃-C-, 6H), δ = 1.24 ppm (-CH₂-(RAFT), 20H), δ = 1.62–1.89 ppm (large, -CH₂-CH-, 120H), δ = 2.30 ppm (large, -CH₂-CH-, 60H), δ = 3.36 ppm (s, CH₃-O-PEO, 126H), δ = 3.62–3.72 ppm (large, -CH₂-CH₂-O- and -CH₂-CH₂-OH, 1512H), δ = 4.16 ppm (large, -C=O-O-CH₂-PEO and -C(=O)-O-CH₂-CH₂-OH, 120H), M_n , NMR = 21 100 g mol⁻¹, DP_n, NMR = 60, M_n , SEC = 22 000 g mol⁻¹, M_w/M_n = 1.16.

P[PEOMA-*st*-HEA] (DP10; 70%/30% mol) with different molecular weights was synthesized using the same experimental procedure, excepted that the RAFT agent to monomer ratio was

adapted accordingly. [PEOMA]/[PA] = 7/3, DP_{th} = 10, [AIBN]/[CTA] = 0.1, ([PEOMA]+[PA])/[CTA] = 10. *M_n*, NMR = 4550 g mol⁻¹, DP_n, NMR = 13, *M_n*, SEC = 7400 g mol⁻¹, *M_w*/*M_n* = 1.16; conversion > 98% (complete for both monomers).

Esterification of P[PEOMA-*st*-HEA] with 4-pentynoic acid. 10 g of P[PEOMA-*st*-HEA] (DP60, 70 mol% PEOMA/30 mol% HEA) (8.5 mmol of OH groups) were dissolved in 120 ml dry CH₂Cl₂ at 0 °C. 920 mg of 4-pentynoic acid (9.35 mmol), 1.89 g of *N,N'*-dicyclohexylcarbodiimide (DCC; 9.35 mmol) and 114 mg of 4-(dimethylamino)pyridine (DMAP; 0.935 mmol) were dissolved in 50 ml of dry CH₂Cl₂, and added dropwise in the copolymer solution at 0 °C. After complete addition, the solution was stirred overnight at room temperature. The organic solution was filtered and the solvent was evaporated under reduced pressure. The residue was dissolved in NaHCO₃ solution (1 M) and the modified copolymer was extracted two times with CH₂Cl₂. After extraction, the organic phase was dried with MgSO₄ and precipitated two times in diethyl ether and dried under vacuum. The P[PEOMA-*st*-AEP] copolymer was analyzed by ¹H NMR and size exclusion chromatography.

¹H NMR (CDCl₃, 250 MHz, TMS): δ = 0.86 ppm (t, -CH₂-CH₃, 3H), δ = 1.16 ppm (CH₃-C-, 6H), δ = 1.24 ppm (-CH₂- (RAFT), 20H), δ = 1.62–1.89 ppm (large, -CH₂-CH-, 120H), δ = 2.05 ppm (-CH, 18H), δ = 2.30 ppm (large, -CH₂-CH-, 60H), δ = 2.47–2.57 ppm (-C(=O)-CH₂-CH₂-CH, 72H), δ = 3.36 ppm (s, CH₃-O-PEO, 126H), δ = 3.62 ppm (large, -CH₂-CH₂-O-, 1428H), δ = 4.16–4.26 ppm (large, -C(=O)-O-CH₂-PEO and -C(=O)-O-CH₂-CH₂-O-C(=O)-, 156H), *M_n*, NMR = 22 600 g mol⁻¹, DP_n, NMR = 60, *M_n*, SEC = 22 500 g mol⁻¹, *M_w*/*M_n* = 1.16.

P[PEOMA-*st*-AEP] (DP13; 70%/30% mol) was synthesized using the same experimental procedure by adapting the monomers to CTA molar ratio. *M_n*, NMR = 4900 g mol⁻¹, DP_n, NMR = 13, *M_n*, SEC = 7500 g mol⁻¹, *M_w*/*M_n* = 1.17.

Synthesis poly(2-hydroxyethyl acrylate) (PHEA) homopolymer. PHEA (DP50) was synthesized by RAFT polymerization of 2-hydroxyethyl acrylate (HEA) as follows. 9 mg of 2,2'-azobis(isobutyronitrile) (AIBN) (0.055 mmol), 200 mg of *S*-1-dodecyl-*S'*-(α,α' -dimethyl- α' -acetic acid)trithiocarbonate (CTA; 0.55 mmol), 3.19 g of 2-hydroxyethyl acrylate (HEA; 27.5 mmol) and 7 ml of DMF were mixed in a 25 ml Schlenk flask. The mixture was degassed by bubbling nitrogen. This reaction mixture was heated in an oil bath at 80 °C for 4 h. The polymer was precipitated two times by addition of the solution into a large volume of a heptane/diethyl ether mixture (50/50). Molecular weight composition and conversion were determined by ¹H NMR in CDCl₃. Relative molecular weights (*M_n*, SEC) and polydispersity was measured by size exclusion chromatography (SEC) in DMF/LiBr using a polystyrene calibration. Conversion > 98%.

¹H NMR (CDCl₃, 250 MHz, TMS): δ = 0.86 ppm (t, -CH₂-CH₃, 3H), δ = 1.16 ppm (CH₃-C-, 6H), δ = 1.24 ppm (-CH₂- (RAFT), 20H), δ = 1.62–1.89 ppm (large, -CH₂-CH-, 110H), δ = 2.30 ppm (large, -CH₂-CH-, 55H), δ = 3.72 ppm (large, -CH₂-CH₂-OH, 110H), δ = 4.16 ppm (large, -C(=O)-O-CH₂-CH₂-OH, 110H), *M_n*, NMR = 6400 g mol⁻¹, DP_n, NMR = 55, *M_n*, SEC = 7000 g mol⁻¹, *M_w*/*M_n* = 1.12.

Grafting of azide bearing molecules to P[PEOMA-*st*-PA] and to P[PEOMA-*st*-AEP]. Azide molecules like rhodamine azide, benzyl azide, DO₃AtBu-azide or DO₃A(Gd³⁺)-azide were grafted to alkyne bearing copolymers using the following optimized conditions: 73 mg of copolymer P[PEOMA-*st*-PA] (70 mol% PEOMA/30 mol% PA, *M_n*, NMR = 3400 g mol⁻¹ or *M_n*, NMR = 16 450 g mol⁻¹) or 78 mg of P[PEOMA-*st*-AEP] (70 mol% PEOMA/30 mol% AEP, *M_n*, NMR = 22 600 g mol⁻¹ or 4900 g mol⁻¹) (62 μmol of alkyne function) were dissolved in 2 ml of dry DMF. 1.2 mg of CuI (6.2 μmol) and azide molecule (62 μmol) were added to the copolymer solution. This mixture was stirred at room temperature overnight. Different purifications were used according to the nature of the azide molecule:

- Benzyl azide and DO₃AtBu modified copolymers were purified as follows: 10 ml of CH₂Cl₂ and 15 ml of EDTA solution (0.5 M, pH = 7) were added to the DMF solution. The mixture was vigorously stirred for 15 min. The blue aqueous solution was removed and 15 ml of EDTA solution was again added, and the mixture was vigorously stirred for a few minutes to remove residual copper. Finally the organic phase was dried with MgSO₄ and the copolymer was recovered by precipitation into a large volume of a diethyl ether/heptane mixture (50/50). Grafting yield of different copolymer was determined by ¹H NMR spectroscopy and is presented in Table 3 (values around 80% for benzyl azide and between 45 and 65% for DO₃AtBu-N₃). Residual copper was quantified by ICP-MS analysis (<100 ppm).

- DO₃A(Gd³⁺)-N₃ modified copolymers were purified as follows: 5 ml of EDTA (0.5 M, pH = 7) was added to the DMF solution. Then the solution was dialyzed (Spectra/Por, molecular weight cut-off, 3500) against water for 48 h. Finally, the copolymers were lyophilized. Grafting yield was determined by ICP-MS spectrometry and was less than 10%. Residual copper was quantified by ICP-MS analysis (<100 ppm).

Synthesis of macrocontrast agent DO₃A(Gd³⁺)-copolymer. Modified DO₃AtBu copolymer (100 mg) previously synthesized was dissolved in neat trifluoroacetic acid (2 ml) and stirred overnight at room temperature to remove the *tert*-butyl groups of protected DO₃AtBu. The solution was evaporated, and the residue was dissolved in water (2 ml). GdCl₃ salt (GdCl₃·6H₂O) (30 mg, 0.081 mmol) was added to this solution and the pH was increased to 6 by addition of a KOH (1 M) solution. The solution was then heated at 40 °C overnight. Uncomplexed Gd³⁺ ions were removed by addition of free DOTA solution (0.1 M, pH = 6) and dialysis (Spectra/Por, molecular weight cut-off 6000/8000) against water for 96 h. After the water was evaporated to dryness under reduced pressure, the complex was recovered as a pale yellow oil (100 mg). The macromolecular contrast agent (20 mg) was dissolved in water (2 ml) and analyzed by relaxometric studies.

Complement consumption testing (CH50 test)

Complement consumption was assessed in normal human serum (NHS) (provided by the Etablissement Français du Sang, CHU, Angers, France) by measuring the residual haemolytic capacity of the complement system after contact with particles. The technique consisted in determining the amount of serum able to lyse 50% of a fixed number of sensitized sheep erythrocytes with

rabbit antisherp erythrocyte antibodies (CH50), according to the procedure described elsewhere.^{22,23} Complement activation was expressed as a function of the surface in order to compare particles with different mean diameters. Nanoparticle surface areas were calculated as described elsewhere,²⁴ using the equation: $S = n4\pi r^2$ and $V = n(4/3)(\pi r^3)$ leading to $S = 3m/r\rho$ where S is the surface area (cm²) and V the volume (cm³) of n spherical beads of average radius r (cm), weight m (μg) and volumetric mass ρ (μg cm⁻³).

Cell culture

The human breast adenocarcinoma cell line MCF-7/BOS was kindly provided by Dr A. M. Soto (Department of Anatomy and Cellular Biology, Tufts University School of Medicine, Boston). MCF-7/BOS cells were grown at 37 °C under humidified air containing 5% CO₂ in DMEM medium low glucose with 10 vol% of Foetal Bovine Serum [Origin: EU Approved (South American) FBS], 1 vol% of penicillin/streptomycin (10 000 units of penicillin (base) and 10 000 μg of streptomycin (base) per ml utilizing penicillin G (sodium salt) and streptomycin sulfate in 0.85% saline) and 0.5 vol% gentamycin (10 mg ml⁻¹). The human melanoma line MEL-5 was obtained from De Giovanni (University of Liege, Belgium). MEL-5 cells were grown at 37 °C in 5% CO₂ in DMEM medium high glucose with 5 vol% of FBS, 1 vol% glutamine 2 mM, 1 vol% HEPES, 1 vol% of penicillin/streptomycin.

Copolymer cytotoxicity

The cytotoxicity of the copolymers was evaluated by determining the viability of the cells (MCF-7/BOS and MEL-5 [10⁴ cells per ml]) after incubation with different concentrations of copolymers (0.05 mg ml⁻¹–30 mg ml⁻¹) for 48 h. The percentage of viable cells was determined by the estimation of their dehydrogenase activity using the MTS tetrazolium [(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)], inner salt and an electron coupling reagent (phenazine ethosulfate). The MTS tetrazolium compound is bio-reduced by living cells into a formazan product.

At the end of incubation period with copolymers, cells were incubated with 20 μl of a MTS solution for 4 h at 37 °C in 5% CO₂. The absorbance of the solubilized formazan was measured spectrophotometrically at 490 nm with a multiplate reader (Powerwave X). Cell viability was expressed as the ratio between the amount of formazan determined for cells treated with the different copolymers and the amount for non-treated cells taken as 100%.

Characterizations

¹H and ¹³C NMR spectra of the different polymers were recorded at 298 K with a Bruker spectrometer (250 MHz; 63 MHz for ¹³C) in CDCl₃ or DMSO ((D₁) 2 s, 16 scans, 5 wt% of polymer or 10 wt% of organic compound).

Size exclusion chromatography (SEC). Size exclusion chromatography (SEC) of the polymers was carried out in dimethylformamide containing 25 mM LiBr (flow rate: 1 ml min⁻¹) at 55 °C with a Waters 600 liquid chromatograph equipped with a 410 refractive index detector and four Waters Styragel columns

[HR 1 (100–5000), HR 3 (500–30 000), HR 4 (5000–500 000), and HR 5 (2000–4 000 000) (7.8 × 300 mm)]. Polystyrene standards were used for calibration.

Electrospray mass spectra. Electrospray mass spectra were obtained on a Bruker Daltonics MicrOTOF (TOF-ESI-MS) spectrometer.

Field cycling relaxometry. The water proton NMRD profiles were measured at 25 °C on a Stellar Fast Field-Cycling Spectrometer FFC-2000 in nondeuterated water. The ¹H T₁ relaxation times were acquired by the standard inversion recovery method with a typical 90° pulse width of 3.5 μs, using 16 experiments of 4 scans. The NMRD profiles were measured in the range of magnetic fields from 0.0002 to 1.88 T (corresponding to 0.01–80 MHz proton Larmor frequencies).

Inductive coupling plasma (ICP). The gadolinium concentration of the modified copolymer was determined by inductively coupled plasma mass spectrometry (ICP-MS), carried out with a spectrometer (DRC II, Perkin Elmer). Samples were prepared by the reaction of 1.5 ml macrocontrast agent solution (1 wt% macrocontrast agent) with 2 ml of HNO₃ (65%) at room temperature for 2 h. The solution was then diluted to 100 ml with bidistilled water prior to ICP-MS analysis.

Results and discussion

Scheme 1 shows the general structure of the statistical copolymers investigated as macromolecular platforms for the design of the MRI blood pool agents. They consist of statistical copolymers of a poly(ethylene oxide) methyl ether acrylate (PEOMA) with an acrylate bearing an alkyne group. The conjugation of the gadolinium based contrast agent with the alkyne functional macromolecule is carried out by the highly efficient and selective Huisgen cycloaddition (“click”) reaction. Two different spacers (Scheme 1) between the polymer backbone and the alkyne group are investigated in order to study the effect of their length on the properties of the corresponding macrocontrast agent (relaxivity and toxicity). The anchoring of the gadolinium complex onto the macromolecule decreases its mobility and should therefore increase its relaxivity by decreasing its tumbling rate. Moreover, hindering this complex by the surrounding PEO chains is also expected to contribute to the enhancement of its contrasting efficiency. To the best of our knowledge, there are various reports describing the controlled radical polymerization of PEO-based (meth)acrylates^{25–29} but not on their copolymerization with alkyne functional acrylates. This copolymerization is therefore discussed in the next section using Reversible Addition Fragmentation chain Transfer (RAFT) polymerization.^{26,30}

Synthesis of poly(poly(ethylene oxide) methyl ether acrylate)-*st*-poly(propargyl acrylate) (P[PEOMA-*st*-PA]; Scheme 4)

In order to prepare well-defined copolymers, they are synthesized by the copolymerization of poly(ethylene oxide) methyl ether acrylate (PEOMA) with propargyl acrylate (PA) by Reversible Addition Fragmentation chain Transfer (RAFT) polymerization,^{26,30} a powerful controlled radical polymerization technique.

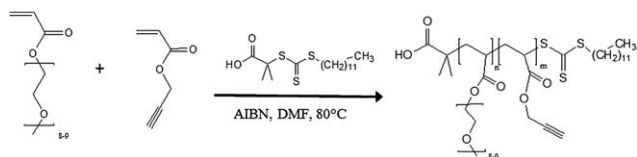
S-1-Dodecyl-*S'*-(α,α' -dimethyl- α'' -acetic acid)trithiocarbonate (CTA)¹⁹ is used as the RAFT agent in the presence of AIBN (10 mol% compared to CTA) in DMF at 80 °C. At a monomer concentration of 33 vol% in DMF, the polymerization is under 70% conversion within 300 min. A complete analysis of the kinetics of copolymerization is carried out in order to evaluate the control character of this copolymerization since, to the best of our knowledge, it has never been reported before. The experimental molecular weights are determined by ¹H NMR spectroscopy by comparison of integrals corresponding to the PEO grafts at 4.16 ppm (–O–CH₂–PEO) and 4.64 ppm (–O–CH₂–alkyne) with the integral of the ω -chain end at 0.87 ppm (CH₃–CH₂–) (Fig. 1).

As shown in Fig. 2, the experimental molecular weight increases linearly with the monomer conversion and is close to the theoretical value. The polydispersity remains low (below 1.2) all along the polymerization process. Moreover, the time dependence of $\ln([M]_0/[M])$ is also linear (Fig. 3). All these observations are consistent with a controlled polymerization.

The composition of the copolymer, determined by ¹H NMR spectroscopy, is also constant during the whole polymerization and is in line with the initial feed composition (Fig. 4), confirming the formation of a statistical copolymer.

$$M_{n, \text{ experimental copolymer}} = \frac{3}{2}(I_i * M_{\text{PEOMA}} + I_j + M_{\text{PA}})$$

where $M_{\text{PEOMA}} = 454$ and $M_{\text{PA}} = 110 \text{ g mol}^{-1}$. M_w/M_n determined by size exclusion chromatography calibrated by PS standards. M_n theor = theoretical molecular weight determined by the following equation



Scheme 4 RAFT copolymerization of PEOMA with PA.

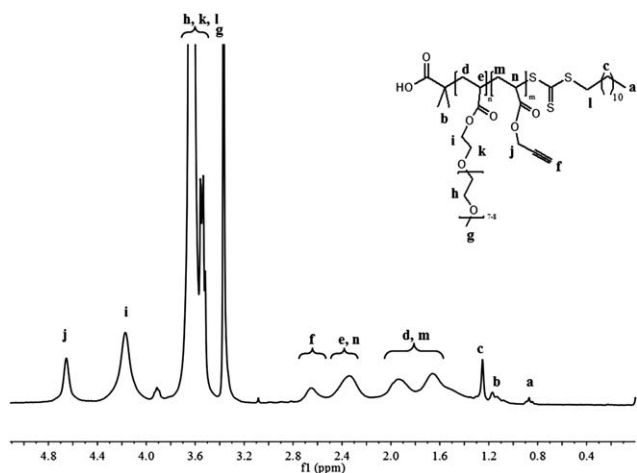


Fig. 1 ¹H NMR spectrum in CDCl₃ of P(PEOMA₃₂-*st*-PA₁₄) (Table 1, entry 2).

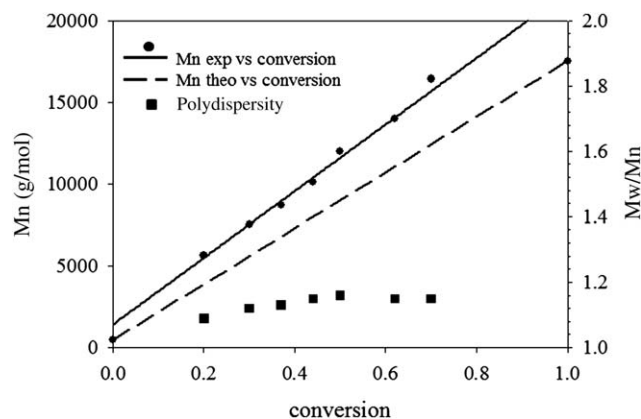


Fig. 2 Experimental molecular weights and polydispersity evolution with the monomer conversion for the PEOMA/PA copolymerization in DMF. Conditions: 80 °C; [monomers]/[CTA] = 50; [PEOMA]/[PA] = 7/3, [CTA]/[AIBN] = 10, (monomers)/DMF = 1/3 v/v. M_n exp = experimental number average molecular weight determined by ¹H NMR spectroscopy using the formula.

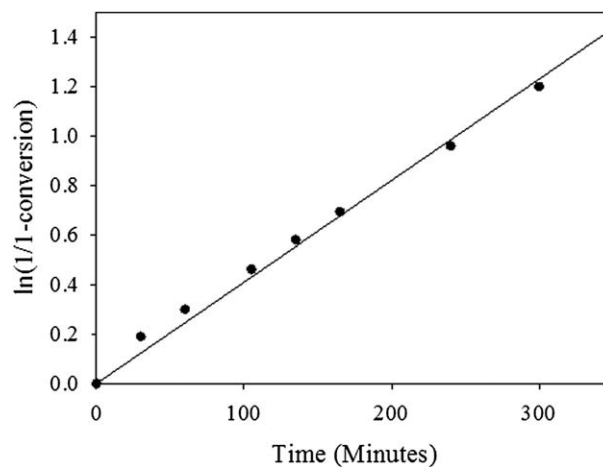


Fig. 3 Time dependence of $\ln([M]_0/[M])$ for the PEOMA/PA copolymerization in DMF. Conditions: 80 °C; [monomers]/[CTA] = 50; [PEOMA]/[PA] = 7/3, [CTA]/[AIBN] = 10, (monomers)/DMF = 1/3 v/v.

$$M_{n, \text{ theoretical copolymer}} = \text{conv} \times \left(\frac{[\text{PA}]_0 \times M_{\text{PA}} + [\text{PEOMA}]_0 \times M_{\text{PEOMA}}}{[\text{CTA}]_0} \right)$$

According to this optimized procedure, two copolymers of different molecular weights (Table 1, entries 1 and 2) are prepared in order to ultimately study the influence of the molecular weight of the polymer on the properties of the macrocontrast agent.

Synthesis of poly(poly(ethylene oxide) methyl ether acrylate)-*st*-poly(2-acryloyloxy)ethyl *pent*-4-ynoate) (P(PEOMA-*st*-AEP); Scheme 5)

The copolymer is prepared by first copolymerizing 2-hydroxyethyl acrylate (HEA) with PEOMA by RAFT, followed by the esterification of the hydroxyl groups with 4-pentynoic acid (Scheme 5). Because CTA was successfully used for the

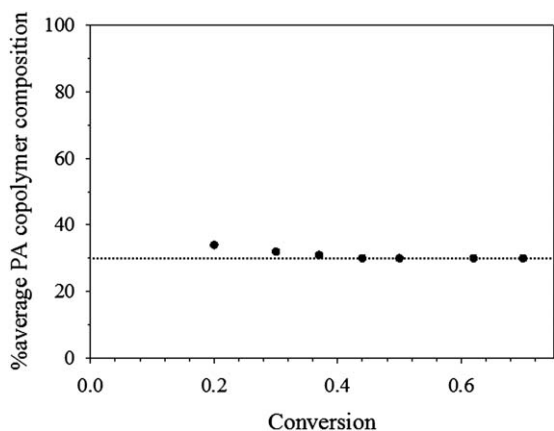


Fig. 4 Propargyl acrylate average composition in the copolymer during the PEOMA/PA copolymerization in DMF at 80 °C ([PEOMA]₀/[PA]₀ = 7/3).

polymerization of both HEA¹⁹ and PEOMA,³¹ it is again used as the RAFT agent in the presence of AIBN (10 mol% compared to CTA) at 80 °C in DMF. At a monomer concentration of 33 vol% in DMF, the polymerization is quasi-complete (>98%) after 4 h with a low polydispersity ($M_w/M_n = 1.16$). After the polymer purification, the experimental molecular weights are determined by ¹H NMR spectroscopy by comparison of integral corresponding to the polymer backbone at 4.14 ppm ($-C=O-O-CH_2-$) with the integral of the ω -chain end at 0.87 ppm (CH_3-CH_2) (Fig. 5). The molecular weight (M_n , NMR = 21 100 g mol⁻¹) is in rather good agreement with the theoretical value as expected for a controlled process (M_n , theor = 17 500 g mol⁻¹). By adapting the monomer to CTA ratio, a P[PEOMA-*st*-HEA] with a different molecular weight is prepared (Table 1, entry 4).

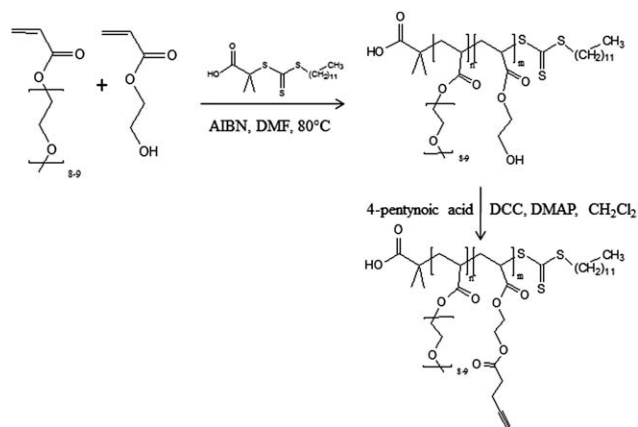
Esterification of the hydroxyl groups of the copolymers with 4-pentynoic acid is carried out in dry CH₂Cl₂ in the presence of *N,N'*-dicyclohexylcarbodiimide (DCC; 1.1 equivalent) and 4-(dimethylamino)pyridine (DMAP; 0.11 equivalent) at 0 °C. Under the investigated conditions, (see Experimental section), the esterification is quantitative after one night as assessed by ¹H NMR analysis (Fig. 5) that evidences the complete disappearance of the signal typical of proton close to hydroxyl group $-CH_2-OH$ at 3.75 ppm and the appearance of the signal characteristic of ester group $-CH_2-O-C(=O)-$ at 4.26 ppm.

Similarly to the previous copolymer, a copolymer with a different molecular weight (Table 2, entries 1 and 2) is also prepared.

Table 1 P[PEOMA-*st*-PA] and P[PEOMA-*st*-HEA] copolymers prepared by RAFT polymerization conditions: [AIBN]/[CTA] = 0.1, 80 °C, [monomer]: 33 vol%, [PEOMA]₀/[PA or HEA]₀ = 7/3, DMF

Entry	[monomer] ₀ /[CTA] ₀	Time/h	Conv. ^a (%)	DP _{PEOM} ^a	DP _{PA or HEA} ^a	M_n , SEC ^b /g mol ⁻¹	M_w/M_n ^b
P[PEOMA- <i>st</i> -PA]							
1	10	5	70%	5	2	7500	1.08
2	50	5	70%	32	14	42 500	1.16
P[PEOMA- <i>st</i> -HEA]							
3	10	4	>98%	9	4	7400	1.16
4	50	4	>98%	42	18	42 000	1.16

^a Determined by ¹H NMR spectroscopy. ^b Determined by size exclusion chromatography (SEC) using polystyrene as standard.



Scheme 5 RAFT copolymerization of PEOMA with AEP, followed by esterification with 4-pentynoic acid.

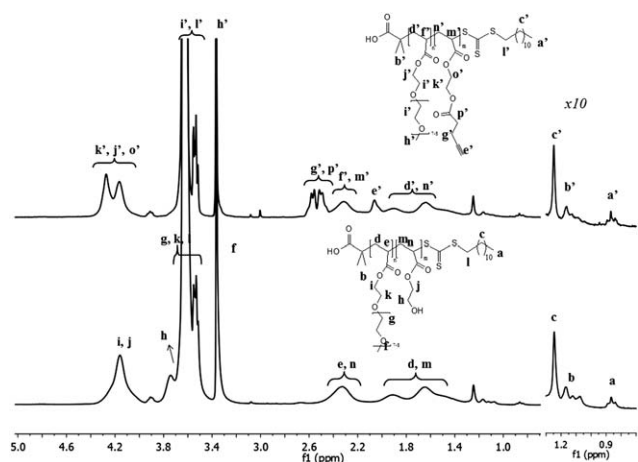


Fig. 5 ¹H NMR spectra of P[PEOMA₄₂-*st*-HEA₁₈] (down) and P[PEOMA₄₂-*st*-AEP₁₈] (up).

Conjugation of azide functionalized molecules to alkynes bearing copolymers (P[PEOMA-*st*-PA] and P[PEOMA-*st*-AEP]) by click reaction (Scheme 1)

Benzyl azide is first used as a model molecule to optimize the conjugation conditions (with or in the absence of sodium ascorbate, bathophenanthroline disulfonic acid disodium salt hydrate, inert atmosphere,...). Optimized conditions are found to be with CuI as the catalyst (10 mol% compared to alkyne) in DMF as solvent at room temperature. As assessed by ¹H NMR

Table 2 Macromolecular characteristics of P[PEOMA-*st*-HEA] and P[PEOMA-*st*-AEP]

Entry	DP (PEOMA)	DP (HEA)	M_n NMR/g mol ⁻¹	DP (PEOMA)	DP (AEP)	M_n NMR/g mol ⁻¹
P[PEOMA- <i>st</i> -HEA] \rightleftharpoons P[PEOMA- <i>st</i> -AEP]						
1	9	4	4550	9	4	4900
2	42	18	21 100	42	18	22 600

analysis (Fig. 6), grafting yields are between 80 and 85% for all the copolymers tested. Due to the small size of benzyl azide, there is no substantial difference between the grafting on the low and high molecular weight copolymers (Table 3, entries 1, 4 and 7, 9). A similar observation is made for the grafting on the two types of copolymers with the different spacers between the alkyne group and the polymer backbone (Table 3, entries 1, 7 and 4, 9).

Following this reaction, the preparation of the MRI blood pool agent is then carried out by two strategies: (1) the grafting of the macroligand precursor (DO₃AtBu-N₃; Scheme 1), followed by the deprotection of the *t*-butyl groups by trifluoroacetic acid

and the subsequent complexation with GdCl₃ at pH = 6; or (2) by direct click reaction of the pre-formed gadolinium complex functionalized by azide group (DO₃A(Gd³⁺)-N₃; Scheme 1).

As expected, when the conjugation of more sterically hindered molecule like DO₃AtBu-N₃ is considered, the grafting yield is decreased to 65% when carried out on the low molecular weight copolymers (Table 3, entries 2 and 8). It drops to about 40–50% when performed on the higher molecular weight copolymers, as the result of increased steric hindrance (Table 3, entries 5 and 10).

When the grafting of DO₃A(Gd³⁺)-N₃ and DO₃AtBu-N₃ is considered, the final product is extracted by EDTA in order to remove the copper catalyst. ICP analysis of the final product confirms that most of the residual catalyst is removed ([Cu]₀ = 5000 ppm, [Cu] after purification = 100 ppm). After reaction, the grafting yield is evaluated by ¹H NMR spectroscopy (for DO₃AtBu-N₃) and ICP analysis (for DO₃A(Gd³⁺)-N₃). The grafting yield of DO₃A(Gd³⁺)-N₃ to P[PEOMA-*st*-PA] with different molecular weights is unfortunately very low (<10%) (Table 3, entries 3 and 6) compared to the DO₃AtBu-N₃ grafting (yield = 40–65%; Table 3, entries 2, 5, 8 and 10). This large difference with DO₃AtBu-N₃ is certainly the result of the folding of the ligand due to the coordination of the gadolinium by the amide and carboxylate groups, rendering DO₃A(Gd³⁺)-N₃ more

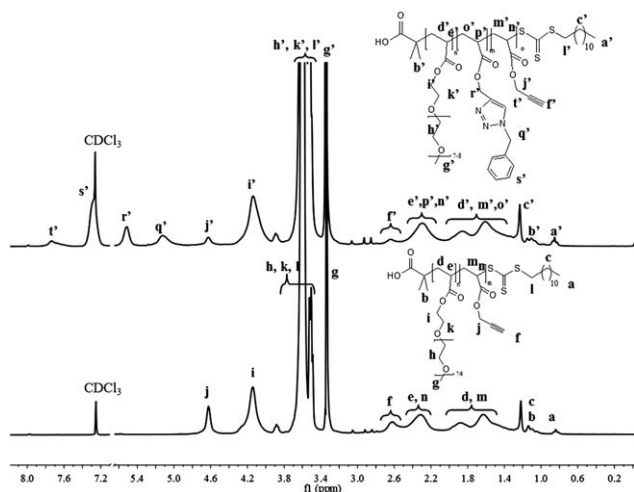
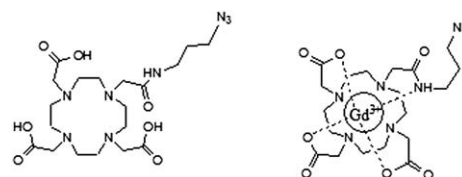


Fig. 6 ¹H NMR spectra of P[PEOMA₃₂-*st*-PA₁₄] ($M_n = 16\,450$ g mol⁻¹) and P[PEOMA₃₂-*st*-PA₁₄] conjugated with benzyl azide (Table 1, entry 5).



Scheme 6 DO₃AtBu-N₃ (left) and DO₃A(Gd³⁺)-N₃ (right).

Table 3 Grafting yields for different azide bearing molecules onto alkyne functionalized copolymers

Entry	Copolymer	Azide bearing molecule	Grafting yield
1	P[PEOMA ₅ - <i>st</i> -PA ₂] $M_n = 3400$ g mol ⁻¹	Benzyl azide	85% ^b
2		DO ₃ AtBu-N ₃	65% ^b
3		DO ₃ A(Gd ³⁺)-N ₃	<10% ^a
4	P[PEOMA ₃₂ - <i>st</i> -PA ₁₄] $M_n = 16\,450$ g mol ⁻¹	Benzyl azide	80% ^b
5		DO ₃ AtBu-N ₃	41% ^b
6		DO ₃ A(Gd ³⁺)-N ₃	<10% ^a
7	P[PEOMA ₉ - <i>st</i> -AEP ₄] $M_n = 4900$ g mol ⁻¹	Benzyl azide	85% ^b
8		DO ₃ AtBu-N ₃	65% ^b
9	P[PEOMA ₄₂ - <i>st</i> -AEP ₁₈] $M_n = 22\,600$ g mol ⁻¹	Benzyl azide	80% ^b
10		DO ₃ AtBu-N ₃	47% ^b

^a Determined by ICP-MS spectrometry. ^b Determined by ¹H NMR spectroscopy.

sterically constrained than the uncomplexed counterpart (Scheme 6).

Because of the low grafting yield of $\text{DO}_3\text{A}(\text{Gd}^{3+})\text{-N}_3$, only the grafting of $\text{DO}_3\text{AtBu-N}_3$ is considered in the following discussion. Macromolecules bearing DO_3AtBu moieties are then reacted with trifluoroacetic acid to remove *tert*-butyl groups, followed by complexation of GdCl_3 at $\text{pH} = 6$. After purifying the copolymers from excess free GdCl_3 , the polymers are analyzed by ICP to determine the amount of Gd^{3+} complexed by the macromolecule. Table 4 summarizes the structure and macromolecular parameters of the macrocontrast agents prepared by this process.

Complement activation tests

Besides imparting water solubility to the macrocontrast agent, the PEO chains have to prevent the recognition of the macrocontrast agent by the immune system that is responsible for its rapid elimination from the blood circulation, restricting timing for studies.

One of the macrocontrast agents ($\text{P}[\text{PEOMA}_{32}\text{-}st\text{-(AEP}_{8-9}\text{-DO}_3\text{A}(\text{Gd}^{3+})_{5-6})]$; Table 4, entry 4) is therefore evaluated by the hemolytic CH50 test and compared to the starting copolymers ($\text{P}[\text{PEOMA}_{32}\text{-}st\text{-AEP}_{14}]$ and $\text{P}[\text{PEOMA}_{42}\text{-}st\text{-HEA}_{18}]$, Table 2, entry 2), and to a poly(2-hydroxyethyl acrylate) (PHEA₅₅) as a positive control.³² Fig. 7 shows that the copolymer bearing PEO grafts and the alkyne groups ($\text{P}[\text{PEOMA}_{32}\text{-}st\text{-PAEP}_{14}]$) has a very low activation of the complement. This activation slightly increases for the copolymer bearing hydroxyl groups ($\text{P}[\text{PEOMA}_{42}\text{-}st\text{-HEA}_{18}]$), known for activating the immune response.³² Importantly, the conjugation of the gadolinium complex onto $\text{P}[\text{PEOMA}_{42}\text{-}st\text{-AEP}_{14}]$ ($\text{P}[\text{PEOMA}_{42}\text{-}st\text{-(AEP}_9\text{-DO}_3\text{A}(\text{Gd}^{3+})_9)]$) does not activate the complement, confirming that the complex is hidden in the PEO shell that renders it stealth to proteins of the immune system. This very low activation means that the macrocontrast agent is expected to have a long blood circulation time and could be ready to be evaluated by *in vivo* test like plasma clearance test.

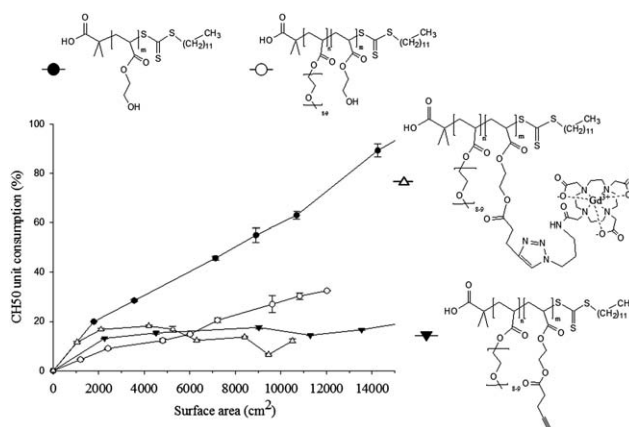


Fig. 7 Consumption of CH50 units in the presence of PHEA₅₅, $\text{P}[\text{PEOMA}_{42}\text{-}st\text{-HEA}_{18}]$, $\text{P}[\text{PEOMA}_{42}\text{-}st\text{-AEP}_{18}]$, $\text{P}[\text{PEOMA}_{42}\text{-}st\text{-(AEP}_9\text{-DO}_3\text{A}(\text{Gd}^{3+})_9)]$ as a function of surface area.

Comparison of CH50 tests realized on $\text{P}[\text{PEOMA}_{42}\text{-}st\text{-AEP}_{18}]$ (Table 2, entry 2) and $\text{P}[\text{PEOMA}_{32}\text{-}st\text{-PA}_{14}]$ (Table 1, entry 2) shows that the length of the spacer between the alkyne group and the polymer backbone has almost no effect on the immune response (Fig. 8). The PEO grafts are thus nicely covering them and render them stealth.

Copolymer cytotoxicity

The cytotoxicity of the different copolymers is evaluated *in vitro* using MCF-7/BOS and MEL-5 cells. Cells (MCF-7/BOS and MEL-5) in appropriate culture media (see Experimental section) are incubated with different concentrations of polymer for 48 h.

Fig. 9 shows the ratio of survival cells population treated with 0.05 mg to 30 mg of copolymer $\text{P}[\text{PEOMA}_{32}\text{-}st\text{-PA}_{14}]$ $M_n = 16\,450\text{ g mol}^{-1}$ for MCF-7 and MEL-5 cell types. Under 10 mg ml^{-1} cell viability is greater than 85%. These results are in good agreement with results of Pissuwan *et al.*³³ ($\text{P}[\text{PEOMA}]$ $M_n = 10\,000\text{ g mol}^{-1}$) and Chang *et al.*³⁴ ($\text{P}[\text{PEOMA}]$ $M_n = 20\,000\text{ g mol}^{-1}$). On the other hand, when the copolymer concentration of

Table 4 Macromolecular characteristics of macromolecular agents prepared in this study

Entry	R	A (DP)	B (DP)	C (DP)	Gd^{3+a} (wt%)
1	$-\text{CH}_2-$	0–1	5	1–2	~7%
2		8–9	32	5–6	~5%
3	$-\text{CH}_2\text{-CH}_2\text{-O-C(=O)-CH}_2\text{-CH}_2-$	1–2	9	2–3	~8%
4		9	42	9	~6%

^a Determined by ICP-MS spectrometry.

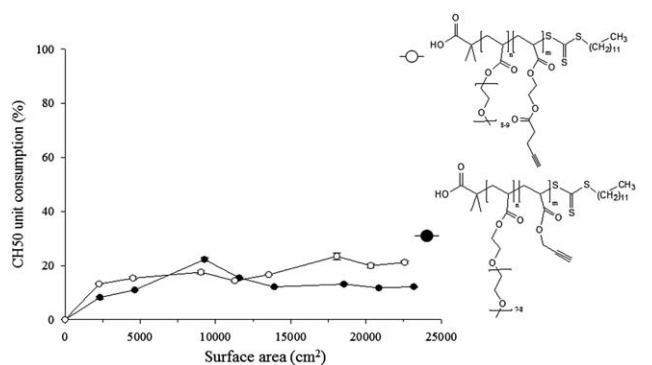


Fig. 8 Consumption of CH50 units in the presence of P[PEOMA₃₂-*st*-PA₁₄] and P[PEOMA₄₂-*st*-AEP₁₈] as a function of surface area.

P[PEOMA₃₂-*st*-PA₁₄] is used at a higher concentration (30 mg ml⁻¹) (higher than Pissuwan *et al.* and Chang *et al.*) cell viability decreases to a value around 50%. The half maximal inhibitory concentration (IC₅₀, the dose to kill half of the cells after 48 h of incubation) is around 30 mg ml⁻¹ for MEL-5 and MCF-7 cells. We have then studied the influence of the structure of the end-group on the copolymer cytotoxicity (Fig. 10). A copolymer with a thiol end group is synthesized by treatment of the copolymer with butyl amine in THF. This thiol end group copolymer has no significant influence on cell viability compared to trithiocarbonate end group (Fig. 10) in agreement with observations made by Pissuwan *et al.*

The cytotoxicity of PEOMA-based copolymers of different functionalities (P[PEOMA₄₂-*st*-HEA₁₈], P[PEOMA₄₂-*st*-AEP₁₈], P[PEOMA₃₂-*st*-PA₁₄] and P[PEOMA₄₂-*st*-(AEP₉-DO₃A(Gd³⁺)₉)] has then been studied under identical conditions (0.5 mg ml⁻¹, 48 h incubation, MEL-5 and MCF-7/BOS) and is compared in Fig. 11.

P[PEOMA₄₂-*st*-HEA₁₈] copolymer is cytotoxic (around 70% of cell viability) at 0.5 mg ml⁻¹ on the two cell types. However

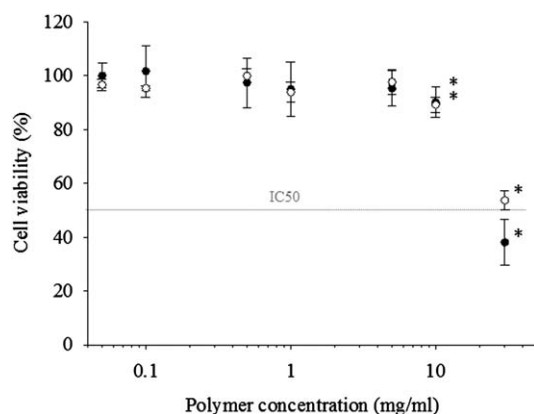


Fig. 9 Cell viability in the presence of different concentrations of copolymer P[PEOMA₃₂-*st*-PA₁₄] in a MEL-5 (○) and MCF-7(●) culture after a 48 h incubation. Data represent mean ± standard error of the mean (S.E.M.) ($n = 4$). Significance indicated by: * $p < 0.05$. On the bottom figure, polymer concentration is represented in logarithm scale conversely on the upper picture which is represented in normal scale. Experiments are set up in *technical replicates*. Cell viability was assessed with the MTS assay.

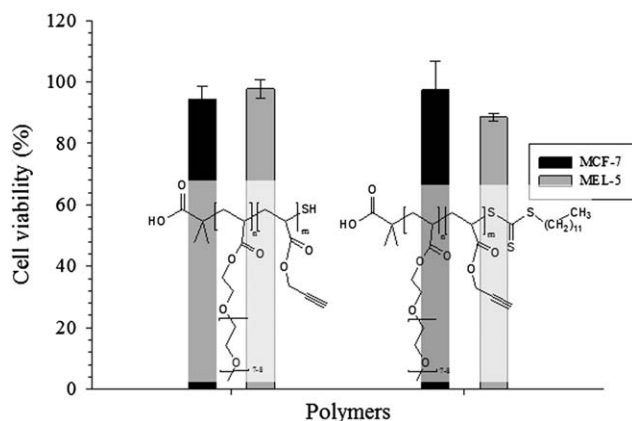


Fig. 10 Cell viability of two copolymers P[PEOMA₃₂-PA₁₄] $M_n = 16\,450\text{ g mol}^{-1}$ with different end groups with same concentration 0.5 mg ml⁻¹. Data represent mean ± standard error of the mean (S.E.M.) ($n = 4$). Experiments are set up in *technical replicates*. Cell viability was assessed with the MTS assay.

esterification of hydroxyl groups with 4-pentynoic acid decreases the copolymer cytotoxicity on both cells.

The cytotoxicity of P[PEOMA₃₂-*st*-PA₁₄] and P[PEOMA₄₂-*st*-(AEP₉-DO₃A(Gd³⁺)₉)] is also compared. DO₃(Gd³⁺) labeled copolymer has only a slightly higher cytotoxicity than bare copolymers. Around 80–85% viable cells are observed, indicating that the grafting of the contrast agent on the copolymer does not induce any significant cytotoxicity to the macromolecule.

Relaxometric studies of the macrocontrast agents

The four different macrocontrast agents prepared by the conjugation of DO₃AtBu-azide by click chemistry on P[PEOMA-*st*-PA] and P[PEOMA-*st*-AEP], followed by the deprotection of the *tert*-butyl esters of the ligand by trifluoroacetic acid and the

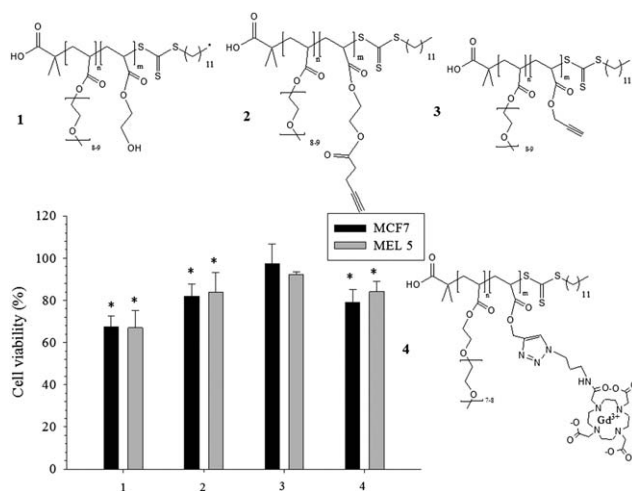


Fig. 11 Cell viability in the presence of different copolymers (1: P[PEOMA₄₂-*st*-HEA₁₈]; 2: P[PEOMA₄₂-*st*-AEP₁₈]; 3: P[PEOMA₃₂-*st*-PA₁₄]; 4: P[PEOMA₃₂-*st*-(AEP₈₋₉-DO₃A(Gd³⁺)₅₋₆)] (0.5 mg ml⁻¹) in MCF-7 and MEL-5 culture after 48 h. Data represent mean ± standard error of the mean (S.E.M.) ($n = 4$). Significance indicated by: * $p < 0.05$. Experiments are set up in *technical replicates*. Cell viability was assessed with the MTS assay.

Table 5 Molecular characteristics of macrocontrast agents and their relaxivities at 20 MHz and 25 °C

Entry	Macrocontrast agents	Relaxivity/mM ⁻¹ s ⁻¹
1	P[PEOMA ₅ - <i>st</i> -(PA ₀₋₁ -DO ₃ A(Gd ³⁺) ₁₋₂)] <i>M</i> _n = 3400 g mol ⁻¹ ,	11.2 (±0.1)
2	P[PEOMA ₃₂ - <i>st</i> -(PA ₈₋₉ -DO ₃ A(Gd ³⁺) ₅₋₆)] <i>M</i> _n = 16 450 g mol ⁻¹	13.0 (±0.1)
3	P[PEOMA ₉ - <i>st</i> -(AEP ₁₋₂ -DO ₃ A(Gd ³⁺) ₂₋₃)] <i>M</i> _n = 4900 g mol ⁻¹	8.4 (±0.1)
4	P[PEOMA ₄₂ - <i>st</i> -(AEP ₉ -DO ₃ A(Gd ³⁺) ₉)] <i>M</i> _n = 22 600 g mol ⁻¹	9.8 (±0.1)

complexation of Gd³⁺ at pH = 6 are characterized (Table 5). Prior to analysis, free Gd³⁺ is removed by adding free DOTA able to strongly complex Gd³⁺, followed by dialysis of the copolymer against water. Freeze-drying of the final product provides the pure macrocontrast agent.

The contrast efficiency of the macrocontrast agents is compared in Table 5. The relaxivity (*r*₁) of these different contrast agents is calculated after measuring longitudinal relaxation time (*T*₁) from:

$$\frac{1}{T_{1\text{obs}}} = r_1 [\text{Gd}^{3+}] + \frac{1}{T_{1\text{H}_2\text{O}}}$$

where *T*_{1obs} and *T*_{1H₂O} are the longitudinal relaxation times in the presence and in the absence of the MR contrast agent [2.86 s] respectively, and [Gd³⁺] is the concentration of the contrast agent in mM.

The relaxivities of the different macrocontrast agents at 20 MHz are found to range from 8.4 to 13 mM⁻¹ s⁻¹ and depend on their structures. For the copolymers of the same chemical nature, increasing the molecular weight of the copolymer slightly increases the relaxivity (comparison of entries 1 with 2, and entries 3 with 4, Table 5). It is a consequence of the slowing down of the rotational motion of the gadolinium complex when the molecular weight increases. Importantly, a substantial difference in relaxivity is observed between the two families of macrocontrast agents. When the length of the spacer between the gadolinium complex and the polymer backbone is decreased, the

relaxivity is higher as a result of improved rigidity of the system (comparison of entries 3 and 4 with entries 1 and 2).

For the sake of comparison, the relaxivities measured for the macrocontrast agents are significantly higher than those of free and low molecular weight contrast agent DO₃A(Gd³⁺)-N₃ (5.1 mM⁻¹ s⁻¹), whose rotational motion is not hindered by a macromolecule. Relaxivities of our best macrocontrast agents (entries 1 and 2, Table 5) are in the same range as that measured for linear polylysine grafted by DOTA (*r*₁ ≈ 15 mM⁻¹ s⁻¹).³⁵

Full relaxometric data are then measured for two macrocontrast agent families over a large magnetic field range (from 0.01 MHz to 100 MHz) and are compared to DO₃A(Gd³⁺)-N₃ (Fig. 12). At low frequency (0.01 to 5 MHz), the relaxivity is about 1.5 higher than that of free DO₃A(Gd³⁺)-N₃. Importantly the effect of the immobilization of gadolinium on macromolecules has an even more pronounced effect on relaxivity at high frequencies (10 to 80 MHz). The maximum relaxivity for our best macrocontrast agent (P[PEOMA₃₂-*st*-(PA₈₋₉-DO₃A(Gd³⁺)₅₋₆)] is obtained at 30 MHz with a 250% relaxivity increase upon grafting of DO₃A(Gd³⁺)-N₃ onto the copolymer.

The grafting of the gadolinium complex onto a hindered macromolecule using a spacer as short as possible is therefore beneficial to the relaxivity of the system. This relaxivity enhancement is a result of an increase of the rotational correlation lifetime of the gadolinium complex due to the bulkiness and rigidity of the whole system. Karfeld-Sulzer *et al.*³⁶ has recently shown that the length between linear polymer backbone and Gd³⁺-complex is very important. Indeed very short spacers prevent tumbling of the complex and long spacers allow the Gd³⁺-complex to move freely.

Conclusion

Novel stealth functional macromolecular platforms were synthesized by RAFT copolymerization of poly(ethylene oxide) methyl ether acrylate with propargyl acrylate or with 2-hydroxyethyl acrylate followed by esterification with 4-pentynoic acid. Gadolinium complexes were grafted to these macromolecular platforms by the Huisgen 1,3-dipolar cycloaddition (“click”) reaction in mild conditions. Gadolinium based macrocontrast agents were best prepared by first grafting the gadolinium ligand precursor (DO₃AtBu-N₃), followed by removing the *tert*-butyl groups by trifluoroacetic acid and finally, by the coordination of Gd³⁺. When the preformed chelate (DO₃A(Gd³⁺)-N₃) was considered, grafting yields were very low due to steric hindrance.

Relaxometry measurements have evidenced an improved relaxivity of the macrocontrast agent by about 250% compared to ungrafted gadolinium chelate. Moreover, this relaxivity was further enhanced when the spacer length between the Gd³⁺-chelate and the polymer backbone was shortened, as a result of its

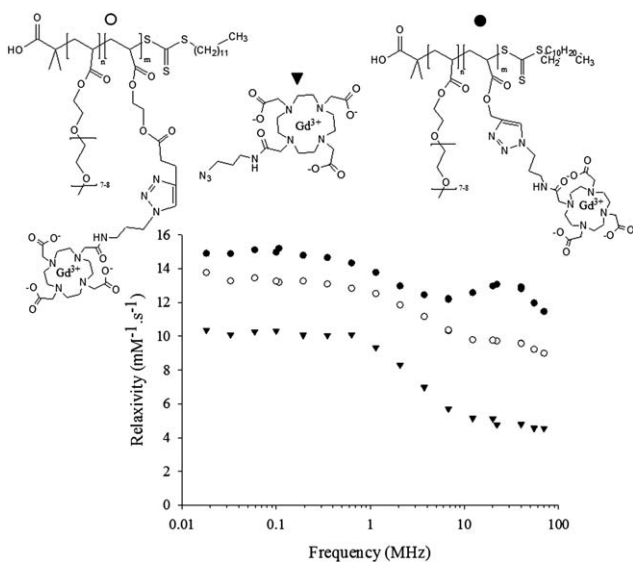


Fig. 12 Comparison of the ¹H NMRD profiles of DO₃A(Gd³⁺)-N₃ (full triangles), P[PEOMA₃₂-*st*-(PA₈₋₉-DO₃A(Gd³⁺)₅₋₆)] (full circles) and P[PEOMA₄₂-*st*-(AEP₉-DO₃A(Gd³⁺)₉)] (empty circles).

decreased tumbling rate. Cytotoxicity and complement activation studies have demonstrated that the macrocontrast agents were free of any cytotoxicity and that the gadolinium complex was hidden in the PEO shell, rendering the macrocontrast agents stealth to proteins of the immune system. This potential long blood circulation half-life time combined with the high relaxivity at high frequency suggests that these novel products are promising candidates for MRI applications at reasonable concentrations.

Because the gadolinium chelates are bonded to a non-degradable polyacrylate chain, the elimination behavior of these macrocontrast agents from the body has now to be studied.

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