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ABSTRACT: New hydrosoluble magnetic resonance imaging (MRI) macrocontrast agents are synthesized by reversible addition fragmentation chain transfer (RAFT) copolymerization of poly(ethylene oxide) methyl ether acrylate (PEOMA) with an acrylamide bearing a ligand for gadolinium, followed by the complexation of Gd^{3+}. This convenient and simple grafting through approach leads to macrocontrast agents with a high relaxivity at high frequency that is imparted by the restricted tumbling of the Gd^{3+} complex caused by its attachment to the polymer backbone. Importantly a very low protein adsorption is also evidenced by the hemolytic CH50 test. It is the result of the poly(ethylene oxide) (PEO) brush that efficiently hides the gadolinium complex and renders it stealth to the proteins of the immune system. Improved contrast and long blood circulating properties are thus expected for these macrocontrast agents. © 2011 Wiley Periodicals, Inc. J Polym Sci Part A: Polym Chem 49: 3700–3708, 2011

KEYWORDS: biological applications of polymers; functionalization of polymers; gadolinium complex; grafting through; MRI; reversible addition-fragmentation chain transfer (RAFT)

INTRODUCTION The search for macromolecular Gd(III) containing contrast agents (CAs)[1] has been an important issue in the past decade in the development of magnetic resonance imaging (MRI). CAs that increase the relaxation rate of water molecules and improve the contrast between tissues of interest, are now widely used in clinical settings but they suffer from a poor sensitivity. The quest for new agents that can be detected by MRI at much lower concentrations thus remains an active area of research.

The CAs presently clinically used comprise a Gd(III) ion chelated by a small organic molecule to reduce the toxicity associated with the free metal ion[2] but they are not optimized to generate maximum contrast. The effective molecular sensitivity of these agents may be improved by attaching multiple Gd^{3+} chelates to macromolecules[1] or to various types of organic[3,4]/inorganic[5,6] nanoparticles. This approach enhances the molecular relaxivity of Gd^{3+} and also results in larger particles that exhibit a prolonged blood circulation that allows a better molecular targeting to tumors or other sites of interest. According to the SBM (Solomon–Bloembergen–Morgan) theory, these macromolecular systems provide an enhanced ability to catalyze solvent proton relaxation rates because of their longer tumbling rates.[7,8] The relaxation effect then becomes a function of the water exchange and of the electronic correlation times, the latter being frequency dependent. This leads to a relaxation maximum between 20 and 100 MHz. Moreover, macromolecular systems increase the lifetime of CAs in the circulating blood by avoiding the extravasation typical of the small-sized Gd(III) complexes commonly used in MRI investigations. Blood lifetime and extravasation are indeed dependent on two main parameters: (1) complement system recognition and (2) kidney clearance. Recognition by the complement system can be strongly decreased by masking CAs by stealth poly(ethylene oxide) (PEO) chains[9], whereas kidney clearance can be limited by the hydrodynamic diameter[10] of CAs. As blood passes through the kidneys, where the glomerulus filters out solutes, waste products, and excess water. These bio-filters consist of a matrix of collagen-like moieties and glycoproteins that form pore size ranging from 4 to 14 nm.[10] Molecules with hydrodynamic diameter smaller than glomerular pores readily permeate and are removed from the body via urine.[11] Fast renal clearance can thus be partially suppressed by grafting gadolinium complexes onto macromolecules with sufficiently high hydrodynamic volumes. The grafting of gadolinium chelates onto linear macromolecules is thus

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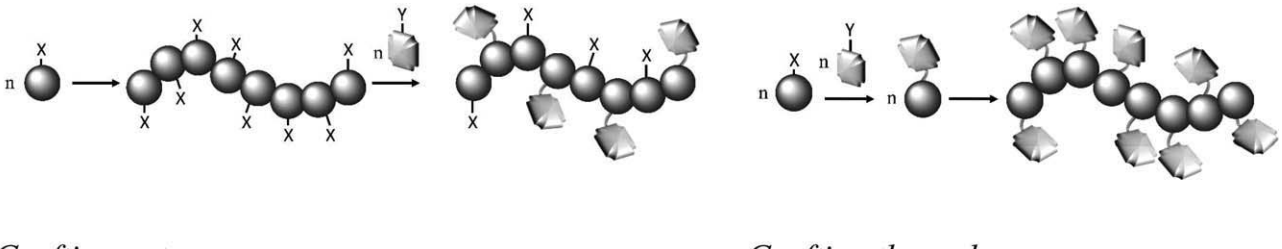


SCHEME 1 Comparison of "grafting onto" and "grafting through" methods used to modify polymers. (*x* and *y* functions in the "grafting onto" strategy are mutually reactive.)

extensively investigated. Various strategies exist such as anchoring bis(anhydride) derivative of diethylenetriaminepentaacetic acid (DTPA) on amine functionalized macromolecules. $(GdDTPA)_n$–albumin,[12] $(GdDTPA)_n$–polylysine[13,14], and dendrimers[15,16] are typical examples. The main drawbacks encountered with these CAs are the difficulty to synthesize large amounts of bis(anhydride) derivative of DTPA and the occurrence of undesirable intra- and intermolecular cross-linking reactions.[17] Macromolecular dendrimer CAs are very well-defined structures with a high relaxivity but purification is particularly difficult.[15] The inverse strategy has also been used by grafting primary amine bearing chelates [1,4,7,10-tetraaza cyclododecane-1,4,7,10-tetraacetic acid (DOTA)] or DTPA onto polymers functionalized by the complementary reactive function such as activated esters[18] or aldehyde functionalized micelles.[19] GdDOTA was also grafted to poly (lysine)s (PLLs) through amidation by reacting free carboxylic acid functions of the metal chelate with primary amines of PLL. Grafting yields around 25% were reported.[20]

Most of these works are based on "grafting to" methods, where the gadolinium chelate is anchored to a preformed macromolecule (Scheme 1). This multistep procedure requires to (i) functionalize the chelate, (ii) synthesize the macromolecule bearing the complementary function, and (iii) anchor the chelate to the polymer. Problems of low grafting yields, side reactions such as cross-linking and/or ill-defined final products may take place thus limiting the possibility of using these macrocontrast agents as effectively as needed.

In the following study we aim at developing a convenient, simple, and efficient method for the production of new well-defined stealth MRI CAs of high relaxivity. Our strategy is based on a "grafting through" method[21] that allows preparing macrocontrast agents by the copolymerization of a chelate bearing a polymerizable function with a comonomer ensuring water solubility and stealthiness (Scheme 1).

EXPERIMENTAL PROCEDURES

Materials

S-1-Dodecyl-*S*′-(α,α′-dimethyl-α″-acetic acid)trithiocarbonate (CTA) was synthesized according to the procedure described elsewhere.[22] Dichloromethane, methanol, ethyl acetate, triethylamine, acetonitrile, methyl bromoacetate, ethylenediamine, acryloyl chloride, poly(ethylene oxide) methyl ether acrylate (PEOMA), potassium carbonate, potassium hydroxide, ammonium chloride, sodium bicarbonate, heptane, gadolinium chloride, deuterated chloroform ($CDCl_3$) with tetramethylsilane (TMS) and sulfate magnesium were purchased from Sigma-Aldrich. The 1,4,7-tris(1,1-dimethylethyl) ester of 1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid (DO_3AtBu) and DOTA were purchased from Chematech. Diethyl ether and dimethylformamide (DMF) were purchased from VWR. 2,2′-Azobis(2-methylpropionitrile) (AIBN) was purchased from Fluka. All products were used as received.

Synthesis

Synthesis of the DO_3A Chelate Functionalized by an Acrylamide Group ($DO_3AtBuAM$)

Synthesis of DO_3A Mono-methyl Tris-tert-butyl Ester, DO_3AtBu-OMe, tert-Butyl 2,2′,2″-(10-(2-Methoxy-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate. DO_3AtBu (9.57 g, 18.6 mmol) was dissolved in 150 mL of acetonitrile. K_2CO_3 (6 g) and 2.12 mL of methyl bromoacetate (24.2 mmol) were added and the resulting solution was stirred for 4 h under reflux. After cooling to room temperature, the solvent was removed under reduced pressure and toluene was added to dissolve the remaining oil. The organic phase was washed three times with water and was dried over $MgSO_4$. The solvent was removed under reduced pressure to give yellow oil (8.75 g, 80% yield).

^1H NMR: ($CDCl_3$, TMS, 250 MHz): δ = 1.42 (s, —C—C**H**$_3$, 27H), δ = 2.79 (m, —N—C**H**$_2$—C**H**$_2$—N, 16H), δ = 3.24 (s, N—C**H**$_2$—C=O—tBu, 6H), δ = 3.38 (s, N—C**H**$_2$—C=O—OMe, 2H), δ = 3.64 (s, —O—C**H**$_3$, 3H). ESI-MS: m/z: 587.41 $[M+H]^+$; 609.39 $[M+Na]^+$.

Synthesis of DO_3AtBu-ethylene Amine Amide (DO_3AtBu-NH_2), tert-Butyl 2,2′,2″-(10-(2-(2-Aminoethylamino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate. DO_3A(tert butyl ester)-methylic ester (8.75 g, 14.9 mmol) was dissolved in ethylene diamine (30 mL, 450 mmol). The solution was stirred at room temperature for 72 h. The efficiency of the reaction between DO_3AtBu-OMe and ethylene diamine was evidenced by mass spectrometry by the complete disappearance of the molecular peak of DO_3AtBu-OMe [(m/z) = 587.41 $(M+H)^+$, 609.39 $(M+Na)^+$] and the appearance of the molecular peak of DO_3AtBu-NH_2 [(m/z) = (M+H), 637.44 $(M+Na)^+$]. After the completion of the reaction (72 h), the excess of ethylene diamine was removed under reduced pressure. The product was purified by silica gel

TABLE 1 ^{1}H NMR and SEC (in $CDCl_3$ and DMF, Respectively) Data of P[PEOMA-st-DO_3AtBuAM] Copolymers Prepared by RAFT Polymerization

Entry	[PEOMA]$_0$/[DO_3AtBuAM]$_0$	Conv. (%)	DP_{th}[a]/DP_{exp}[b] PEOMA	DP_{th}[c]/DP_{exp}[d] DO_3AtBuAM	$M_{n,SEC}$ (g mol^{-1})	M_w/M_n[e]
1[f]	85/15	66	23/29	4/4	11,500	1.08
2[f]	70/30	65	18/25	8/10	12,000	1.10
3[g]	70/30	90	8/10	3/3	4500	1.09

[a] Theoretical DP of PEOMA determined by the equation DP_{PEOMA} = ([PEOMA]$_0$ × conversion)/[CTA]$_0$.
[b] Experimental DP of PEOMA determined by ^{1}H NMR.
[c] Theoretical DP of DO_3AtBuAM determined by the equation $DP_{DO_3AtBuAM}$ = ([DO_3AtBuAM]$_0$ × conversion)/[CTA]$_0$.
[d] Experimental DP of DO_3AtBuAM.
[e] Experimental molecular weight distribution determined by SEC.
[f] [Monomer]$_0$/[CTA]$_0$ = 40, 15 h of polymerization.
[g] [Monomer]$_0$/[CTA]$_0$ = 10, 8 h of polymerization.

chromatography (gradient from 100% CH_2Cl_2 to CH_2Cl_2/MeOH 1:1) and the pure compound (6.40 g, 70% yield) isolated as white foam.

^{1}H NMR ($CDCl_3$, TMS, 250 MHz): δ = 1.42 ppm (s, —C—C**H**$_3$, 27H), 1.80–3.84 ppm (very broad signals with a total integration corresponding to 30H), 8.18 ppm (broad, 1H; C(O)N**H**). ^{13}C NMR (63 MHz, TMS, $CDCl_3$): δ = 27.58 ppm (—C—**C**H$_3$), δ = 41.4 ppm (—**C**H$_2$—NH$_2$), δ = 42.03 ppm (C=O—NH—**C**H$_2$—), δ = 51.9, 52.91, 54.26, 55.80 ppm (—**C**H$_2$—**C**H$_2$—N—), δ = 56.4 ppm (—**C**H$_2$—C=O—OtBu) δ = 57.73 ppm (—**C**H$_2$—C=O—NH—), δ = 80.33 ppm (—**C**—CH$_3$), δ = 170.06 ppm (**C**=O), δ = 171.97 ppm (—**C**=O—NH—). ESI-MS: m/z: 637.44 [M+Na]$^+$.

Synthesis of DO_3AtBuAM,* tert-*Butyl 2,2′,2″-(10-(2-(2-Acrylamidoethylamino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate. DO_3AtBu-NH$_2$ (7.15 mmol, 4.4 g) and triethyl amine (10.75 mmol, 1.1 g) were dissolved in 100 mL of dry CH_2Cl_2. Acryloyl chloride (8.6 mmol, 0.775 g) dissolved in 5 mL of dry CH_2Cl_2 was added dropwise under vigorous stirring to the reaction mixture that was maintained at 0 °C with an ice bath. After completing the addition, the solution was stirred for 4 h at room temperature. The product was purified by flash chromatography (10%/90% MeOH/CH_2Cl_2). The eluate was washed twice with a saturated NH_4Cl solution and was dried over $MgSO_4$. Finally, the mixture was filtered and was concentrated under reduced pressure to give 3.75 g of off-white foam (yield 79%).

^{1}H NMR (TMS, $CDCl_3$, 250 MHz) δ = 1.42 ppm (s, —C—C**H**$_3$, 27H), 1.60–3.84 ppm (very broad signals with a total integration corresponding to 28H), δ = 5.8 and 6.5 ppm (C**H**$_2$—C**H**—C=O—, 3H), 8.7 ppm (broad, 1H; C(O)N**H**) and 9.0 ppm (broad, 1H, C(O)N**H**). ^{13}C DEPT 135 (TMS, $CDCl_3$, 63 MHz): δ = 27.95 ppm (—C—**C**H$_3$), δ = 39.07 and 39.20 ppm (—C=O—NH—**C**H$_2$—**C**H$_2$—), δ = 52.16 ppm (—**C**H$_2$—**C**H$_2$—N—), δ = 56.49 ppm (—**C**H$_2$—C=O—OtBu), δ = 125.16 ppm (**C**H$_2$=CH—C=O), δ = 131.63 ppm (CH$_2$=**C**H—C=O). ESI-MS: m/z: 691.42 [M+Na]$^+$.

Copolymerization of Poly(ethylene oxide methyl ether acrylate) with DO_3AtBuAM

P[PEOMA-st-DO_3AtBuAM], the degree of polymerization (DP) 40; 70 mol % PEOMA/30 mol % DO_3AtBuAM statistical copolymer was synthesized by reversible addition fragmentation chain transfer (RAFT) polymerization of PEOMA and DO_3AtBuAM as follows. AIBN (0.9 mg, 0.0055 mmol), 20 mg of CTA (0.0548 mmol), 697 mg of PEOMA (1.54 mmol), 440 mg of DO_3AtBuAM (0.66 mmol), and 3 mL of DMF were mixed in a 10-mL Schlenk flask. The mixture was degassed by bumbling nitrogen for 5 min. This reaction mixture was heated in an oil bath at 80 °C for 15 h. The resulting polymer was precipitated twice by adding the solution to large amounts of a heptane/diethyl ether (50/50) mixture under vigorous stirring. The molecular weight composition and conversion were determined by ^{1}H NMR in $CDCl_3$. Relative molecular weight and polydispersity were measured by size exclusion chromatography (SEC) in DMF using a polyethylene oxide calibration.

^{1}H NMR ($CDCl_3$, 250 MHz, TMS): δ = 0.86 ppm (t, —CH$_2$—C**H**$_3$, 3H), δ = 1.16 ppm (C**H**$_3$—C—, 6H), δ = 1.24 ppm (—C**H**$_2$—(RAFT), 20H), δ = 1.43 ppm (s, C**H**$_3$—C—, 270H), δ = 3.36 ppm (s, C**H**$_3$—O—PEO, 75H), δ = 3.65 ppm (large, —C**H**$_2$—C**H**$_2$—O—, 850H), δ = 4.16 ppm (large, —C=O—O—C**H**$_2$—PEO, 50H). $M_{n,NMR}$ = 18,000 g mol^{-1}, $DP_{n,NMR}$ = 35, $M_{n,SEC}$ = 12,000 g mol^{-1}, M_w/M_n = 1.10, and conversion = 65%.

P[PEOMA-st-DO_3AtBuAM] with different molecular weights and different compositions were synthesized using the same experimental procedure, except that the RAFT agent to monomer ratio and the ratio between the two monomers were adapted as follows:

P[$PEOMA_{10}$-st-$DO_3AtBuAM_3$] (Table 1, Entry 3): [PEOMA]/[DO_3AtBuAM] = 7/3, [PEOMA + DO_3AtBuAM]/[CTA] = 10, [AIBN]/[CTA] = 0.1. $M_{n,NMR}$ = 6500 g mol^{-1}, $DP_{n,NMR}$ = 13, $M_{n,SEC}$ = 4500 g mol^{-1}, M_w/M_n = 1.09, conversion 90%.

P[$PEOMA_{29}$-st-$DO_3AtBuAM_4$] (Table 1, Entry 1): [PEOMA]/[DO_3AtBuAM] = 85/15, [PEOMA + DO_3AtBuAM]/[CTA] = 40, [AIBN]/[CTA] = 0.1. $M_{n,NMR}$ = 15,800 g mol^{-1}, $DP_{n,NMR}$ = 33, $M_{n,SEC}$ = 11,000 g mol^{-1}, M_w/M_n = 1.08, and conversion 66%.

Formation of the Macromolecular Contrast Agent

Typically, 1 g of the copolymer P[$PEOMA_{25}$-st-$DO_3AtBuAM_{10}$] (Table 1, Entry 2) was dissolved in trifluoroacetic acid (TFA; 10 mL) and stirred overnight at room temperature to remove the *tert*-butyl groups of protected DO_3AtBu. The solution was evaporated to dryness and the residue was dissolved in water (15 mL). $GdCl_3 \cdot 6H_2O$ (230 mg, 0.620 mmol,

1.1 equiv compared with the DO_3AAM function) was added to this solution and the pH was adjusted to 6 by the addition of 1 M KOH solution. The reaction mixture was heated overnight at 40 °C. The uncomplexed Gd^{3+} ions were removed by the addition of DOTA solution (0.1 M, pH = 6) followed by dialysis (Spectra/Por, molecular weight cut-off 6000/8000) against water for 96 h. Water was then evaporated to dryness under reduced pressure and the macromolecular complex ($P[PEOMA_{25}$-st-$DO_3AAM(Gd^{3+})_{10}]$) was obtained as pale yellow oil (1.1 g). The macromolecular CA (20 mg) was dissolved in water (2 mL) and analyzed by relaxometry.

Preparation of the Gd^{3+}Complex with 1-(5-Amino-3-aza-2-oxopentyl)-4,7,10-tris(carbonylmethyl)-1,4,7,10-tetraazacyclododecane, $GdDO_3A$-NH_2

The ligand (DO_3AtBu—NH_2; 200 mg, 0.325 mmol) was dissolved in TFA (3 mL) and was stirred overnight at room temperature. The solvent was evaporated under reduced pressure and the ligand was dissolved in water (5 mL). $GdCl_3 \cdot 6H_2O$ (133 mg, 0.36 mmol) was added to this solution and the pH was adjusted to 6 by the addition of 1 M KOH solution. The solution was heated overnight at 40 °C. Finally, the uncomplexed Gd^{3+} 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 $GdDO_3A$—NH_2 complex was recovered as pale yellow solid in almost quantitative yield (>95%). MS (ESI): m/z: 602.15 $[M\text{-}H]^+$.

Polymerization of 2-Hydroxyethyl acrylate (PHEA)

PHEA (DP50) was synthesized by RAFT polymerization of 2-hydroxyethyl acrylate (HEA) as follows. 2,2′-Azobis(isobutyronitrile) (AIBN; 9 mg, 0.055 mmol), 200 mg of CTA (0.55 mmol), 3.19 g of HEA (27.5 mmol), and 7 mL of DMF were mixed in a 25-mL Schlenk flask. The mixture was degassed by bumbling nitrogen. This reaction mixture was heated in an oil bath at 80 °C for 4 h. The polymer was precipitated twice by adding the solution into a large volume of a heptane/diethyl ether mixture (50/50). The molecular weight composition and conversion percentage were determined by ^{1}H NMR in $CDCl_3$. Relative molecular weights ($M_{n,SEC}$) and polydispersity were measured by SEC in DMF/LiBr using a polyethylene oxide calibration. Conversion >98%.

^{1}H NMR ($CDCl_3$, 250 MHz, TMS): δ = 0.86 ppm (t, —CH_2—CH_3, 3H), δ = 1.16 ppm (CH_3—C—, 6H), δ = 1.24 ppm (—CH_2—(RAFT), 20H), δ = 1.62–1.89 ppm (large, —C**H**$_2$—CH—, 110H), δ = 2.30 ppm (large, —CH_2—C**H**—, 55H), δ = 3.72 ppm (large, —CH_2—C**H**$_2$—OH, 110H), δ = 4.16 ppm (large, —C(=O)—O—C**H**$_2$—CH_2—OH, 110H), $M_{n,NMR}$ = 6400 g mol^{-1}, $DP_{n,NMR}$ = 55, $M_{n,SEC}$ = 6000 g mol^{-1}, M_w/M_n = 1.12.

Characterizations

^{1}H and ^{13}C NMR spectra of the different polymers were recorded at 298 K with a Bruker spectrometer (250 MHz; 63 MHz for 13C) in $CDCl_3$ or d_6-DMSO [($D1$) 2 s, 16 scans, 5 wt % of polymer or 10 wt % of organic compound].

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 mm × 300 mm)]. Polyethylene oxide standards were used for calibration.

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

Field cycling relaxometry. The water proton nuclear magnetic relaxation dispersion (NMRD) profiles were measured at 25 °C in nondeuterated water on a Stelar Fast Field-Cycling Spectrometer FFC-2000 equipped with a permanent magnet for the relaxation measurements in the 15–80 MHz range. The ^{1}H T_1 relaxation times were acquired by the standard inversion recovery method with a typical 90° pulse width of 3.5 μs, using 16 experiments of four 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 reaction of 1.5 mL of a macrocontrast agent solution with 2 mL of HNO_3 (65%) at room temperature for 2 h. The solution was then diluted to 100 mL with bidistilled water before ICP-MS analysis.

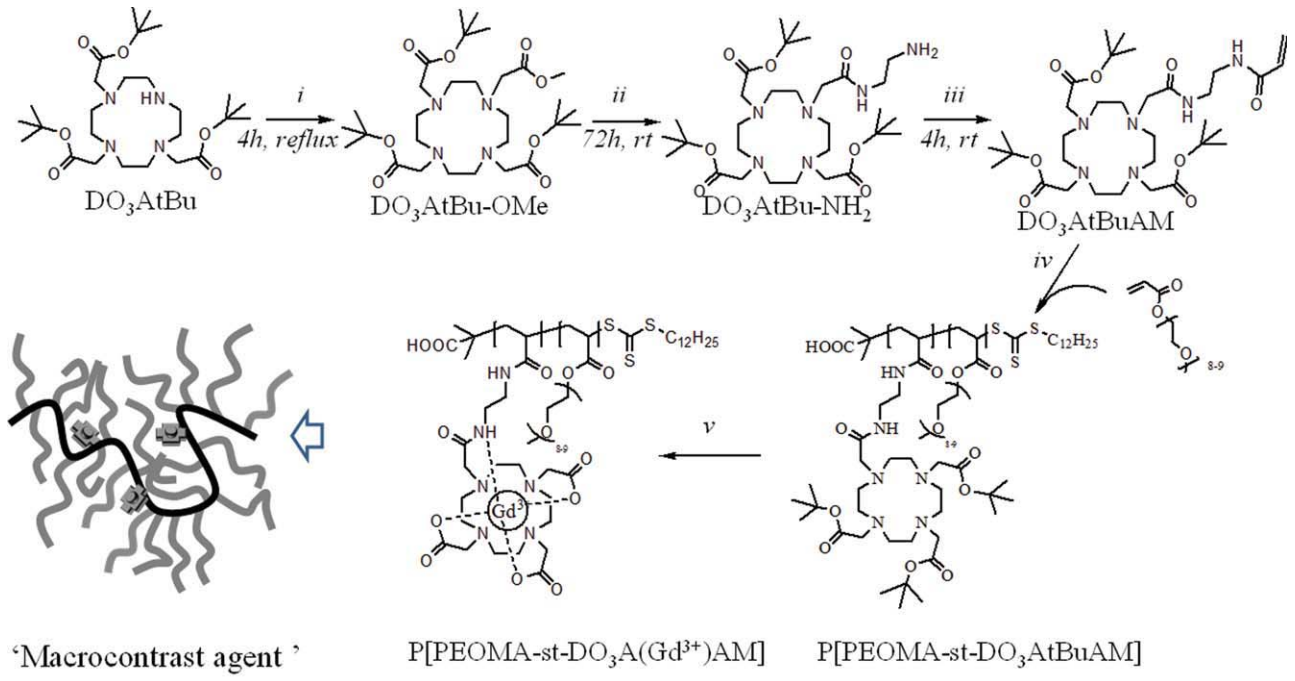
Dynamic light scattering (DLS). Copolymers were analyzed for their size distribution using a Malvern Zetasizer® Nano Series DTS 1060 (Malvern instruments S.A., Worcestershire, UK). Concentrations of copolymers were adjusted to 2.5 mg mL^{-1} in a solution composed of 750 μL of deionized water and 250 μL of Veronal-buffered saline containing 0.15 mM Ca^{2+} and 0.5 mM Mg^{2+} to ensure a convenient scattered intensity on the detector.

Complement consumption testing (CH50 test). The complement consumption was assessed in normal human serum (provided by the Etablissement Français du Sang, CHU, Angers, France) by measuring the residual hemolytic capacity of the complement system. The technique consisted in determining the amount of serum able to lyse 50% of a fixed number of sensitized sheep erythrocytes with rabbit antisheep erythrocyte antibodies (CH50), according to the procedure described elsewhere.[23,24] The complement activation was expressed as a function of the surface to compare polymeric particles with different mean diameters. Nanoparticle surface areas were calculated as described elsewhere,[25] using the equations: $S = n4\pi r^2$ and $V = n(4/3)(\pi r^3)$ leading to $S = 3m/r\rho$, where S is the surface area (cm^2) and V the volume (cm^3) of n spherical beads of average radius r (cm), weight m (μg), and volumetric mass ρ (μg cm^{-3}).

RESULTS AND DISCUSSION

Synthesis of the Macrocontrast Agent P[PEOMA-st-$DO_3A(Gd^{3+})$AM]

Scheme 2 summarizes the general procedure adopted for the synthesis of the macrocontrast agent. First, the *tert*-butyl



SCHEME 2 General procedure for the synthesis of the macromolecular CA by "grafting through" method.

ester of 2,2′,2″-(1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (DO_3AtBu) was functionalized with a *N*-(2-propionamidoethyl)acrylamide group in three steps to obtain $DO_3AtBuAM$. This new macrocyclic ligand was then copolymerized with PEOMA using RAFT process. CTA was the RAFT agent,[22] and the polymerization was performed in the presence of 10 mol % AIBN at 80 °C in DMF. Finally, the *tert*-butyl groups were removed with TFA and the complexation of Gd^{3+} ions was carried out at pH 6 and 40 °C for 10 h. The excess of free Gd^{3+} ions was removed by the addition of 2,2′,2″,2‴-(1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayl) tetraacetic acid (DOTA), a ligand known to strongly complex lanthanide ions. This was followed by dialysis of the copolymer against water and by freeze-drying the final product. The content of gadolinium immobilized onto the copolymers was determined by ICP-MS.

Two different PEOMA/$DO_3AtBuAM$ molar ratios (85/15 and 70/30) were considered as well as two different DPs = 40 and 10 (Table 1). The monomer conversion, copolymer composition, and molecular weights were obtained by ^1H NMR spectroscopy. The experimental molecular weights of the purified copolymers were determined by comparison of the resonance integrals at 4.19 ppm of (—C(=O)—O—C**H**$_2$—PEO) and 1.43 ppm of *tert*-butyl (—C—(C**H**$_3$)$_3$) of $DO_3AtBuAM$ with the integrals at 0.86 ppm corresponding to the ω chain-end (Fig. 1) [—$(CH_2)_{11}$—C**H**$_3$]. They were in good agreement with the theoretical values (Table 1), and the polydispersity of the copolymers determined by SEC was low ($M_w/M_n < 1.1$; Table 1) as expected for a controlled process. The SEC chromatograms are also monomodal (Fig. 2).

Relaxivities of the Macrocontrast Agents P[PEOMA-st-$DO_3A(Gd^{3+})$AM]

The contrast efficiencies of all the synthesized macrocontrast agents are compared in Table 2. The relaxivity (r_1) of each CA is calculated after measuring longitudinal relaxation time (T_1) according to

$$\frac{1}{T_{1\,\mathrm{obs}}} = r_1[Gd^{3+}] + \frac{1}{T_{1\,H_2O}}$$

where $T_{1\ \mathrm{obs}}$ and T_{1H_2O} are the longitudinal relaxation times in the presence and absence (2.86 s) of the MRI CA, respectively, and $[Gd^{3+}]$ is the concentration of the CA in millimolar.

The relaxivities (r_1) of the different macrocontrast agents at 20 MHz range from 12.2 to 14.5 $mM^{-1}\ s^{-1}$ and are about 2.3–2.8 times higher than that of the free $DO_3A(Gd^{3+})$—NH_2 complex taken here as a reference (Table 2). This relaxivity increase is due to the reduced tumbling rates of Gd chelates attached to the macromolecule.[26]

Full relaxometric data were measured for P[$PEOMA_{25}$-st-$DO_3A(Gd^{3+})AM_{10}$] (Table 2, Entry 2) over a large magnetic field range (from 0.01 MHz to 100 MHz) and are compared with $DO_3A(Gd^{3+})$—NH_2 in Figure 3. At low frequencies (0.01–5 MHz), the relaxivity is about 1.5 higher than that of free $DO_3A(Gd^{3+})$—NH_2. The effect of the immobilization of

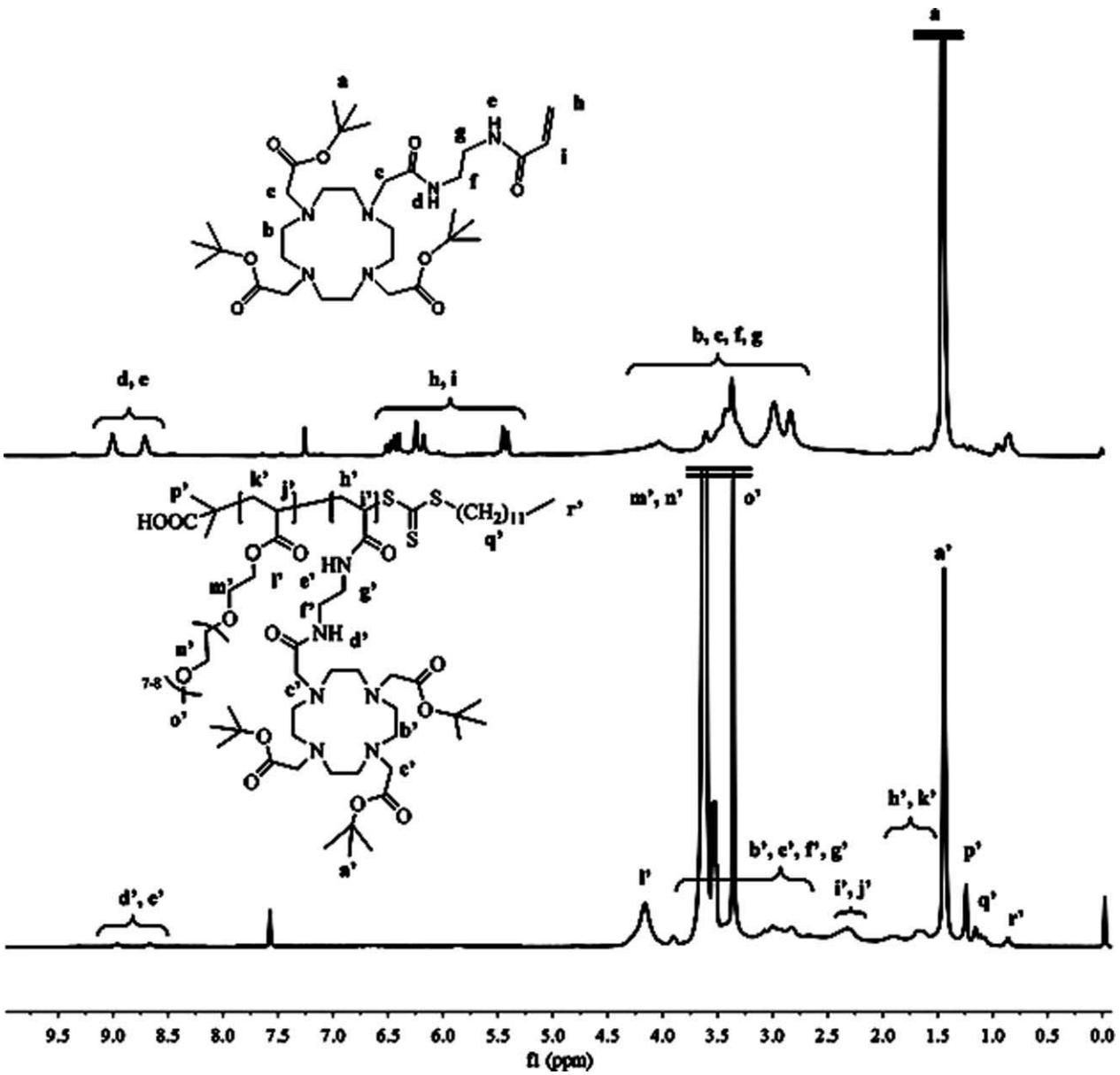


FIGURE 1 ^{1}H NMR spectra of $DO_3AtBuAM$ (up) and $P[PEOMA_{25}\text{-st-}DO_3AtBuAM_{10}]$.

gadolinium on macromolecules has an even more pronounced effect on the relaxivity at high frequencies (10–80 MHz). As shown in Figure 3, the maximum relaxivity for the best macrocontrast agent $P[PEOMA_{25}\text{-st-}DO_3A(Gd^{3+})AM_{10}]$ (Table 2, Entry 2) is obtained at 40 MHz ($r_1 = 14.9\ mM^{-1}\ s^{-1}$) with a 300% relaxivity increase compared with free $DO_3A(Gd^{3+})$—NH_2 ($r_1 = 4.6\ mM^{-1}\ s^{-1}$). This relaxivity value is in the same range as that measured for macrocontrast agents based on micelles ($r_1 = 11.9\ mM^{-1}\ s^{-1}$),[19] twice-higher compared with Gd chelates immobilized on polyacrylic nanoparticles ($r_1 = 6.8\ mM^{-1}\ s^{-1}$),[27] and remains slightly lower than that of dendrimer based Gd chelates ($r_1 = 17.3\ mM^{-1}\ s^{-1}$ at 37 °C).[28]

It should be noted here that the percentage of DO_3A (and thus of Gd^{3+}) in copolymers with similar molecular weights has little influence on the relaxivity at 20 MHz (Table 2, Entries 1 and 2). On the other hand, an increase of the molecular weight of the copolymers slightly improves the relaxivity as expected because the tumbling of the macrocontrast agents becomes slower, in agreement with the work of Karfeld-Sulzer et al.[20] This modest relaxivity increase suggests that the DO_3A ligand attached to the macromolecule possesses a fairly high mobility. The spacer between the ligand and the macromolecule backbone might be shortened to improve this relaxivity.

Complement Activation Test

The improvement of relaxivity by attaching a ligand to a macromolecule is a first way to improve the CA efficiency. A second one consists in avoiding its recognition by the immune system to avoid a rapid elimination from the blood

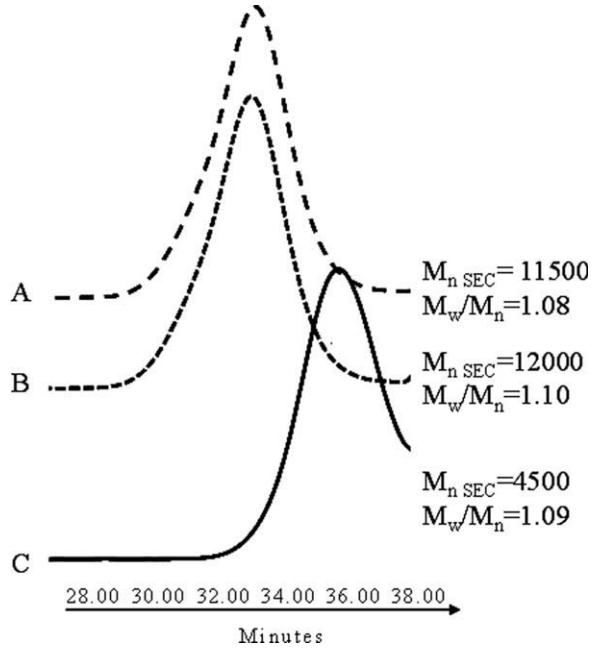


FIGURE 2 Size exclusion chromatograms in DMF of P[PEOMA-st-DO_3AtBuAM]; A: Table 1, Entry 1; B: Table 1, Entry 2; and C: Table 1, Entry 3.

circulation. PEO grafts on our P[PEOMA-st-$DO_3A(Gd^{3+})$AM] macrocontrast agents should therefore ensure this role due to their well-known stealth character.

P[$PEOMA_{25}$-st-$DO_3A(Gd^{3+})AM_{10}$] macrocontrast agent and its precursor P[$PEOMA_{25}$-st-$DO_3AtBuAM_{10}$] were first dissolved in water (2.5 mg mL^{-1}; see the section "Experimental Procedures") and analyzed by DLS. Although P[PEOMA-st-DO_3AtBuAM] forms micelles with an average diameter of 200 nm due to the presence of hydrophobic domains (DO_3AtBuAM) inside the hydrophilic polymer, the macrocontrast agent is fully water soluble with an average size of 25 nm (Fig. 4).

TABLE 2 Data of P[PEOMA-st-$DO_3A(Gd^{3+})$AM] and Their Relaxivities Compared with Free $DO_3A(Gd^{3+})$—NH_2 as Reference at 20 MHz and 25 °C

Entry	(Macro)contrast Agents	Relaxivity per Gd^{3+a} (mM^{-1} s^{-1})	Relaxivity per Polymer (mM^{-1} s^{-1})
1	P[$PEOMA_{29}$-st-$DO_3A(Gd^{3+})AM_4$]	14.2 (±0.1)	56.8 (±0.1)
	M_n = 15,800 g mol^{-1}		
2	P[$PEOMA_{25}$-st-$DO_3A(Gd^{3+})AM_{10}$]	14.5 (±0.1)	145 (±0.1)
	M_n = 18,000 g mol^{-1}		
3	P[$PEOMA_{10}$-st-$DO_3A(Gd^{3+})AM_3$]	12.2 (±0.1)	36.6 (±0.1)
	M_n = 6500 g mol^{-1}		
4	$DO_3A(Gd^{3+})$—NH_2	5.2 (±0.1)	5.2 (±0.1)

[a]At 20 MHz and 25 °C.

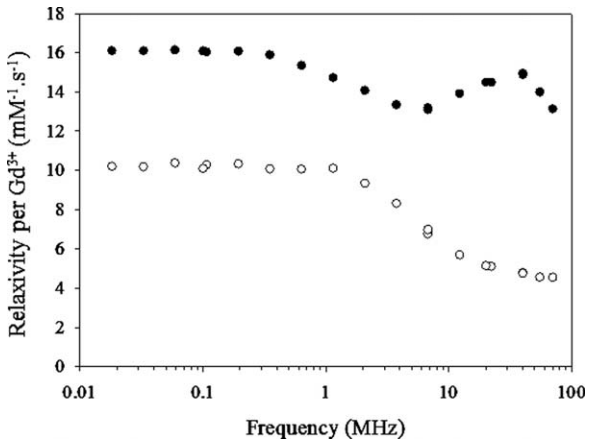


FIGURE 3 Comparison of the ^1H NMRD profiles of $DO_3A(Gd^{3+})$—NH_2 (empty circles) and P[$PEOMA_{25}$-st-$DO_3A(Gd^{3+})AM_{10}$] (Table 2, Entry 2; full circles).

The recognition of the macromolecular CA by the immune system was then evaluated by the hemolytic CH50 test and was compared with poly(2-hydroxyethyl acrylate) used here as a positive control. Interestingly, Figure 5 clearly shows that the macromolecular CA synthesized by the grafting through method does not activate the complement. This very low activation means that the macromolecular CA is expected to have a long blood circulation time, resulting from the stealth PEO brush that hides efficiently the gadolinium complex (Scheme 2).

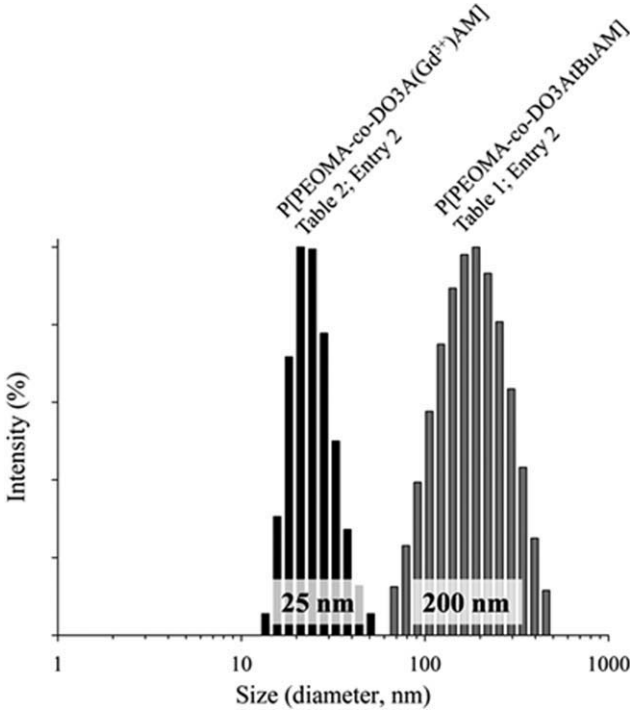


FIGURE 4 DLS of P[$PEOMA_{25}$-st-$DO_3A(Gd^{3+})AM_{10}$] (Table 2, Entry 2) and its precursor P[$PEOMA_{25}$-st-$DO_3AtBuAM_{10}$] (Table 1, Entry 2).

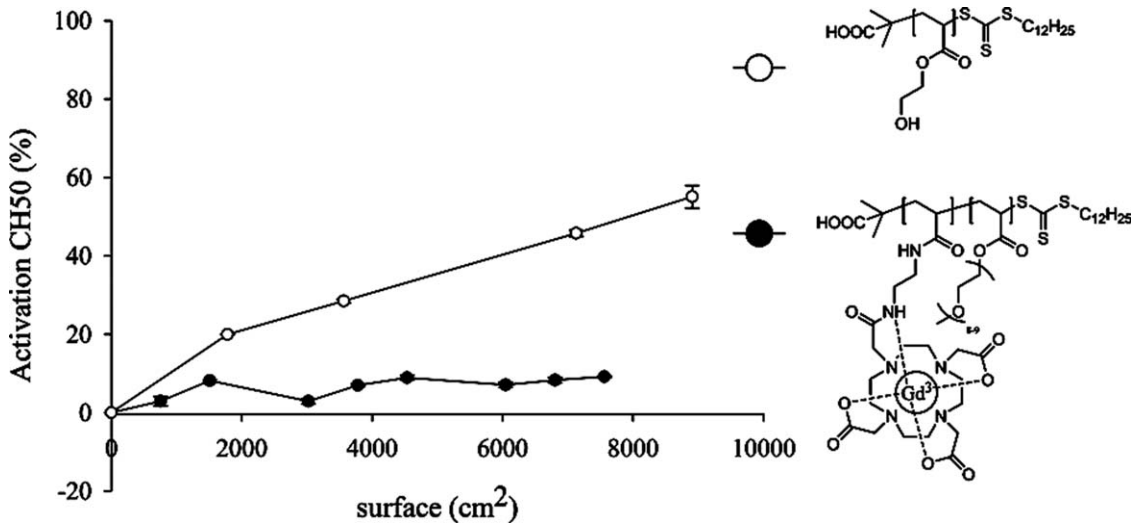


FIGURE 5 Consumption of CH50 units in the presence of $PHEA_{55}$ and $P[PEOMA_{25}\text{-st-}DO_3A(Gd^{3+})AM_{10}]$ as a function of surface area.

Furthermore, it is very important to note that the stability of macrocyclic compounds such as DO_3A is characterized by slow kinetics of decomplexation (dissociation half-life at pH 7.4, $t_{1/2} > 1000$ years).[29] This very slow decomplexation is therefore suitable for safely biological uses.

CONCLUSIONS

Well-defined stealthy MRI CAs were synthesized by a grafting through method by RAFT copolymerization of an acrylamide functionalized ligand ($DO_3AtBuAM$) with PEOMA. By adjusting the copolymerization conditions, the molecular weight of the macrocontrast agent and their content in gadolinium chelates can be easily tuned. Relaxivity was enhanced by 300% compared with the free gadolinium chelate ($DO_3A(Gd^{3+})—NH_2$) at high frequencies. These results suggest that the restricted tumbling of the Gd^{3+} complexes (caused by their attachment to the polymer backbone) contributes to an improved relaxivity at high frequency in consistency with the theory of relaxivity. Moreover, the hemolytic CH50 test has demonstrated that PEO grafts on the macrocontrast agent prevent its recognition by the immune system by hiding the gadolinium chelates. This observation suggests that the PEO-modified macrocontrast agent should have long blood circulation.

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