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Synthesis and characterization of a new lanthanide based MRI contrast agent, potential and versatile tracer for multimodal imaging

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ABSTRACT

In the present work a modular pathway towards the synthesis of a new versatile MRI contrast agent is reported and its physico-chemical properties are described. Two different functional groups were attached on two arms of the gadolinium 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetate (DOTA) in order to get a platform able to bind one probe designed to target specific biological marker and a fluorescent molecule likely to be used for optical imaging. The nuclear magnetic relaxation dispersion (NMRD) profile, the oxygen-17 relaxometric NMR study and stability assessment versus transmetalation of the Gd-complex show that this new contrast agent has a relaxivity and transmetalation stability similar to Gd–DOTA.

1. Introduction

In past two decades, clinical Magnetic Resonance Imaging (MRI) has been developing very rapidly. However unambiguous medical diagnosis often requires the help of MRI contrast agents (CAs). These contrast agents are mainly paramagnetic complexes of Gd(III) or superparamagnetic nanoparticles. The chemistry of numerous paramagnetic complexes has been described in several reviews and books[1,2] and derivatives designed to optimize chemical and magnetic properties have been proposed.[3] The efficacy of a contrast agent is measured by its relaxivity (r_i with i=1,2), the paramagnetic relaxation rate of water proton normalized to a 1 mM concentration of Gd^{3+}. Several tactics were devised to increase the relaxivity of the paramagnetic metal complexes typically by increasing: (i) the molecular size of the paramagnetic system by covalent or non-covalent interaction with macromolecules to slow down the rotational motion in the medium,[4] (ii) the number of coordinated water molecules (q),[5] (iii) the number of paramagnetic metal centres, linking the single complexes in multimeric systems. The significant parameters determining the relaxivity of the complexes are the rotational correlation time (τ_R), the water residence time (τ_M) and the electron spin relaxation times ($\tau_{S1,2}$).

Ligand design has poor influence on the electron spin relaxation, whereas τ_M can be tuned over several orders of magnitude by imposing steric hindrance around the water-binding site in Gd^{3+} complexes of both linear (DTPA-type) and macrocyclic (DOTA-type) ligands.[1] Introduction of an extra methylene group on the backbone of the ligands DOTA and DTPA leads to chelators TRITA and EPTPA, respectively, whose Gd^{3+} chelates display a water exchange around two orders of magnitude faster than the parent Gd–DOTA and Gd–DTPA.[6–8] The development of multifunctional ligands for Gd^{3+} complexation has largely contributed to the advances of magnetic resonance imaging (MRI) in biomedical research.[2,3] These ligands, which could be more versatile, allow for conjugation of the Gd-chelate with specific biological vectors or for the optimization of their efficacy. Ideally, a multifunctional chelator should integrate optimal properties for metal complexation with easy and versatile synthetic possibility for conjugation. Grafting vectors on Gd–DOTA, as mono or diamide chelates has been reported. However, it is well known that complexes bearing amide-chelating units are less stable and exhibit slower water exchange rates that are detrimental to relaxivity.[9–11] Taking into account all these features of Gd-chelates (stability and physico-chemical properties), we preferably design to prepare DOTA derivatives due to their well-recognized kinetic inertness and thermodynamic stability, even if the synthesis of macrocyclic chelates is more difficult and more time consuming than the preparation of non-cyclic analogues. We synthesized and

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characterized a new trendy and versatile agent Gd–DOTA–[Amino Pentyl-Succinic Acid] APSA (Gd–DOTA–APSA), which provides pendant functional groups for conjugation to selected probes. These conjugations are preferably carried out via selective chemistry among amine and acid functionalities, which provide stable compounds. This new derivative is obtained by selective alkylation of two of the nitrogens of the macrocycle by acetate arms bearing different functional groups allowing conjugation through amide or urea linkage.

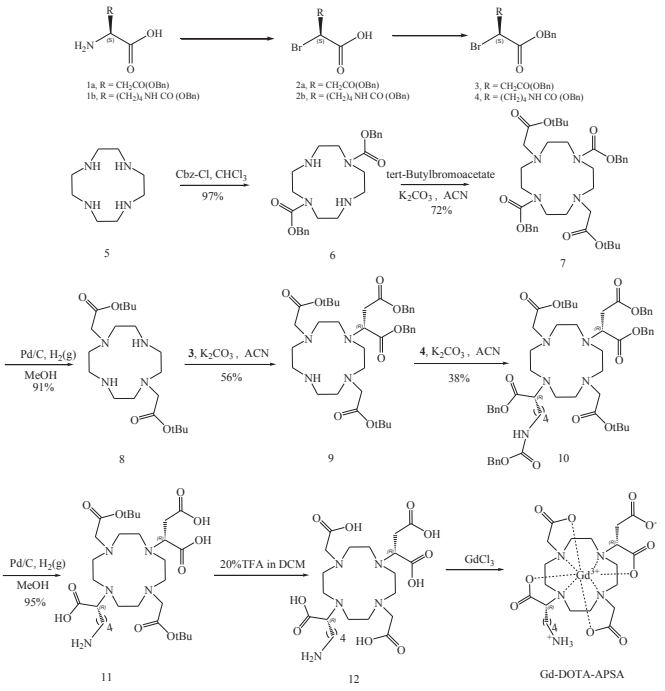
2. Results and discussion

The *trans*-DO2A **8** was synthesized by selective protection of *N*-1 and *N*-7 nitrogens of cyclen by benzylchloroformate and further alkylation and deprotection as previously described[12,13]. The bromo derivatives **2a** and **2b** were synthesized from the benzyl esters of L-aspartic acid and benzylurea derivative of L-lysine by a method analogous to Holmberg followed by Steglich esterification, which provided *S*-dibenzyl 2-bromosuccinate **3** and benzyl (2*S*)-6-{[(benzyloxy) carbonyl]amino}-2-bromohexanoate **4**.[14] The synthetic building blocks **3** and **4**, proved to be efficient alternatives to (unstable) reagents, such as alkene or tosylate-derivatives. Building blocks **3** and **4** were obtained in high yield. They react easily with the *trans*-DO2A **8** via S_N2-type alkylation but the second alkylation needs a little longer reaction time due to steric hindrance of the fourth amine. The mono alkylation of **8** has been optimized by varying the equivalents of bromide **3** in the presence of different bases. Although using K_2CO_3 resulted in the worst selectivity of mono versus bis alkylation, it resulted in the best overall yields for the monoprotection reaction to yield **9**. The final alkylation of **9** was performed alike but by using excess of the bromo analogue under refluxing condition and yielded **10**. Compounds **9** and **10** were obtained with an excellent optical purity of 100% (determined by chiral HPLC). The debenzylation of **10** was performed under hydrogenolysis yielding **11** quantitatively. The final deprotection procedure with TFA afforded the DOTA-(APSA) chelator **12**. Purification of **12** performed by RP-chromatography yielded the trifluoroacetate salt (analytical purity) with a reasonable yield. It is to be noted that the ethyl esters of the bromo analogues of **3** and **4** were synthesized and tested but during the final basic hydrolysis, a semi-hydrolyzed product precipitation occurred, leading to incompletion of reaction.

Finally complexation was performed with $GdCl_3 \cdot 6H_2O$ by maintaining pH between 6.2 and 6.7. The excess of gadolinium ions was removed by using chelex. The absence of excess ions was confirmed by xylenol orange test. The complex was purified by reverse phase chromatography.

The functional groups (e.g., $-NH_2$, COOH) of our complex allow selective conjugation to a wide variety of organic moieties or (bio) macromolecules, via amine as well as carboxylic acid functions (Scheme 1), for purposes of targeting and/or optimization of τ_R. It is to be noted that a metal chelator with one amine group, DO3A-*N*-α-aminopropionate (α-amino-DOTA), has already been reported.[15] The straightforward synthesis and the versatility of further conjugation of our new chelator make this system an excellent multifunctional ligand for the development of imaging agents. The full synthesis and characterization of the ligand are described in the Experimental section. We should note, however, that the non-complexed ligand is not compatible with an extended use in conjugation reactions like peptide couplings.

Proton relaxivity measurements in water revealed that the Gd–DOTA–APSA chelate is stable in the pH range extending from 3 to 8. The magnetic field dependence of the proton longitudinal water proton relaxivities (r_1 NMRD profile) measured at 310 K shows that the relaxivity of our new complex is slightly higher than that of the parent compound Gd–DOTA (Fig. 1).



Scheme 1. Synthesis of Gd–DOTA–APSA.

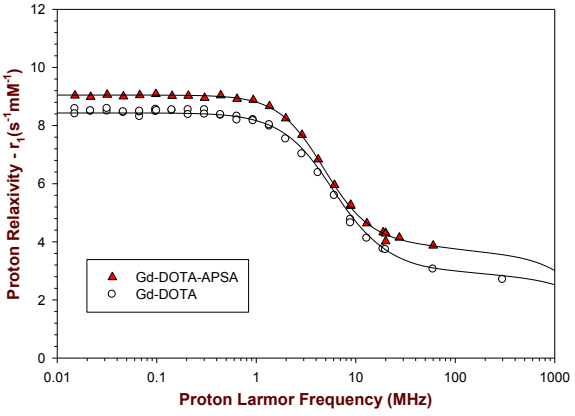


Fig. 1. ^{1}H NMRD profiles of Gd–DOTA–APSA (triangles) & Gd–DOTA (white circles). The lines represent simulations using the best-fit parameters (see Table 1).

The water residence time was determined from variable-temperature ^{17}O NMR studies. The temperature dependence of the reduced ^{17}O transverse relaxation rates ($1/T_{2r}$) (Fig. 2) is typical of a water residence time of the order of 100 ns at 310 K. The analysis of the data using the usual equations[16] gives a τ_M value of 73 ns, a value lower than that of Gd–DOTA (Table 1) but larger than that reported for Gd–DOTMA, a more crowded macrocyclic Gd complex.[17] The NMRD profile was fitted using the inner sphere (Solomon–Bloembergen–Morgan) and outer sphere (Freed) theories. As expected considering the molecular weight of Gd–DOTA–APSA, its value of τ_R is increased as compared to Gd–DOTA (Table 2). Finally the value of τ_{SO} is decreased and similar to that of Gd–$HPDO_3A$.

2.1. Transmetalation

The Gd^{3+} chelates can be sensitive to transmetalation by endogenous ions, such as Cu^{2+}, Ca^{2+} and Zn^{2+}. Among the three metals mentioned, Zn^{2+} has a high affinity for the Gd complexes.

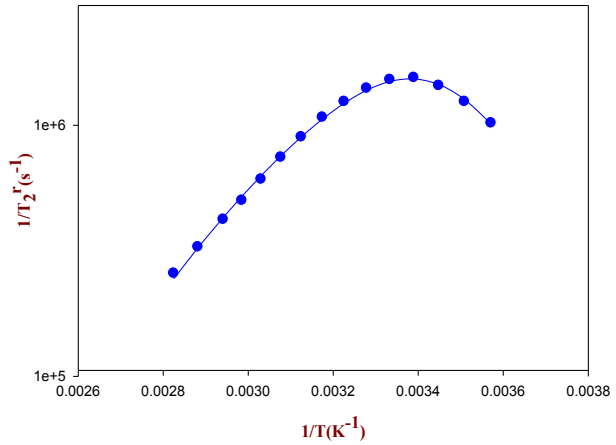


Fig. 2. Temperature dependence of the reduced ^{17}O transverse relaxation rate of Gd DOTA–APSA (26.156 mM).

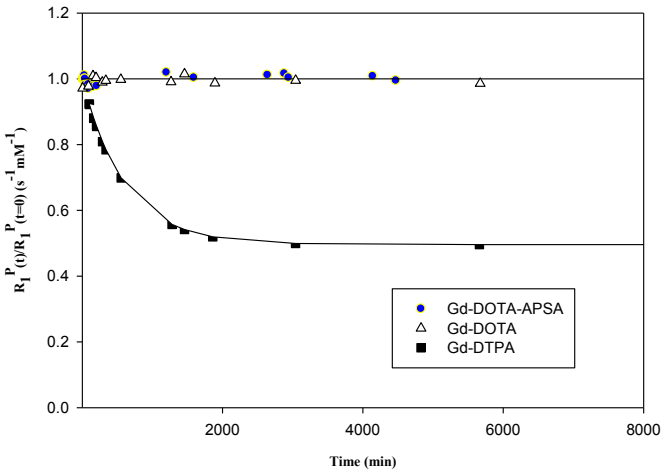


Fig. 3. Relaxation rate $R_1^P(t)/R_1^P(t_0)$ versus time of 2.5 mM Zn^{2+}aqueous solution for Gd–DOTA–APSA (2.5 mM), Gd–DOTA (2.5 mM) and Magnevist (2.5 mM).

Table 1
Parameters obtained by the theoretical fitting of the ^{17}O experimental NMR data (q was fixed to 1)

Parameters	Gd–DOTA–APSA	Gd–DOTA[a]
τ_m^{310} [ns]	73±11	122±10
$\Delta H^{\neq}$ [kJ mol^{-1}]	52.7±0.23	50.1±0.2
$\Delta S^{\neq}$ [kJ mol^{-1}]	61.4±0.46	48.7±0.2
$A/\hbar$ (10^6 rad/s^{-1})	−3.64±0.13	−3.42±0.03
B [10^{20} s^{-2}]	1.79±0.12	1.94±0.09
τ_v^{298} [ps]	2.7±0.2	11.4±0.5
E_V [kJ mol^{-1}]	20.0±3.1	4.0±4.4

[a] Ref. 18.

Table 2
Parameters obtained by ^1H NMRD relaxivity profile of Gd-complex in water at 37 °C[a]

Parameters	Gd–DOTA–APSA[a]	Gd–DOTA[a,b]
τ_v [ps]	8.4±1.3	7±1
τ_R [ps]	73.5±3.5	53±1.3
$\tau_{(so)}$ [ps]	193±18	404±24

[a] The following parameters were fixed: (D is the diffusion coefficient=3×10^{-9} m^2 s^{-1}, d is the distance of closest approach=0.36 nm, r is the distance between the proton of the inner sphere water molecule and Gd ion=0.31 nm, q is fixed to 1 and τ_m was fixed to the value determined by ^{17}O NMR).
[b] Ref. 18.

Therefore, this metal ion is able to replace a significant amount of Gd^{3+}, which may result in release of the toxic Gd^{3+} aqua ion in the body. To investigate the kinetic stability of Gd–DOTA–APSA, the proton longitudinal relaxation rate of a mixture of Gd^{3+} complex and an equal amount of $ZnCl_2$ in phosphate buffer was as usually monitored at 20 MHz and 37 °C.[11] Upon transmetalation by a diamagnetic Zn^{2+} ion in phosphate buffer, the released Gd^{3+} precipitates as $GdPO_4$, which does not contribute to the relaxivity. Therefore, the overall relaxivity of the solution decreases over time with a rate depending on the rate of transmetalation. This decrease in relaxivity is a good estimation of the kinetic lability of the Gd^{3+} complexes (Fig. 3). For example, Magnevist shows significant decomplexation during a time period of 5 days. On the contrary, Gd–DOTA–APSA is virtually inert towards transmetalation with the Zn^{2+} ion at pH 7.0 for extended periods of time, appearing therefore similar to Gd^{3+} complexes like Gd–DOTA and Gd–HPDO$_3$A[8,11] (Fig. 3). These data show that the replacement of two acetate by aminobutylacetate and succinate arms does not lower significantly neither the thermodynamic nor the kinetic stabilities as compared to Gd–DOTA.[19]

3. Conclusion

A new contrast agent was synthesized and characterized. This contrast agent shows promising relaxivity, which could further be optimized by reducing τ_M and provides versatile functional groups for getting multimodal contrast agent by grafting targets and chromophores. The modular synthetic pathway described can be further implemented in the synthesis of wide variety of specific MRI contrast agents.

4. Experimental section

4.1. General

Cyclen was purchased from Chematech (France) while all chemicals were purchased from Sigma–Aldrich (Belgium), Across (Belgium). All chemicals were used without further purification. All ^1H and ^{13}C NMR spectra were recorded on Bruker Avance 500 MHz spectrometer at 25 °C using 5 mm sample tubes. The chemical shifts are given in (δ) parts per million. The purification of the compounds was performed on KP-silica cartridges from Biotage over Biotage flash chromatography instrument, Uppsala, Sweden. For the ligand purification KP-C_{18} Biotage cartridges were used. The pH values of the solution were adjusted using aqueous solution 1 M NaOH and HCl. Mass spectra were obtained on Q-TOF Ultima mass-spectrometer (Micromass, Manchester UK). Samples were dissolved in H_2O/MeOH and injected at flow rate 5 μl min^{-1}. The cone voltage was 40 V (T=90 °C). The compounds **2a**, **2b**, **6**, **7** and **8** were synthesized as reported in Ref. 13. Chiral HPLC was performed on Alliance Waters 2695, ChiralPak® AD-RH column 5 μm (4.6×150 mm), mobile phase: acetonitrile/water (40/60), flow rate: 0.5 ml/min, detection by Waters PDA 486.

4.2. Experimental procedures

*4.2.1. Synthesis of (S)-dibenzyl 2-bromosuccinate (**3**).* A solution of **2a** (9.3 g, 32.1 mol), benzyl alcohol (5 mL, 48.2 mol), *N*,*N*-dicyclohexylcarbodiimide (DCC) (10.0 g, 48.5 mol) and 4-dimethylaminopyridine (DMAP) (catalytic) in CH_2Cl_2 (25 mL) was stirred at room temperature for 3 h. The precipitated dicyclohexylurea was filtered off and the filtrate was washed with water, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude product was purified by flash chromatography to give **3** (7.29 g) as colourless oil yield 60%. ^1H NMR (500 MHz, $CDCl_3$) δ: 7.35–7.28 (m,

10H), 5.23–4.99 (m, 4H), 4.69–4.43 (m, 1H), 3.30 (dd, *J*=17.2, 8.9 Hz, 1H), 3.00 (dd, *J*=17.2, 6.1 Hz, 1H); ^{13}C NMR (500 MHz, $CDCl_3$) δ: 169.38, 168.81, 135.32, 135.07, 128.69, 128.57, 128.59, 128.40, 128.26, 67.90, 67.04, 39.81, 38.18; ESI-MS ($C_{18}H_{17}BrO_4$); *m*/*z*: 378 $[M+H]^+$.

*4.2.2. Syntheis of benzyl (2S)-6-{[(benzyloxy) carbonyl]amino}-2-bromohexanoate (**4**).* A solution of compound **2b** (1.03 g, 2 mmol), benzyl alcohol (0.33 mL, 3 mmol), *N*,*N*′-dicyclohexyl carbodiimide (DCC) (0.678 g, 3.2 mmol) and 4-dimethylaminopyridine (0.03 g, 0.3 mmol) in CH_2Cl_2 (10 mL) was stirred at room temperature for 3 h. The precipitated dicyclohexylurea was filtered off and the filtrate was washed with 5% aq AcOH (10 mL) and water (3×10 mL), dried over anhydrous Na_2SO_4 and evaporated under vacuum. The crude product was purified by column chromatography to give **4** (0.77 g, 60%) as pale yellow oil. ^{1}H NMR (500 MHz) δ: 7.51–7.27 (m, 10H), 5.26–5.03 (m, 4H), 4.71 (d, *J*=11.9 Hz, 1H), 4.24 (t, *J*=7.2 Hz, 1H), 3.17 (dd, *J*=12.4, 6.1 Hz, 2H), 2–2.1 (m br, 2H), 1.7 (s br, 2H), 1.5 (s br, 2H) ESI-MS ($C_{21}H_{24}BrNO_4$) *m*/*z*=434 $[M+H]^+$.

*4.2.3. Synthesis of dibenzyl (2R)-2-[4,10-bis(2-tert-butoxy-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl]succinate (**9**).* Compound **3** (0.532 g, 1.4 mmol) and (0.3 g, 2.26 mmol) K_2CO_3 are added to a solution of (562 mg, 1.4 mmol) **8** in 100 mL ACN, after which the mixture is heated at 50 °C for 8 h. The solids are filtered off over a pad of Celite and the solvent is removed in vacuo. Chromatographic purification (silica gel, 98/1.5/0.5 DCM/MeOH/TEA) yielded 555 mg of **9** as slight yellow oil. Yield 56% with 100% optical purity. H NMR (500 MHz, $CDCl_3$) δ: 7.48–7.12 (m, 8H), 4.04 (dd, *J*=10.0, 4.0 Hz, 1H), 3.41–3.11 (m, 4H), 2.92–2.4 (m, 14H), 2.49 (dd, *J*=23.6, 9.9 Hz, 4H), 1.46 (s br, 18H); ^{13}C (500 MHz, $CDCl_3$), δ: 171.89, 171.41, 170.91, 135.78, 135.73, 128.52, 128.48, 128.33, 128.28, 128.26, 128.17, 127.12, 126.75, 80.88, 66.47, 66.42, 57.13, 56.08, 53.41, 52.03, 51.69, 50.00, 49.86, 46.81, 28.20. HRMS (ESI): calcd for $C_{38}H_{57}N_4O_8$ $[M+H]^+$ 697.4176; found 697.4150.

*4.2.4. Synthesis of dibenzyl(2R)-2-[7-((1R)-1-[(benzyloxy)carbonyl]-5-{[(benzyloxy)carbonyl]amino}pentyl)-4,10-bis (2-tert-butoxy-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl]succinate (**10**).* Compound **4** (0.465 g, 1.08 mmol) and K_2CO_3 (0.2 g, 1.4 mmol) are added to a solution of (500 mg, 0.72 mmol) **8** in (60 mL) ACN, after which the mixture is heated under reflux conditions for 18 h. The solids are filtered off over a pad of Celite and the solvent is removed in vacuo. Chromatographic purification (silica gel, 83/15/2 Acetone/MeOH/TEA) yielded 286 mg of **10** as yellow oil. Yield 38% with 100% optical purity. H NMR (500 MHz, $CDCl_3$) δ: 7.37–7.22 (m, 20H), 5.33–4.91 (m, 9H), 4.27 (dd, *J*=7.5, 4.3 Hz, 1H), 3.43–3.03 (m, 9H), 2.83–2.13 (m, 17H), 1.92–1.33 (m, 23H); ^{13}C (500 MHz, $CDCl_3$) δ: 175.96, 174.17, 173.30, 173.24, 173.17, 171.41, 156.51, 136.83, 135.38, 135.23, 134.88, 128.68, 128.65, 128.53, 128.41, 128.26, 128.03, 127.93, 82.16, 82.06, 67.47, 67.04, 66.82, 66.37, 61.03, 58.39, 56.04, 53.95, 52.84, 52.59, 48.93, 48.77, 47.67, 47.23, 46.05, 44.92, 44.81, 44.35, 41.90, 40.52, 29.88, 29.26, 28.98, 28.18, 27.93, 26.94, 26.18, 24.95, 24.47, 22.52, 22.08. HRMS (ESI): calcd for $C_{59}H_{80}N_5O_{12}$ $[M+H]^+$ 1050.5803; found 1050.5787.

*4.2.5. Synthesis of (2R)-2-[7-[(1R)-5-amino-1-carboxypentyl]-4,10-bis(carboxymethyl)-1,4,7,10-tetraazacyclododecan-1-yl] succinic acid (**12**).* 10% Pd/C (50 mg) is added to a solution of (280 mg, 0.27 mmol) **10** in (20 mL) MeOH and the resulting suspension is shaken for 6 h under H_2 atmosphere. The suspension was filtered over a Celite pad and the solvent was removed in vacuo. Crude **11** (163 mg) was obtained as slight yellow oil. The crude was proceeded for TFA hydrolysis without further purification. The crude was dissolved in (10 mL) 20% TFA in CH_2Cl_2 stirred for 16 h at room temperature. The reaction mixture was concentrated and washed with Et_2O (2×25 mL) and the residue was dried under reduced pressure and yielded **12** quantitatively. ^{1}H NMR (500 MHz, D_2O) δ: 4.32–4.21 (m, 1H), 4.1–3.42 (m, 8H), 3.09–2.9 (m, 15H), 2.89–2.61 (m, 5H), 1.65–1.52 (m, 4H), 1.38 (s br, 2H). ^{13}C (500 MHz, D_2O) δ: 176.07, 170.43, 169.23, 60.43, 57.49, 57.04, 56.56, 56.25, 52.41, 51.15, 50.56, 49.84, 46.61, 45.46, 45.37, 44.12, 43.21, 38.68, 38.42, 28.19, 25.94, HRMS (ESI): calcd for $C_{22}H_{40}N_5O_{10}$ $[M+H]^+$ 534.2775; found 534.2799.

4.2.6. Preparation of Gd^{3+} complex of DOTA-[APSA]. The complex was obtained by adding portion wise $GdCl_3 \cdot 6H_2O$ to a solution of (0.1 g, 0.18 mmol) in (5 mL) H_2O, maintaining the pH between 6.2 and 6.7 by addition of 1 M NaOH. The final pH of the solution after stirring 52 h was 6.3. The excess of the free lanthanide was removed as $Gd(OH)_3$ precipitate, which appeared at pH 9 after addition of 1 M NaOH. The resulting solution was treated with chelex-100 to remove free Gd^{3+} ions. The absence of free Gd^{3+} ions was confirmed by a xylenol orange test. The pH of the supernatant was decreased to 7 and the solution was freeze-dried. The complex was dissolved in water, purified by reverse phase flash chromatography and freeze-dried, yielding 80 mg of Gd–DOTA–APSA as a white powder. HRMS (ESI): calcd for $C_{22}H_{36}GdN_5O_{10}Na$ $[M+Na]^+$ 707.1569; found 707.1545.

4.2.7. Transmetalation. The stability of the Gd^{3+} complexes was determined by a transmetalation method monitoring the ^{1}H longitudinal relaxation rates of water during 5 days at 37 °C.[11] The measurements were performed on a Bruker Minispec mq-20 spin analyzer at 20 MHz (Bruker, Karlsruhe, Germany) using 7 mm sample tubes containing 2.5 mM of Gd^{3+} complexes and 2.5 mM of $ZnCl_2$ in 300 μl of phosphate buffer solution (26 mM KH_2PO_4, 41 mM Na_2HPO_4, pH=7).

4.2.8. ^{17}O relaxometric measurements. ^{17}O NMR measurements were performed at 11.75 T on 350 μL samples contained in 5 mm o.d. tubes on a Bruker Avance 500 spectrometer (Karlsruhe, Germany). Temperature was regulated by air or nitrogen flow controlled by a Bruker BVT 3200 unit. ^{17}O transverse relaxation times of distilled water (pH 6.5–7) were measured using a CPMG sequence and a subsequent two-parameter fit of the data points. The 90° and 180° pulse lengths were 27.5 and 55 μs, respectively. The ^{17}O T_2 of water in complex solution was obtained from line width measurements. All spectra were proton decoupled. The data are presented as the reduced transverse relaxation rate $\{1/T_2^R = 55.55/([\text{Gd complex}] \times q \times T_2^q)$, where: [Gd complex] is the molar concentration of the complex, q is the number of coordinated water molecules and T_2^q is the paramagnetic transverse relaxation rate}. The fitting of the experimental data was performed as previously described.[16] The sample concentration was determined by ICP-AES on a JobinYvon JY 70$^+$ instrument (Longjumeau, France) and was further confirmed by ^{1}H relaxometry of a decomplexed sample.

4.2.9. Proton NMRD. Proton nuclear magnetic relaxation dispersion (NMRD) profiles were measured on a Stelar Spinmaster FFC (Mede, Italy) fast field cycling NMR relaxometer over a magnetic field range from 0.24 mT to 1.0 T. Measurements were performed on 0.6 mL samples contained in 10 mm o.d. Pyrex tubes. Additional relaxation rates at 20 and 60 MHz were obtained on a Minispec mq20 and a Minispec mq60, respectively.

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