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#### Recommended Citation

Bhuiyan MP, Aryal MP, Janic B, Karki K, Varma NR, Ewing JR, Arbab AS, and Ali MM. Concentration-independent MRI of pH with a dendrimer-based pH-responsive nanoprobe. *Contrast Media Mol Imaging* 2015; 10(6):481-486.

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# Concentration-independent MRI of pH with a dendrimer-based pH-responsive nanoprobe

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The measurement of extracellular pH ( $\text{pH}_e$ ) has significant clinical value for pathological diagnoses and for monitoring the effects of pH-altering therapies. One of the major problems of measuring  $\text{pH}_e$  with a relaxation-based MRI contrast agent is that the longitudinal relaxivity depends on both pH and the concentration of the agent, requiring the use of a second pH-unresponsive agent to measure the concentration. Here we tested the feasibility of measuring pH with a relaxation-based dendritic MRI contrast agent in a concentration-independent manner at clinically relevant field strengths. The transverse and longitudinal relaxation times in solutions of the contrast agent (GdDOTA-4AmP)<sub>44</sub>-G5, a G5-PAMAM dendrimer-based MRI contrast agent in water, were measured at 3 T and 7 T magnetic field strengths as a function of pH. At 3 T, longitudinal relaxivity ( $r_1$ ) increased from 7.91 to 9.65  $\text{mM}^{-1} \text{s}^{-1}$  (on a per  $\text{Gd}^{3+}$  basis) on changing pH from 8.84 to 6.35. At 7 T,  $r_1$  relaxivity showed pH response, albeit at lower mean values; transverse relaxivity ( $r_2$ ) remained independent of pH and magnetic field strengths. The longitudinal relaxivity of (GdDOTA-4AmP)<sub>44</sub>-G5 exhibited a strong and reversible pH dependence. The ratio of relaxation rates  $R_2/R_1$  also showed a linear relationship in a pH-responsive manner, and this pH response was independent of the absolute concentration of (GdDOTA-4AmP)<sub>44</sub>-G5 agent. Importantly, the nanoprobe (GdDOTA-4AmP)<sub>44</sub>-G5 shows pH response in the range commonly found in the microenvironment of solid tumors. Copyright © 2015 John Wiley & Sons, Ltd.

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**Keywords:** responsive agent; noninvasive pH measurement; magnetic resonance imaging; dendritic agent

## 1. INTRODUCTION

The extracellular tumor microenvironment is characterized by an acidic pH (denoted  $\text{pH}_e$  to identify it as the extracellular pH), and an intracellular  $\text{pH}_i$  (1–5) that is neutral to alkaline. A similar transcellular pH gradient is not observed in normal tissues. Contributing factors to an acidic tumor  $\text{pH}_e$  are increased glycolysis, even in the presence of sufficient oxygen (the Warburg effect) (6). Hydrolysis of ATP is also a significant contributor to acidosis in tumors during acute hypoxia. The tumor microenvironment is intrinsically acidic, mainly due to accumulation of lactic acid as a result of increased aerobic and anaerobic glycolysis by tumor cells (7,8). Poor tissue perfusion and reduced buffering capacity in the extracellular tumor microenvironment aggravates the decrease in tumor  $\text{pH}_e$  (9,10). A lower  $\text{pH}_e$  in tumors has been correlated with increased gene mutation (11) and gene rearrangement rates (12,13), and altered gene expressions (14–21) that can lead to spontaneous transformation of non-metastatic tumors into metastatic tumors (22–26). A lower tumor  $\text{pH}_e$  can cause ion trapping and provide resistance to chemotherapies that act as weak bases (27–30), such as doxorubicin (31). In some cases, the lower tumor  $\text{pH}_e$  can enhance the efficacy of chemotherapies that act as weak acids (32–35). Thus, a method to estimate tissue pH that could both point to potentially resistant cases, and guide supplementary therapeutic strategies, might significantly improve clinical outcomes.

Previous work has shown that magnetic resonance imaging (MRI) can map  $\text{pH}_e$  throughout a tumor volume at high spatial resolution with good detection sensitivity in clinically reasonable

time frames (36–38). However, the change in  $T_1$  relaxation caused by a pH-responsive MRI contrast agent can also depend on the concentration of the agent. The tissue concentration of a pH-responsive agent can be estimated by using a second pH-unresponsive agent as a surrogate (36). However, this serial injection substantially prolongs the time of the study, a major disadvantage for clinical translation. Although a pH-unresponsive  $T_2^*$  relaxation contrast agent can be co-injected with a pH-responsive  $T_1$  relaxation contrast agent, and the different  $T_2^*$  and  $T_1$  effects can then be used to selectively detect each agent within the same tumor tissue, the strong correlations between  $T_1$  relaxation and  $T_2^*$  relaxation create difficulties in quantifying each agent in the same tissue, making this approach extremely challenging (39). In brief, a concentration-independent technique for estimating tissue pH would significantly improve efforts to estimate  $\text{pH}_e$  by changes in  $T_1$  relaxation rates.

To quantify the agent's concentration, the positron emission tomography (PET) isotope F-18 has been incorporated with a

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pH-responsive relaxation agent (GdDOTA-4AmP<sup>5-</sup>) to measure pH *in vitro* (40). PET was used to quantify the concentration of relaxation-based pH-responsive MRI probe (GdDOTA-4AmP<sup>5-</sup>). This approach required a multimodal MR-PET device, and reduced spatial resolution to that of the PET scanner. The <sup>19</sup>F/1H ratiometric method has also been used to measure pH (41). <sup>19</sup>F MRS also suffers from coarse resolution and lack of clinically available MRI coils.

Recently, small molecule CEST and PARACEST contrast agents have been used to estimate *in vivo* pH by taking the ratio of the CEST effect from two different chemical shifts originating from the same molecule. Although these MRI CEST methods appear promising, they suffer from the low detection sensitivity of CEST agents in an *in vivo* application (42,43).

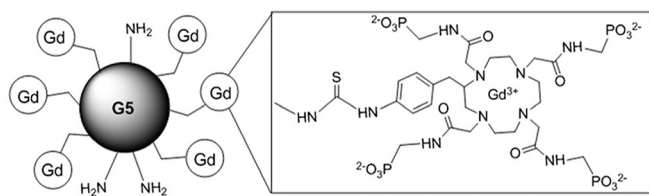
A concentration-independent method for the (GdDOTA)<sub>33</sub>-poly-L-ornithine macromolecular MRI contrast agent has been proposed (44). This method used the ratio of  $R_1$  and  $R_2$  relaxation rates, which is independent of concentration, but sensitive to pH for a macromolecular system. However, (GdDOTA)<sub>33</sub>-poly-L-ornithine is sensitive only to a pH greater than 7, and shows limited dynamic range and sensitivity in the pH range from pH 6.5 to 8, the range most relevant *in vivo* to tumor physiology.

A macromolecule is needed that is pH sensitive in the range 6.5–8, and with an  $R_2/R_1$  ratio that has a linear relationship to pH<sub>e</sub>, independent of Gd<sup>3+</sup> concentration. Previously, we succeeded in synthesizing a nanoscale, pH-responsive MRI contrast agent (Fig. 1) (45). Conjugation of the small molecule pH-responsive agent GdDOTA-4AmP<sup>5-</sup> to the surface amines of a Generation 5 (G5) PAMAM dendrimer improved  $T_1$  relaxivity with pH significantly at 0.5 T, 25 °C. Importantly, the nanoprobe (GdDOTA-4AmP<sup>5-</sup>)<sub>96</sub>-G5 showed pH response in the physiological range of 6.0–8.0.

In this report, we present (GdDOTA-4AmP<sup>5-</sup>)<sub>44</sub>-G5, a modified version of the previous G5 PAMAM dendrimer. This new compound has a reduced negative surface charge, thus higher biocompatibility, and a higher potential for drug conjugation than previous compounds, thus improving its theranostic potential. For a relevant *in vivo* application in preclinical models, it is necessary to investigate pH response at the higher field strengths, where animals are usually scanned. In this report, we study the longitudinal and the transverse relaxation properties of this (GdDOTA-4AmP<sup>5-</sup>)<sub>44</sub>-G5 conjugate at 3 T and 7 T.

## 2. RESULTS AND DISCUSSION

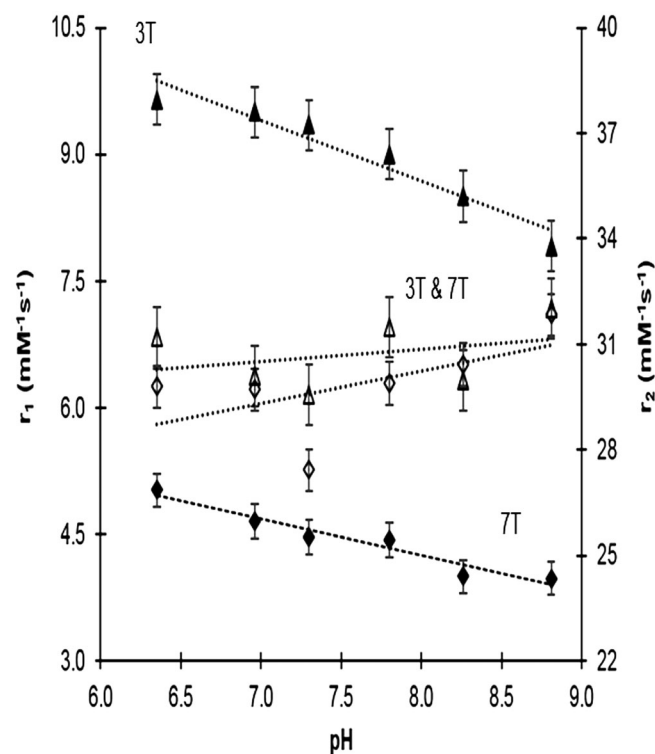
A pH-responsive GdDOTA-4AmP<sup>5-</sup> analogue was conjugated to the surface amines of a G5-PAMAM dendrimer via an isothiocyanatobenzyl group using methods previously reported (45). In that report, we conjugated 96 (GdDOTA-4AmP<sup>5-</sup>) chelates on the surface of a G5 PAMAM dendrimer, thus leading to a highly negative charged paramagnetic nanoparticle. The



**Figure 1.** Schematic view of Gd<sup>3+</sup> chelated with 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraaminophosphonate (DOTA-4AmP<sup>5-</sup>) in a G5 PAMAM dendrimer.

present study presents a modification of that synthetic method. A MALDI-TOF analysis showed an average of 44 (GdDOTA-4AmP<sup>5-</sup>) chelates in the nanostructure (Supplementary Fig. 1). The GdDOTA-4AmP<sup>5-</sup> complex has four appended phosphonate groups, with variable pK<sub>a</sub> values ranging from 6.0 to 8.0 (37,38). When the protons of the phosphonate group dissociate at their respective pK<sub>a</sub> values, the chelate gains a negative charge up to a value of −5, while it has either neutral or positive charge below these pK<sub>a</sub> values (38). That is to say, a phosphonate-based MRI contrast agent has charge reversal properties.

We observed the pH response of this dendrimeric agent. The absolute detection sensitivity of the nanoscale MRI contrast agent showed a robust improvement relative to the monomeric agent (38) at 3 T. The average  $r_1$  relaxivity of Gd<sub>44</sub>-G5 conjugate per dendrimer was a sizable 348 mM<sup>-1</sup> s<sup>-1</sup> at pH 8.84, rising to 425 mM<sup>-1</sup> s<sup>-1</sup> at pH 6.35 (Fig. 2). At 7 T, the  $r_1$  relaxivity of Gd<sub>44</sub>-G5 decreased significantly for all pH values (Fig. 2); this is the typical behavior of the  $r_1$  relaxivity of large molecules with increasing field strength (46). Nevertheless, compared with the monomer, the  $r_1$  relaxivity of Gd<sub>44</sub>-G5 still showed pH response at high field strength (38), and the detection sensitivity of the Gd<sub>44</sub>-G5 dendrimeric conjugate improved by a factor of two at 3 T for all pH values. Thus, compared with a monomeric agent, a lower injection dose of Gd<sub>44</sub>-G5 dendrimeric conjugate will be required for *in vivo* applications. GdDOTA-4AmP has been tested in biological fluid (40). In the presence of Ca(II), Cu(II), and Zn(II) ions (38) there was no change in pH-dependent relaxivity profiles. In addition, the agent has been applied in

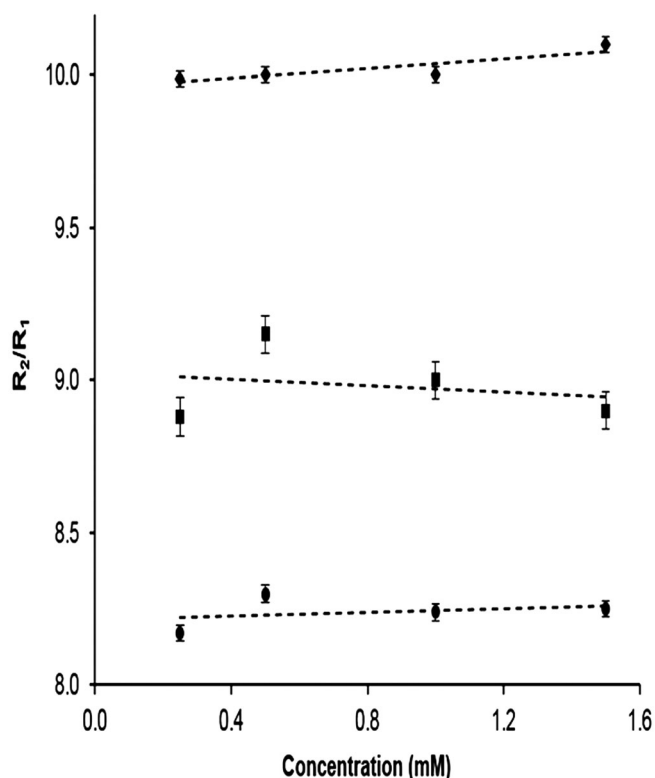


**Figure 2.** Longitudinal and transverse relaxivities ( $r_1$  and  $r_2$ , mM<sup>-1</sup> s<sup>-1</sup>) as a function of pH (6.35–8.84) in liquid phantoms measured at 3 T (triangles) and 7 T (diamonds), with linear regression fits to the data. Transverse relaxivity does not vary significantly with pH, or with field strength. The slopes of the longitudinal relaxivities do not differ between the field strengths.

tissues (47), perfused tissues or organs or *in vivo* (48), with the result that GdDOTA-4AmP did not interact in biological fluids or in the presence of ions.

The  $r_1$  relaxivity of Gd<sub>44</sub>-G5 showed pH response at both field strengths, while  $r_2$  relaxivity remained independent of pH and magnetic field strength (Fig. 2). The  $R_2$  values at 3 T were not significantly larger than those at 7 T, and the  $r_2$  profiles (slopes) at the two field strengths were essentially the same. As for  $r_1$  versus pH, the slopes of  $r_1$  versus pH at 3 T and 7 T are essentially the same.

The pH-responsive  $r_1$  relaxation properties of GdDOTA-4AmP<sup>5-</sup> were first reported by the Sherry group (38). This agent responds to pH by changes in proton exchange (variable  $\tau_M$ ). The compound has four appended phosphonate groups that have



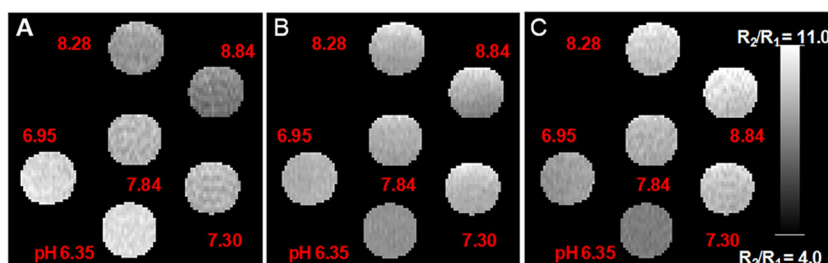
**Figure 3.** The calculated ratio of  $R_2/R_1$  for (Gd-DOTA-4AmP)<sub>44</sub>-G5 at four different concentrations and three different pH values at 7 T: top line, pH = 8.84, middle line, pH = 7.40, bottom line, pH = 6.96. The ratio  $R_2/R_1$  does not vary significantly with concentration of contrast agent at the lower pH values, but there is a small and significant variation at the higher pH, which lies outside the range of values usually encountered in tumor tissues.

$pK_a$  values in the range 6.0–8 (37,38), and as these phosphonate groups become protonated below pH  $\approx$  8 the monoprotonated phosphonate groups hydrogen bond (38) with the single Gd<sup>3+</sup>-bound water molecule and catalytically exchange the highly relaxed bound water protons with protons of bulk water (45). To understand the origin of pH-sensitive  $r_1$  relaxation properties of dendrimer-based GdDOTA-4AmP conjugate, nuclear magnetic resonance dispersion (NMRD) profiles were studied in our previous report (45). NMRD profiles revealed that multiple factors are involved in pH sensing  $r_1$  relaxation properties of this agent. The pH response was the result of a complex interplay between the rate of proton exchange between the bulk solvent and water molecules in the inner and second hydration spheres (45). In contrast to  $r_1$  relaxation properties of (GdDOTA-4AmP)<sub>44</sub>-G5 agent, the  $r_2$  relaxation properties remain essentially independent of pH, as shown in Fig. 2. We did not observe a significant difference in  $r_2$  relaxation properties at either 3 T or 7 T, which is consistent with the reported  $r_2$  relaxation properties of manganese complexes (46).

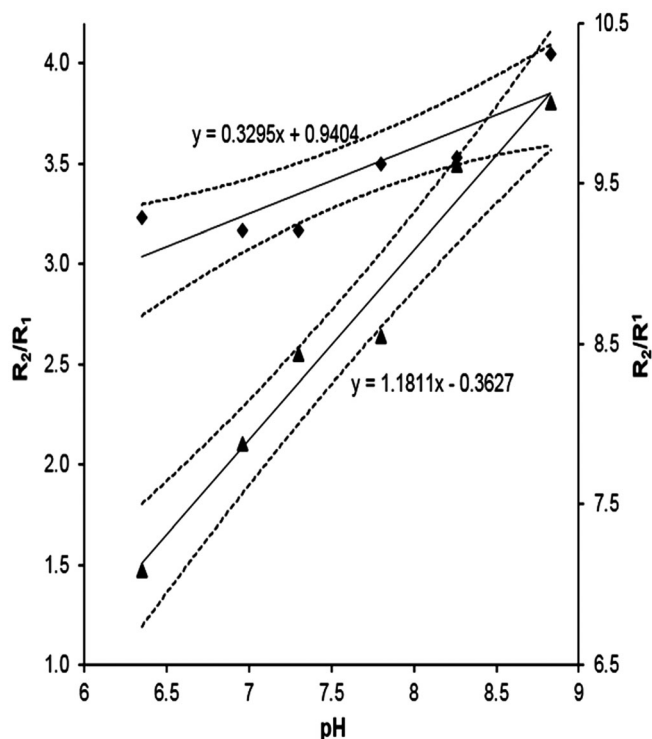
Silvio Aime and co-workers (44) reported a novel method based on a ratiometric approach that consists of measuring the ratio between the transverse and the longitudinal paramagnetic contribution to the water proton relaxation rate, i.e.  $R_{2p}/R_{1p}$  of a macromolecule at magnetic field strength higher than 0.2 T. The ratio,  $R_{2p}/R_{1p}$ , of water protons becomes independent of Gd<sup>3+</sup> concentration for a motionally restricted agent, (GdDOTA)33-poly-L-ornithine ( $\tau_R > 1$  ns), but remains dependent on  $\tau_M$ ,  $\tau_R$  and other magnetic parameters that normally affect relaxation in these complexes. We applied this  $R_2/R_1$  ratiometric approach to our dendrimer-based (Gd-DOTA-4AmP)<sub>44</sub>-G5 system.

In order to examine our hypothesis that (Gd-DOTA-4AmP)<sub>44</sub>-G5 measures pH as a single agent without knowledge of the agent's concentration, we measured the ratio of  $R_2$  and  $R_1$  relaxation rates of (Gd-DOTA-4AmP)<sub>44</sub>-G5 at four different Gd<sup>3+</sup> concentrations with three different pH values at 7 T (Fig. 3). For three concentrations, the mean values of  $R_2/R_1$  are  $8.24 \pm 0.05$ ,  $8.98 \pm 0.12$ , and  $10.02 \pm 0.05$  for pH values of 6.96, 7.40, and 8.84, respectively. The slopes of the three plots of  $R_2/R_1$  are  $0.03 \pm 0.06$ ,  $-0.05 \pm 0.15$ , and  $0.08 \pm 0.03$ , respectively. The slope of  $R_2/R_1$  is significantly nonzero at the highest pH (pH = 8.84). However, this pH is outside the range of physiological value in tumors, and the variation across even the range of concentrations reported is only about 6%. Thus, we conclude that the  $R_2/R_1$  ratio is essentially independent of concentration for each pH value.

In order to further examine the ratiometric approach,  $R_1$ ,  $R_2$ , and  $R_2/R_1$  (Fig. 4(A)–(C), respectively) maps for six different pH values at 7 T for a given Gd<sup>3+</sup> concentration were generated. The ratio of  $R_2/R_1$  maps of the phantoms containing solutions



**Figure 4.**  $R_1$  (A) and  $R_2$  (B) maps generated from  $T_1$  and  $T_2$ -weighted images of phantoms containing 0.16 mM Gd<sub>44</sub>-G5 at six different pH values. Average  $R_1$  and  $R_2$  values were calculated from hand-drawn ROIs.  $R_2/R_1$  ratio maps at six different pH values were also generated (C).



**Figure 5.**  $R_2/R_1$  versus pH plots. Left-hand ordinate, 3 T values; right-hand ordinate, 7 T values. The pH dependence of the relaxation rate ratio is shown with regression lines. Upper line (diamonds), 3 T data; bottom line (triangles), 7 T data. The 95% confidence intervals are plotted for both linear regressions.

of different pH with added (Gd-DOTA-4AmP)<sub>44</sub>-G5 were plotted versus pH for both 7 T and 3 T data, along with the 95% confidence intervals for their regressions. Figure 5 demonstrates that the ratio  $R_2/R_1$  was pH responsive at both field strengths. The two slopes are  $1.18 \pm 0.11$  and  $0.330 \pm 0.091$  for 7 T and 3 T curves, respectively, and the y-intercepts are  $-0.36 \pm 0.81$  and  $-0.94 \pm 0.69$ , again for 7 T and 3 T. Neither of the intercepts is significantly different from zero. The ratio  $R_2/R_1$  increased from 3.23 to 4.05 and from 7.04 to 10 pH units, at 3 T and 7 T respectively, on changing the solution pH from 6.35 to 8.84. It is apparent that the accuracy of the 3 T methods is lower than that of the 7 T methods. We do not believe this to be intrinsic to the field strengths, but it is clear that a careful calibration of both  $T_1$  and  $T_2$  methods, particularly with regard to flip angles, is required for accuracy in reading out the ratio of  $R_2/R_1$  as an estimate of pH.

### 3. CONCLUSION

In this study, a novel pH-responsive dendrimer-based MRI contrast agent (Gd-DOTA-4AmP)<sub>44</sub>-G5 was evaluated as a potential pH imaging agent at 3 T and 7 T magnetic field strengths. In general, the compound exhibited good dynamic range in a concentration-independent parameter ( $R_2/R_1$ ) across physiologically significant pH values. The improvement of  $R_2/R_1$  values for this nanostructure, 1.19 per pH unit, is almost double that of the previously reported  $R_2/R_1$  values for a ratiometric pH-responsive probe (44). These findings support the expectation that the agent can be used to estimate tumor pH *in vivo* by

means of estimates of changes in the  $R_2/R_1$  ratio after administration of (Gd-DOTA-4AmP)<sub>44</sub>-G5. The ratiometric approach to measure pH does not require knowledge of the concentration of the agent. (Gd-DOTA-4AmP)<sub>44</sub>-G5 with  $T_1$  and  $T_2$  effects constitutes a single MRI contrast agent that can accurately measure pH<sub>e</sub> in a concentration-independent manner at clinically relevant magnetic field strengths.

## 4. EXPERIMENTAL

### 4.1. Synthesis

We have synthesized a modified version of the pH-responsive MRI nanoprobe GdDOTA-4AmP-G5 by following our published synthetic method (45). The final conjugate was purified by diafiltration using a Centricon C-30 cell with a 30 kDa molecular weight cut-off, after which the solvents were removed by lyophilization to afford a colorless solid (0.19 g). The MW of the p-SCN-DOTA-4AmPE conjugated G5 dendrimer was estimated at 79 082 g/mole by MALDI-TOF analysis (Supplementary Fig. 1). This corresponds to a G5-dendrimer with an average of 44 chelated Gd<sup>3+</sup> ions per dendrimer. The number of chelates per dendrimer unit was also obtained using the same method as in our previous report (45). To examine our hypothesis, we prepared phantoms of [(GdDOTA-4AmP)<sub>44</sub>-G5] at four or five different concentrations with six different pH values. Gd<sup>3+</sup> concentration was determined by ICP analyses.

### 4.2. MRI methods

MRI experiments were performed on a 3.0 T clinical system (Signa Excite, GE Health) using 50 mm diameter × 108 mm RF rung length (Litzcage small animal imaging system, Doty Scientific, Columbia, SC). Multi-echo  $T_2$ -weighted ( $T_2W$ ) images and  $T_1$ -weighted ( $T_1W$ ) images using three dimensional spoiled gradient echo (3D SPGR) were acquired.  $T_2W$  images were obtained using standard two-dimensional Fourier transformation (2DFT) multi-slice (15 slices) multi-echo MRI sequences with an echo time ( $TE$ ) of 11, 22, 33, and 44 ms and a repetition time ( $TR$ ) of 2000 ms, field of view (FOV) of 30 mm, 1 mm slice thickness, and matrix size of 128 × 128. The  $T_1W$  images were acquired using 3D SPGR with the following parameters:  $TR = 9.46$ ms,  $TE = 1.732$ ms, 128 × 128 matrix size, FOV = 40 × 40 × 40 mm<sup>3</sup>, effective slice thickness = 1 mm, and flip angles = 2°, 4°, 8°, 12°, 15°, 20°, 25°, 30°, 35°. The  $R_2$  ( $1/T_2$ ) maps were calculated from the acquired  $T_2W$  images at eight different  $TE$  values using a log-linear least square fit on a pixel-by-pixel basis by following the reported method (49). The  $T_1$  maps were calculated from the acquired  $T_1W$  images at nine different flip angles using a linear least square fit (on a pixel-by-pixel basis) (50). The calculated value of  $T_1$  was inverted to generate the  $R_1$  map.

For measurements of  $T_1$  and  $T_2$  relaxation times on a 7 T Varian system, axial spin echo (SE) sequences were obtained with multiple  $TR$  (50, 100, 200, 300, 500, 750, 1000, 1500, 2000, 3000, and 5500 ms) and  $TE$  (8.4, 16.8, 25.2, and 33.6 ms) values. All sequences were acquired with FOV = 24 mm<sup>2</sup>, matrix size = 128 × 64, one slice, thickness = 1 mm, and  $N_{EX} = 1$ . MR images were transferred to an off-line server and reconstructed using home-written software.  $T_1$  and  $T_2$  maps of the samples were calculated by fitting the appropriate relaxation equation to the image data, assuming monoexponential signal decay (for both  $T_1$  and  $T_2$ ) on a pixel-by-pixel basis. For  $T_1$  maps, all echoes were

summed for each  $TR$  and then fitted using the equation  $M_2(t) = M_0[1 - \exp(-TR/T_1)]$ .  $T_2$  maps were generated by summing data across all  $TR$  values, and then fitting the summed values using the equation  $M_{xy}(t) = M_0[\exp(-TE/T_2)]$ . The longitudinal and transverse relaxation times of the  $Gd_{44}$ -G5 in solution were then measured in ROIs selected in the calculated sample maps.

## Acknowledgements

The authors acknowledge research support from the National Institutes of Health (NIH) grants K25CA129173 (to MMA) and 1R01CA160216 (to ASA). We thank Professor Mark D. Pagel for helpful discussion.

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