PERKIN 1

Use of sodium triacetoxyborohydride in the synthesis of nitroxide biradicals

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A family of nitroxide biradicals was synthesized for the purpose of creating terminal groups with high relaxivity for linkage to dendrimers for use as targeted MR contrast agents for articular cartilage. The structure of the nitroxide biradicals strongly influenced their MR relaxivities. The addition of a carbon atom between the nitroxyl rings and the bridging nitrogen increased the relaxivity of the nitroxide biradical by ~14% over what was expected by doubling the relaxivity of the monomeric nitroxide. Conversely in nitroxide biradicals without the additional carbon atoms, steric hindrance restricted the relaxivity to ~35% below what was expected by doubling the relaxivity of the monomeric nitroxide. Nitroxide biradicals that have a carbon atom incorporated between the nitroxyl rings and the bridging nitrogen exhibit MR relaxivity values ~223% greater than that of the corresponding monomeric nitroxide. By linking these nitroxide biradicals to a dendrimer, it should be possible to synthesize targeted magnetic resonance contrast agents with higher relaxivities than dendrimer-linked nitroxides with single nitroxyl rings as terminal groups. These dendrimer-linked dinitroxides should be capable of differentiating normal and abnormal articular cartilage on the basis of the glycosaminoglycan concentration.

Introduction

Paramagnetic inorganic ion chelates, such as gadolinium diethylenetriaminepentaacetic acid (Gd–DTPA), play an important role as magnetic resonance (MR) contrast agents evaluating the physiological and pathological changes caused by disease. Although stable organic free radicals, such as nitroxides (aminoxyls), should be of clinical interest for specific targets,[1] their ease of one-electron reduction catalyzed by either enzymes or biological reductants, such as ascorbate, has greatly limited their *in vivo* application.[1b,2] Nevertheless, nitroxides with excellent *ex vivo* stability at physiological pH and temperature have been documented and used as contrast agents for MR imaging.[3] For instance, it has been shown that charged nitroxides can detect abnormalities in the blood brain barrier[3a] and also monitor renal function.[3b] Unfortunately enthusiasm for these new applications has been tempered because of the exceedingly high nitroxide dose required for adequate contrast change in MR images.

One potential use of MR contrast agents is in the evaluation of osteoarthritis, which is a group of related joint diseases characterized by the degeneration and loss of articular cartilage.[4] Early on in the disease, there is depletion of glycosaminoglycan (GAG) from the articular cartilage, which progresses with severity of the disease.[5] Such biochemical changes within the articular cartilage matrix are manifested by a loss of the normal biomechanical properties of the articular cartilage, which leads to visible tissue damage and loss.[5] Since matrix alterations occur in the early stages of the disease, the development of medications capable of reversing pre-existing abnormalities in articular cartilage or prevent their progression may be possible if therapy is started early in the course of the disease. Therefore, a minimally invasive technique, such as MR imaging,[6] that can accurately detect the presence of early cartilage abnormalities, such as the loss of GAG content, and progression of those abnormalities in individuals with osteoarthritis would be desirable.

The use of MR imaging with intraarticular or intravenous injection of Gd–DTPA has been proposed for the evaluation of articular cartilage. This approach allows the GAG concentration in articular cartilage to be measured[6d,7] from changes in the spin–lattice relaxation time (T1 value) after administration of Gd–DTPA.[7a] Since the GAG molecules in the matrix of normal articular cartilage contain a large number of negatively charged side groups, the negatively charged gadolinium chelate, Gd–DTPA^{2-}, is relatively excluded from areas of normal cartilage compared to those depleted of GAG.[7a] At equilibrium the abnormal cartilage will have a greater Gd–DTPA^{2-} concentration than normal cartilage and will thus have lower T1 values.[6d,7] However with this technique, the overall concentration of contrast agent within the cartilage is low, and measurement and calculation of T1 images for determination of Gd–DTPA^{2-} uptake are required.[7a]

In theory, a positively charged MR contrast agent would have a greater affinity for GAG than would Gd–DTPA^{2-} and would therefore produce greater T1 shortening within cartilage for a given concentration. This may be advantageous for imaging normal cartilage, especially when the cartilage is very thin. However, of greater importance is the ability of the contrast agent to differentiate normal cartilage from abnormal cartilage. For a charged contrast agent, the equilibrium distribution between highly charged GAG-rich cartilage and uncharged GAG-depleted cartilage should follow the Donnan equilibrium theory.[8] This theory states that the magnitude of the charge of

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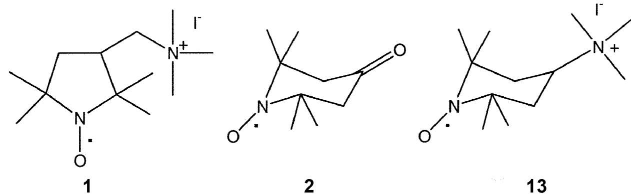
the contrast agent will determine the relative concentration distribution, *i.e.* the number of charges on the molecule, independent of the sign of the charge. In fact, the concentration difference of the contrast agent between charged and uncharged regions has been shown to be proportional to the power of the number of charges on the contrast agent.[8] Therefore, the more highly charged the contrast agent, the more sensitive it should be to small differences in GAG concentration within the cartilage.

The positively charged nitroxide, 3-trimethylammoniomethyl-2,2,5,5-tetramethylpyrrolidin-1-yloxyl iodide **1**, has been shown to have an affinity for the negatively charged GAG molecules and diffuses into articular cartilage.[3d,e] Unfortunately, the poor relaxivity of this nitroxide, ~0.18 s^{-1} mM^{-1},[9] meant high concentrations were required to achieve a detectable change in MR contrast.[3e] These relatively high concentrations of nitroxide increase the potential for toxicity.



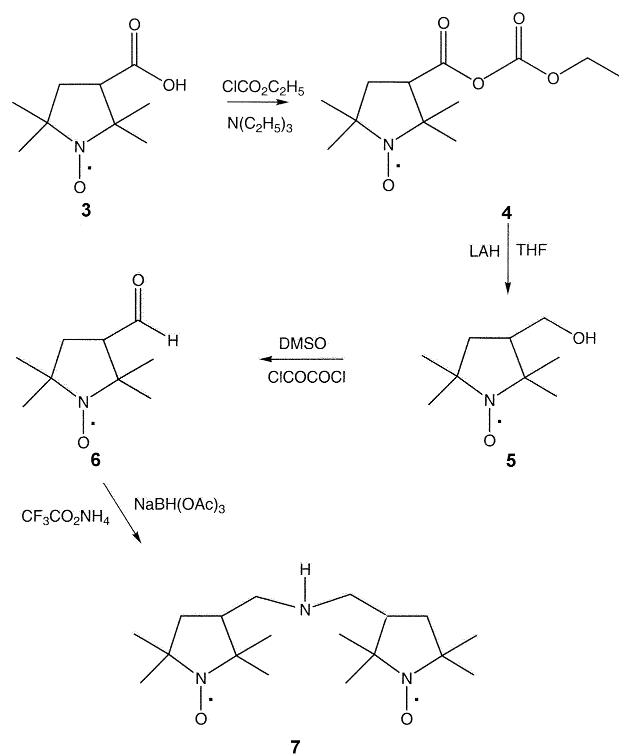
To address these relaxivity limitations, dendrimer-linked nitroxides were prepared based on the work of Bosman *et al.*[10] As the number of nitroxyl rings attached to a dendrimer was increased, the relaxivity of these compounds increased linearly and for 3rd and 4th generation dendrimers, the dendrimer-linked nitroxides were able to exceed the relaxivity of Gd–DTPA (~4.8 s^{-1} mM^{-1}).[9] Since each nitroxide moiety was electrically neutral, the dendrimer-linked nitroxides maintained the chemical characteristics of the dendrimer core and allowed, in a pilot MR study, the highly positively charged DAB †-dendrimer-linked nitroxides to selectively enhance healthy rabbit articular cartilage *in vivo*.[9] However, the dendrimer-linked nitroxides that exhibited high relaxivity were quite large, possibly limiting their diffusion into cartilage.

To maintain the relaxivity of the higher generation dendrimer-linked nitroxides and yet improve their diffusivity into tissue (*i.e.* decrease their molecular weight), it is necessary to increase the number of nitroxyl groups attached to the dendrimer termini. Our approach was to synthesize a family of nitroxide biradicals, or dinitroxides, to which a linking group to the dendrimers could be incorporated. In this manuscript, sodium triacetoxyborohydride is used in the synthesis of several dinitroxides. The structure of the resulting nitroxide biradicals strongly influenced their relaxivities.

Results and discussion

Sodium cyanoborohydride has been used previously to reductively aminate 4-oxo-2,2,6,6-tetramethylpiperidin-1-yloxyl **2** without reducing the nitroxyl group.[11] More recently, we expanded upon those earlier studies to include the reduction of aldehydes.[3d] However, even with aldehydes, sodium triacetoxyborohydride was a considerably easier reductant to work with than sodium cyanoborohydride.[12] While the reduction amination proceeded well at ambient temperature in a number of different solvents such as acetonitrile and 1,2-dichloroethane, tetrahydrofuran (THF) proved to be the most versatile solvent; reactants were readily soluble and the products were easily isolated from the reaction mixtures in high yields. Activated molecular sieves (3A), to absorb water, increased the yield of the desired products.

† DAB is a polypropyleneimine dendrimer.



Scheme 1

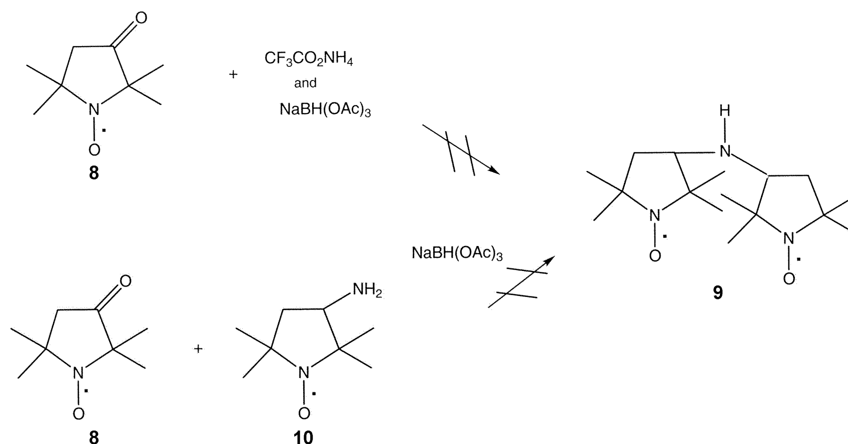
The first dinitroxide prepared was *N*,*N*-bis(1-ylooxy-2,2,5,5-tetramethylpyrrolidin-3-ylmethylene)amine **7** (Scheme 1). The initial step in the synthesis of **7** was the formation of 3-formyl-2,2,5,5-tetramethylpyrrolidin-1-yloxyl **6**.[3d,13] Reductive amination of **6** with sodium triacetoxyborohydride and ammonium trifluoroacetate afforded the dinitroxide **7**.

All attempts at synthesizing *N*,*N*-bis(1-ylooxy-2,2,5,5-tetramethylpyrrolidin-3-yl)amine **9** by reductively aminating 3-oxo-2,2,5,5-tetramethylpyrrolidin-1-yloxyl **8** with either ammonium trifluoroacetate or 3-amino-2,2,5,5-tetramethylpyrrolidin-1-yloxyl **10** were unsuccessful (Scheme 2). We speculate that steric hindrance at the 3-position as well as the diminished reactivity of a ketone compared to an aldehyde resulted in our inability to obtain the desired dinitroxide **9**.

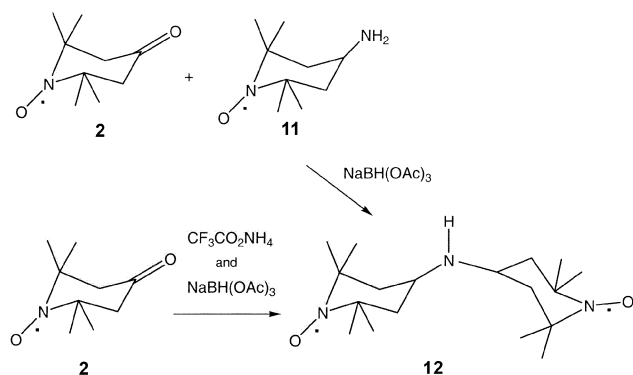
N,*N*-Bis(1-ylooxy-2,2,6,6-tetramethylpiperidin-3-yl)amine **12** was prepared in reasonable yields by reacting 4-oxo-2,2,6,6-tetramethylpiperidin-1-yloxyl **2** with 4-amino-2,2,6,6-tetramethylpiperidin-1-yloxyl **11** in the presence of sodium triacetoxyborohydride (Scheme 3). While direct reductive amination of ketone **2** with ammonium trifluoroacetate furnished the dinitroxide **12**, yields of the desired nitroxide biradical were unacceptably poor. This result suggests that the nucleophilicity of a primary amine, although not essential, clearly enhances the reductive amination of cyclic ketones.

The EPR spectrum of nitroxide **7** was typical of a symmetrical nitroxide biradical that contains five lines with intensities of 1 : 2 : 3 : 2 : 1.[14] The EPR spectrum shown in Fig. 1 exhibits a strong spin exchange with intramolecular motion, modulating the exchange integral. From the EPR spectrum, it is suggested that the spin exchange is considerably greater than the nitrogen-coupling constant (Fig. 1). Surprisingly, in the case of nitroxide **12**, where the motion of the two nitroxides is further restricted due to the proximity of each of the two piperidinyloxyls, the EPR spectrum was essentially the same as that of the nitroxide biradical **7**.

The T1 relaxivities, r_1, of the nitroxides **1**, **7**, **12** and **13**, and Gd–DTPA were measured at 1.5 T and at room temperature (22 °C). The r_1 values of nitroxide biradicals **7** and **12** were then compared to those of the mononitroxides **1** and **13**, and



Scheme 2



Scheme 3

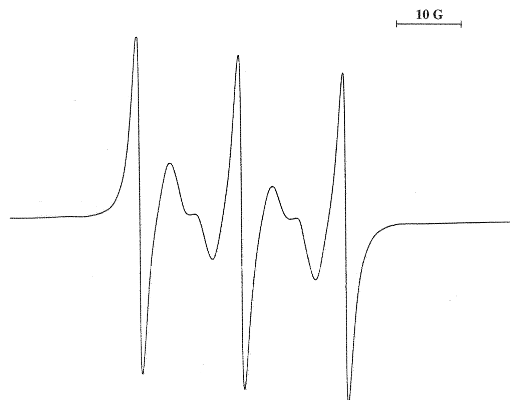


Fig. 1 A typical EPR spectrum of nitroxide biradical **7** in sodium phosphate buffer, pH 7.4. Nitroxide biradical **12** exhibited the same EPR spectrum.

Gd–DTPA (Table 1). Two rather surprising observations were noted from these experiments. First, the MR relaxivity of nitroxide biradical **7** was 0.41 s^{-1} mM^{-1}, ~14% above what was expected by doubling the relaxivity of monomeric nitroxide **1** at 0.18 s^{-1} mM^{-1} (Table 1). Second, the MR relaxivity of nitroxide biradical **12** was 0.22 s^{-1} mM^{-1}, ~35% below what was expected by doubling the relaxivity of nitroxide **13** at 0.17 s^{-1} mM^{-1}. The relaxivity values of the monomeric nitroxides **1** and **13** were in good agreement and equal to previously measured values.[9]

The greater than expected increase in MR relaxivity for dinitroxide **7** is likely caused by an increase in the hydration sphere of the nitroxide biradical. The hypothesized increase in volume of water exposed to the free radical results from the high mobility of the 5-membered rings due to the introduction of a carbon atom between the nitrogen atom and the two nitroxyl rings. Conversely, the dramatic decrease in the relaxivity of dinitroxide **12** compared to the expected relaxivity probably results from a fairly rigid nitroxyl ring–nitrogen–nitroxyl ring structure. Steric hindrance most likely limits the flexion angle of this bond, which in turn results in the hydration sphere being defined by molecular tumbling and conformational changes within the 6-membered rings. It was not possible to confirm this theory because synthesis of a compound with two 5-membered ring nitroxides directly bound to the nitrogen was not feasible. Finally, the very large difference in MR relaxivities between the nitroxide biradicals **7** and **12** is likely to be due to the increase in the degrees of freedom for the molecular motion of dinitroxide **7** allowed by the carbon between the ring structures and the bridging nitrogen atom.

Table 1 T1 relaxivity values, r_1, for nitroxide monomers, nitroxyl biradicals, and Gd–DTPA measured at room temperature (22 °C) and 1.5 T

Compound	r_1/s^{-1} mM^{-1}
Nitroxide **1**	0.18 ± 0.004
Nitroxide **13**	0.17 ± 0.003
Dinitroxide **7**	0.41 ± 0.003
Dinitroxide **12**	0.22 ± 0.002
Gd–DTPA	4.63 ± 0.13

Experimental

Reagents

4-Oxo-2,2,6,6-tetramethylpiperidin-1-yloxyl **2** was prepared as described by Rozantsev.[15] 3-Trimethylammonio-2,2,5,5-tetramethylpyrrolidin-1-yloxyl iodide **1** was synthesized as presented in the literature.[3d] 4-Trimethylammonio-2,2,6,6-tetramethylpiperidin-1-yloxyl iodide **13** was prepared as described by Rosen and Abou-Donia.[11b] 3-Oxo-2,2,5,5-tetramethylpyrrolidinyloxyl **8** was synthesized as described in the literature.[13a] All reagents were obtained from commercial vendors. Elemental microanalyses were determined by Atlantic Microlabs, Inc (Norcross, GA). EPR spectra were recorded on a Varian Associates, model E-109 (Palo Alto, CA). IR spectra were recorded on a FT-IR spectrometer (Perkin-Elmer, Norwalk, CT) in $CHCl_3$. Melting points were obtained on a Thomas Hoover capillary melting point apparatus and are corrected.

3-Formyl-2,2,5,5-tetramethylpyrrolidin-1-yloxyl 6

The Swern[16] oxidation was used to convert alcohol **5** to aldehyde **6**. Dimethyl sulfoxide (12.82 mmol, 1 g, 0.91 mL, Aldrich Chemical Co.) in CH_2Cl_2 (5 mL) was added to a solution of oxalyl chloride (6.38 mmol, 0.81 g, 0.56 mL, Aldrich) in CH_2Cl_2 cooled to −60 °C. The addition of DMSO was at a rate that maintained the temperature of the reaction at less than −50 °C.

Once completed, the reaction was stirred at this temperature for 5 min, at which point a CH_2Cl_2 solution of alcohol **5** (5.82 mmol, 1 g)[13a] was added, keeping the temperature of the reaction at −50 °C. This reaction was maintained at this temperature for another 20 min whereupon triethylamine (29 mmol, 2.93 g, 4.0 mL, freshly distilled) in CH_2Cl_2 (10 mL) was added at −50 °C. After another 10 min at this temperature, the reaction was allowed to warm to room temperature. Water (10 mL) was added, the layers separated and the organic solution was dried over anhydrous Na_2SO_4, and filtered and evaporated, *in vacuo*, to dryness. The red oil was chromatographed using silica gel (200–400 mesh, Aldrich) and hexane : diethyl ether, 7 : 3, which removed a small amount of an orange material that was not identified. Changing the solvent to hexane : diethyl ether, 1 : 2, aldehyde **6** was isolated as a red oil (760 mg, 78%), which was stored at −20 °C until use: IR ($CHCl_3$) ν_{max} 1720 cm^{-1}.[13b]

N,*N*-Bis(1-ylooxy-2,2,5,5-tetramethylpyrrolidin-3-ylmethylene)-amine 7

To a THF (50 mL) solution containing aldehyde **6** (3.5 mmol, 600 mg), ammonium trifluoroacetate (1.18 mmol, 154 mg, Aldrich), sodium triacetoxyborohydride (4.9 mmol, 1.03 g, Aldrich) and activated molecular sieves, 3A, were added, sequentially. The reaction was run at room temperature under N_2 for 36 h and then quenched with saturated $NaHCO_3$ (5 mL). Methylene chloride (25 mL) was added and the layers separated, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The remaining oil was cooled in an ice bath, acidified with dilute HCl and extracted with $CHCl_3$. The water phase was basified with NH_4OH, and extracted with $CHCl_3$. After drying with anhydrous Na_2SO_4, the $CHCl_3$ solution was evaporated to dryness. The remaining orange oil was chromatographed using silica gel (200–400 mesh, Aldrich) and chloroform : acetone, 92 : 8. The isolated dinitroxide **7** solidified upon standing at room temperature and was recrystallized from hexane, mp 116–118 °C (250 mg, 65%). The EPR spectrum is characteristic of a nitroxide biradical (Fig. 1). Anal. ($C_{18}H_{35}N_3O_2$): Theory: C = 66.42; H = 10.84; N = 12.91. Found: C = 66.41; H = 10.84; N = 12.73%.

N,*N*-Bis(1-ylooxy-2,2,6,6-tetramethylpiperidin-3-yl)amine 12

To a THF (50 mL) solution containing ketone **2** (prepared as described in the literature,[15] 3.21 mmol, 550 mg) and amine **11** (2.92 mmol, 500 mg, Aldrich) was added sodium triacetoxyborohydride (4.38 mmol, 930 mg, Aldrich) and activated molecular sieves, 3A. The reaction was run at room temperature under N_2 for 30 h and then quenched with saturated $NaHCO_3$ (5 mL). Methylene chloride (25 mL) was added and the layers separated, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The remaining oil was cooled in an ice bath, acidified with dilute HCl and extracted with $CHCl_3$. The water phase was basified with NH_4OH, and extracted with $CHCl_3$. After drying with anhydrous Na_2SO_4, the $CHCl_3$ solution was evaporated to dryness. The orange solid was washed several times with cold hexane to remove amine **11**. The solid that remained in the flask was recrystallized from hot hexane yielding dinitroxide **12**, mp 156–158 °C (710 mg, 75%). The EPR spectrum is characteristic of a nitroxide biradical. Anal. ($C_{18}H_{35}N_3O_2$): Theory: C = 66.42; H = 10.84; N = 12.91. Found: C = 66.65; H = 11.19; N = 12.63%.

Relaxivity measurements

The spin–lattice relaxivity, r_1, of nitroxides **1**, **7**, **12** and **13** and Gd–DTPA (Magnevist; Berlex Laboratories, Wayne, NJ) were calculated from T1 measurements made from imaging experiments at 1.5 T and at room temperature (22 °C). Various concentrations of each compound in Dulbecco's phosphate-buffered saline (PBS) (Gibco BRL, Grand Island, NY) were made: 0.25, 0.5, 1.0, 2.0, and 4.0 mM (nitroxides **1**, **7**, **12** and **13**) and 0.125, 0.25, 0.5, 1.0, and 2.0 mM (Gd–DTPA). Lower concentrations were required for the higher relaxivity Gd–DTPA. Three sets of 1.5 mL aliquots of each solution and PBS without a contrast agent were imaged simultaneously in plastic centrifuge tubes using a standard quadrature head coil in a 1.5 T MR system (SIGNA; General Electric Medical Systems, Milwaukee, WI). Gradient echo localizer images were used to identify a single slice that included all the solution tubes. Solution T1 values were measured using an inversion recovery fast spin echo sequence with repetition time (TR) of 6000 ms, and inversion times (TI) of 50, 100, 200, 400, 700, 1400 and 2800 ms, an echo time (TE) of 12.2 ms, and an echo train length of 8. All images were obtained from a coronal slice with a 20 cm field of view, 3 mm slice thickness, 256 × 256 matrix and 1 excitation. The images were then transferred to a Sun Blade 100 workstation (Sun Microsystems, Mountain View, CA) for analysis.

Calculation of T1 relaxivity (r_1)

The T1 for each solution and PBS were calculated using MATLAB (The Mathworks, Inc., Natick, MA) for each pixel from image data using a three parameter fit to the equation $M_{TI} = M_o[1 - Ae^{(-TI/T1)} + e^{(-TR/T1)}]$, where M_{TI} is the pixel signal intensity from the image obtained with an inversion time of TI, M_o is the signal intensity that would be obtained from the solution in the fully relaxed state, and $A = 1 - \cos\phi$, where ϕ is the flip angle of the inversion pulse. The T1 values for a 4 × 4 set of pixels from the center of each tube were then averaged. The r_1 values of each solution and PBS were calculated, using a least squares fit from the slope of $[1/T1_{solution} - 1/T1_{PBS}]$ *versus* concentration of each nitroxide **1**, **7**, **12** and **13**, or Gd–DTPA. $T1_{solution}$ is the T1 of the nitroxide solution or Gd–DTPA and $T1_{PBS}$ is the T1 of the PBS without contrast agent. The mean r_1 of the three sets of identical solutions for each nitroxide **1**, **7**, **12** and **13**, and Gd–DTPA was calculated (Table 1).

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