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Toward the Design of MR Agents for Imaging β-Cell Function

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Abstract

The chemistry of Gd³⁺-based MRI agents has advanced considerably during the past decade toward agents with higher relaxivity and agents that respond to physiology and/or metabolism. This review describes various approaches that have been taken toward the development of responsive contrast agents and discusses the importance of fast water exchange for advancement of targeted Gd³⁺-based agents with higher sensitivity. The recent discovery of Eu³⁺ complexes having extraordinarily slow water exchange has opened a new avenue in contrast agent design based upon the chemical exchange saturation transfer (CEST) mechanism. These new paramagnetic complexes called PARACEST agents offer new possibilities of imaging biological functions such as tissue pH and metabolite levels. The lower detection limits that may apply to each class of contrast agent (Gd³⁺-based versus PARACEST) are discussed and the extent to which they may be applied to the imaging of β-cells is considered.

Keywords

Magnetic resonance imaging; Targeted contrast agents; Responsive contrast agents; β-cells.

1. ENHANCING CONTRAST IN MR IMAGES

The wide range of magnetic, photophysical and nuclear properties offered by the lanthanide or “rare earth” elements render them ideal as imaging agents in many branches of diagnostic medicine [1,2]. Foremost among these for MRI applications is the Gd³⁺ ion. Lying at the mid-point of the lanthanide series, Gd³⁺ has the most unpaired electrons (seven) of all lanthanide ions and this, coupled with a long electronic relaxation time, renders Gd³⁺ an ideal candidate for use in contrast media for magnetic resonance imaging (MRI). Since the introduction of MR contrast media in 1983, the majority of clinically approved contrast agents have been complexes of Gd³⁺ (Chart (1)) [3,4]. Owing to the toxicity of all lanthanide ions and their poor solubility at physiological pH they must be administered in the form of thermo-dynamically stable and kinetically robust chelates. In all commercially approved agents, Gd³⁺ is chelated by a single octadentate ligand and has one water molecule completing its coordination sphere.

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Paramagnetic contrast agents increase the rate of longitudinal water proton relaxation and so doing increase MR image signal intensity in typical imaging sequences. The increase in the water proton relaxation rate per unit concentration of contrast agent is known as relaxivity so the higher the relaxivity of an agent, the more effective it will be at shortening $T_1$. The overall relaxivity of an agent can have contributions from inner-sphere, second-sphere and outer-sphere components. The outer-sphere component arises from the diffusion of the agent through the solvent. Due to the strong distance dependence of the relaxation effect ($r^{-6}$), the outer sphere effect is small and usually accounts for about 2 mM$^{-1}$s$^{-1}$ of the relaxivity of a small paramagnetic complex. The second-sphere effect arises from water molecules hydrogen bonded to the periphery of the complex. These molecules are much closer to the paramagnetic center and so have greater potential to be relaxed. Second-sphere water molecules also exchange rapidly with bulk solvent so they are often not efficiently relaxed before leaving the vicinity of the complex. Most complexes do not exhibit a measurable second-sphere contribution since they lack the suitable hydrogen bonding sites required for such an effect, but there are a few examples where a sizable second-sphere contribution has been observed [5–8]. Inner-sphere relaxation occurs when a water molecule is directly coordinated to the paramagnetic ion. This is nearly always the largest contribution for typical Gd$^{3+}$ complexes because of the close proximity of the protons on this water molecule to the paramagnetic center. Exchange of this coordinated water molecule with the bulk water then transfers the relaxation effect to bulk solvent. In all currently approved Gd$^{3+}$-based contrast agents, exchange takes place by a dissociative mechanism (D) wherein the coordinated water molecule leaves the inner coordination sphere to form an octadentate complex before a another water molecule reoccupies the vacant coordination site. Inner-sphere relaxation is well described by Solomon, Bloembergen and Morgan theory for paramagnetic systems, [9–13], summarized by eqn. 1–eqn.3.

$$r_{1,I.S.} = \frac{C q}{55.6} \left( \frac{1}{T_{1M} + \tau_M} \right)$$  \hspace{1cm} \text{(Eqn. 1)}

$$T_{1M}^{-1} = \frac{2/15S(S+1)g^2 \beta^2 \gamma_i^2}{r^6} \left\{ \frac{7 \tau_c}{(1+\omega_2^2 \tau_c^2)} + \frac{3 \tau_c}{(1+\omega_1^2 \tau_c^2)} + \frac{2A^2}{3h^3} S(S+1) \right\} \left\{ \frac{1}{(1+\omega_2^2 \tau_c^2)} \right\}$$ \hspace{1cm} \text{(Eqn. 2)}

$$\frac{1}{\tau_c} = \frac{1}{\tau_S} + \frac{1}{\tau_R} + \frac{1}{\tau_M}$$ \hspace{1cm} \text{(Eqn. 3)}

where, $r_{1,I.S.}$ is the longitudinal inner-sphere relaxation rate, $C$ is the concentration of the agent, $q$ is the number of water molecules coordinated to the paramagnetic center (usually one), $T_{1M}$ is the longitudinal proton relaxation time and $r$ is the distance of the proton from the paramagnetic center. Typically for these complexes, $T_{1M} > \tau_M$ (eqn 1) so the relaxation effectiveness of a complex is determined by a characteristic correlation time, $\tau_C$. $\tau_C$ can have contributions from three other physical features of a complex, the bound water residence lifetime ($\tau_M$), the rotational correlation time ($\tau_R$) and the electronic relaxation time ($\tau_S$). The electronic relaxation time is an inherent feature of the paramagnetic ion in its coordination environment and is not readily altered in a predictable way by chemistry. At very low magnetic fields ($< 1$ MHz), $\tau_S$ is the prime determinant of $\tau_C$ but as the field is increased to those more typical for clinical imaging, the significance of $\tau_S$ diminishes and the rotational correlation time, $\tau_R$, typically dominates relaxation. The practical result is that if a paramagnetic complex can be made to rotate more slowly, then the relaxivity of the complex
will be more favorable at typical imaging fields. An example is given by the agent MS-325, currently under development at EPIX Medical [14]. Although the agent itself is a low molecular weight Gd$^{3+}$ chelate, once administered to a patient the diphenylcyclohexyl group binds to hydrophobic binding sites on human serum albumin (HSA) and this slows molecular rotation, increases $\tau_R$, and substantially increases the effective relaxivity of the agent. The hydrophobic interaction imparts good binding affinity for HSA ($K_{al} = 1.1 \times 10^4$ M$^{-1}$) [15] such that greater than 70% of the agent is bound in serum. This in turn increases the relaxivity of the HSA-bound complex to 48.9 mM$^{-1}$s$^{-1}$ (20 MHz, 37°C), [15] a 10-fold increase over conventional low molecular weight chelates [16]. The binding to HSA not only increases the relaxivity or effectiveness of the agent, but it also helps to retain the agent in the vascular space longer. This may prove advantageous in imaging of larger blood vessels (angiography) and in dynamic contrast enhancement studies of smaller vascular beds.

However, the potential improvements in relaxivity that can be achieved by optimizing $\tau_R$ may be limited if the effect of water exchange ($\tau_M$) is not taken into account. If the inner-sphere water molecule does not reside on the paramagnetic center long enough, then its protons will not be effectively relaxed before the water molecule departs. On the other hand, if the molecule resides on the Gd$^{3+}$ too long (beyond the time necessary to be fully relaxed), then it needlessly occupies a coordination site that could be employed to relax other water molecules. Surprisingly, most Gd$^{3+}$ complexes investigated as potential contrast agents to date have suffered from water exchange that is too slow and certainly not optimal [3]. By ensuring that the water residence lifetime is optimal ($\tau_M \sim 10 – 40$ ns) the potential gains in relaxivity from slowing rotation would be even more substantial. Theoretically, the relaxivity of a $q = 1$ Gd$^{3+}$ complex could approach 110 – 120 mM$^{-1}$s$^{-1}$ upon slowing rotation (Fig. (1)).

2. IMPROVING CONTRAST AGENT SENSITIVITY

All current Gd$^{3+}$-based contrast agents distribute through-out all extracellular space, have comparatively low relaxivities of 4–5 mM$^{-1}$s$^{-1}$ [3], and require comparatively high doses, ~0.5 mmol kg$^{-1}$. To detect specific biological targets by MRI, the sensitivity of Gd$^{3+}$-based agents must be enhanced substantially. Given the assumption that any targeted agent will exhibit slow rotation upon binding to a large biological target molecule, this means one must either optimize water exchange in these complexes or develop agents that can alter tissue contrast via entirely different mechanisms. Water exchange in Gd$^{3+}$ complexes is essentially determined by two factors and to some extent these can be controlled by design. Let’s consider each factor in turn. The first and most easily controlled factor is the electron density on the central lanthanide ion. The effect of electron density upon water exchange is easily understood, the more electron deficient the metal ion, the greater its demand for electron density from the lone pair of the water oxygen. This greater electrostatic attraction will shorten the lanthanide-water bond, slowing dissociation of a bound water molecule. Intuitively, water exchange is likely to be fastest when the ligand donor atoms are anionic groups capable of substantial electron donation to the Gd$^{3+}$. Thus, it is expected that ligands containing carboxylate donors will favor faster water exchange whereas neutral ligands like amides or alcohols would favor slower water exchange. This has been illustrated in our laboratory by a series of complexes in which Gd$^{3+}$ is coordinated by a macrocyclic ligand with four pendant arms (Fig. (2)). The rate of water exchange in each complex was measured by a temperature dependent analysis of the $^{17}$O NMR linewidth of bulk water of a solution of each complex. As amide ligating groups are replaced by the ionic acetate ligands, the temperature profiles of the $^{17}$O transverse relaxation rate changes dramatically corresponding to a change in the water exchange rate. The data of Table 2 show that the water residence lifetime ($\tau_M$) shortens as the number of acetate ligands is increased so
clearly water exchange can be increased by incorporating more electron donating groups in the ligand.

Water exchange may also be made more rapid by increasing the steric encumbrance around the water coordination site. In practice, this can be a difficult to achieve but may be necessary to optimize water exchange rates. Substituting more bulky phosphonate groups for acetates in DOTA increases the steric encumbrance around the water coordination site to such an extent that water cannot approach the lanthanide ion closely enough to coordinate and GdDOTP$^{5-}$ is eight coordinate. So if a suitable compromise can be reached, it seems that the coordinated water molecule can be held far enough from the lanthanide that it can only form a weak interaction and thereby exchange rapidly. Aime and co-workers who studied a number of ligands based around the pyridine containing cyclen analogue, pyclen, have provided just such an example [24–27]. The Gd$^{3+}$ complex of the trimethylenephosphonate derivative of pyclen, PCTP, is eight coordinate, the three phosphonates crowding out the ninth coordination site [25]. A similar situation arises when one of these phosphonates is replaced by a carboxylate as in GdBn-PC2PA$^{2-}$ which is also $q = 1$ [27]. The water residence lifetime is shortened from 75 ns in GdBn-PC2PA$^{2-}$ (two phosphonates) to 6 ns in GdPCTP$^{3-}$ (three phosphonates) [25,27]. Likewise, when just one of the acetates of GdDOTA$^-\ $is replaced by a phosphonate (GdDO3A-P$^{2-}$) the complex retains its inner sphere water molecule but the rate of water exchange is much faster [28].

So ultimately, although phosphonates can be successful in increasing the rate of water exchange, they represent a crude tool for the chemist since they tend to increase the rate too much. It has been estimated that if introduction of a phosphonate (or phosphonates) causes an O-Ln-O bond angle smaller than 136° then it (they) will force the water molecule off the complex altogether [29]. However, acetate pendant arms can be used to increase the steric encumbrance around the water coordination site and with rather more control. In LnDOTA$^-\ $complexes the central lanthanide ion is sandwiched between four co-planar nitrogens of the macro-cycle and four co-planar oxygens of the acetates, the water molecule occupying an apical position above the four acetate oxygens. The torsion angle ($\omega$) between these two planes of donor atoms defines the coordination geometry of the complex. A larger angle (~39°) defines a square antiprismatic (SAP) coordination geometry whereas a smaller angle (~25°) defines a twisted square antiprismatic (TSAP) geometry. Both these coordination isomers are observed in solution because they interconvert [30]. This interconversion takes place by either a flip in the conformation of the macrocyclic ring ($\lambda\lambda\lambda\lambda\leftrightarrow\delta\delta\delta\delta$) or by arm rotation ($\Lambda\leftrightarrow\Delta$). So in a solution of an LnDOTA$^-\ $complex four isomeric structures, related as two enantiomeric pairs, exist in dynamic equilibrium. Sequential arm rotation and ring flip interconverts enantiomers (Fig. (3)).

A number of recent studies have shown that the rate of water exchange in LnDOTA-type complexes is up to two orders of magnitude faster in the TSAP isomer than in the SAP isomer [31–34]. The O-Gd-O angle in the SAP isomer of GdDOTA$^-\ $is a wide 146°, allowing unfettered access of the water molecule to the lanthanide ion [29]. This suggests that using a complex that exists as the TSAP isomer in solution would offer substantial advantages when trying to achieve higher relaxivities. Unfortunately, it is difficult to predict the population of each isomer adopted by a complex and furthermore, the TSAP isomer is usually only preferred for the very early lanthanide ions [35–37]. What is required is a means by which the coordination geometry can be selectively controlled. A recent study by Woods et al. [32,33,38] showed that $\alpha$-substitution of the acetate arms could increase the energy barrier to arm rotation such that arm rotation was effectively “frozen out”. The orientation of the arms is then determined by the configuration at the chiral carbon, an $R$-configuration giving rise to a $\Lambda$ conformation and $S$- to $\Delta$. Experiments in our laboratory [39] showed that introducing a substituent onto the macrocyclic ring had a similar effect in
“freezing-out” the ring flip. These two effects where combined to design a ligand that, when complexed with a lanthanide ion, would adopt only one of the two coordination geometries [40]. Furthermore, since it is known which orientation of the ring and arms are required for a given coordination geometry, then by appropriate selection of the chirality of each chiral centre within the ligand it should be possible to determine which coordination geometry should dominate. Substituting DOTA with a nitrobenzyl group on the macrocyclic ring and a methyl group on each acetate affords the ligand NO$_2$BnDOTMA, the lanthanide complexes of which were found to adopt exclusively one coordination isomer. The configuration at the macrocycle was $S$- and so by altering the configuration of the center at each acetate the coordination isomer obtained could be controlled. An $R$- configuration at the pendant arms defines a $\Delta(\lambda\lambda\lambda\lambda)$ (SAP) conformation, in contrast an $S$-configuration defines the desired $\Delta(\delta\delta\delta\delta)$ (TSAP) conformation [40]. This can be clearly seen in the extended spectral width $^1$H NMR spectra of the europium complexes of these two ligands (Fig. (4)). The more compact SAP structure exhibits much greater lanthanide induced shifts than that of the more open TSAP.

The presence of one inner sphere water molecule was confirmed by luminescence measurements [40] before the water exchange rates were measured by variable temperature $^{17}$O NMR. The water residence lifetime in the SAP isomer was indeed found to be longer, one order of magnitude longer than that observed for the TSAP isomer. This is because as the pendant arms rotate from the larger 39° angle in the SAP isomer to the narrower 25° angle of the TSAP isomer the oxygen atoms of the carboxylates are pushed upwards over the central lanthanide ion in order to accommodate the intervening atoms of the pendant arm (Fig. (5)). This has the effect of increasing the steric demands around the water coordination site, pushing the water molecule slightly further away from the lanthanide ion and thus weakening the water-lanthanide interaction. The value obtained for the TSAP isomer of GdNO$_2$BnDOTMA$^-$ (15 ns) is close to the optimal value for achieving the highest relaxivities suggesting that TSAP coordination geometries obtained in this way may form the ideal basis for targeted agents.

Merbach and co-workers employed a different approach to increase the steric encumbrance around the water coordination site [41–43]. The attraction of their approach is its simplicity, it just involves inserting another methylene group into a part of the ligand. One advantage of this approach is that it can not only be applied to macrocyclic tetraacetate complexes but also to those derived from DTPA. As long as only one methylene group is added, either to the backbone or within a pendant arm, the complex retains much of its former stability and its one inner sphere water molecule. The effect of adding further methylene groups is deleterious on both of these factors with the complexes becoming increasingly less stable and removal of all inner-sphere water molecules [44,45]. However, when a single extra methylene group is inserted into the complex the water exchange rate is improved by two orders of magnitude. Best results appeared to be achieved when the methylene group was inserted into the pendant arm of DTPA [43]. The water residence lifetime of about 30 ns is right on the mark of a theoretical optimum value. Introducing the methylene into the backbone of the ligand was marginally less successful, with the water exchange rate accelerated beyond optimum in all cases studied (Table (3)). Unfortunately, the most suitable complex in terms of water exchange kinetics, GdDTTA-prop$^{2-}$, is also found to have the lowest stability [46]. With stability constants in the region log $K_{GdL}$ = 16 – 23 it is far from clear that these complexes are stable enough for in vivo applications in which the complex is not rapidly excreted. Despite the drop in stability of these complexes the two most stable were functionalized with the same nitrobenzyl substituent used in the NO$_2$BnDOTMA complexes discussed above [41,43]. Nitrobenzyl substituents are favored because they are readily modified into an aryl-isothiocyanate for conjugation of the complex under mild conditions. The introduction of a substituent into these complexes does not
radically affect the observed value of $\tau_M$ and, at around 7–8 ns, a sizeable increase in relaxivity may reasonably be expected once the complexes are conjugated and rotation has been slowed. In contrast, substitution of a benzyl group onto GdEPTPA$^{2-}$ weakens the stability of the complex yet further. So ultimately this approach to improving water exchange rates will only be practical in situations where the contrast agent is not required to spend prolonged periods in the body.

A partially solved crystallographic structure of GdTRITA$^-$ helps to explain the rapid exchange rates observed in this complex. The O-Gd-O angle was found to be 136.7°, [41] extremely close to the 136° cut off at which the bound water is pushed off the complex [29]. This suggests that water exchange rates in octadentate complexes cannot be accelerated much beyond that observed here. At this limit another factor that potentially influences water exchange comes to light. By measuring water exchange as a function of pressure Merbach and his group were able to determine the activation volume $\Delta V^\ddagger$ of the exchange process. A large positive volume of activation characterizes a dissociative exchange mechanism whereas an associative mechanism exhibits a negative volume of activation. GdEPTPA$^{2-}$ has an activation volume of $+6.6 \text{ cm}^3\text{mol}^{-1}$, cf. $\Delta V^\ddagger = +10.5 \text{ cm}^3\text{mol}^{-1}$. GdDTPA$^{2-}$, and the value for GdTRITA$^-$ at $-1.5 \text{ cm}^3\text{mol}^{-1}$ is even lower. This suggests that exchange is occurring by a mechanism with more associative character in these rapidly exchanging complexes. This is probably best described as a dissociative interchange (I_d) mechanism in which the departure of the coordinated water molecule is assisted by the approach of the incoming water molecule. This change in mechanism may be partly responsible for the near optimal exchange rates observed in these complexes and is probably the result of a longer water-lanthanide bond distance.

By reducing the denticity of the ligand and opening one more coordination site to water, the exchange mechanism becomes even more associative in nature. This could accelerate water exchange without the necessity to tinker with the groups surrounding the water coordination sites. Furthermore, allowing a second water molecule into the inner coordination sphere doubles the number of protons available for relaxation, so the potential exists for a large relaxivity enhancement to be observed if a $q = 2$ complex is employed as the basis of a targeted agent. As can be seen from Table (3), the effect of increasing $q$ is quite positive in terms of improving the water residence lifetime. Although in general the rate of water exchange is a little slower than the optimal calculated value, this is likely to be offset by the additional coordinated water molecule. The relaxivities of these types of complexes are quite promising: GdDO3A – 6.8 mM$^{-1}$s$^{-1}$ (25 °C) [53], GdDO3MA – 4.4 mM$^{-1}$s$^{-1}$ (40 °C) [49], GdPCTA – 6.9 mM$^{-1}$s$^{-1}$ (25 °C) [25], GdPCP2A – 8.3 mM$^{-1}$s$^{-1}$ (25 °C) [26], all at 20 MHz. However, removing one of the ligating groups from the ligand necessarily leads to a reduction in the stability of the complex and this raises questions about the safety of using these complexes in vivo. However, these complexes still exhibit higher stability constants than some of the octadentate ligands discussed earlier such as GdTRITA. Furthermore, steps can be taken to enhance the stability of these complexes. More rigid chelates are generally associated with more stable complexes and by substituting the acetate arms of DO3A with methyl groups the ligand may be rendered more rigid. This is reflected in an increase of 4 orders of magnitude in the thermodynamic stability constant [49].

In a radical departure from the polyaminocarboxylate ligands, Ken Raymond and colleagues have taken the approach that Gd$^{3+}$ prefers “harder” oxygen donor ligands over nitrogen donors and developed a series of hexadentate oxygen donor ligands that form astonishingly stable complexes with Gd$^{3+}$ (Table (3)). Based around three 3-hydroxy-2(H)-pyridinone (HOPO) units appended to a tris(2-aminoethyl) amine (TREN) scaffold, the Gd$^{3+}$ complex of TREN-3, 2-Me-HOPO is more stable than its corresponding complex with TRITA and the complex has two inner-sphere water molecules [50, 54]. The result is a complex that
should, in theory, exhibit rapid associative water exchange and this is borne out by a measured relaxivity of 10.5 mM$^{-1}$s$^{-1}$ (37 °C 20 MHz) [55]. This is a high relaxivity value even for a $q = 2$ complex. It was difficult to assess the extent to which this high relaxivity is due to optimal exchange rate because the complex is poorly soluble in water which made it difficult to measure the rate of water exchange by the conventional method of variable temperature $^{17}$O NMR. As a result, more permutations of the ligand were synthesized and studied to improve the water solubility and the already remarkably high stability constant. Altering the podand skeleton was generally found to have a negative effect on stability [56] but altering the chelating groups appended to it enabled compounds with improved water solubility and stability [51, 52, 57–59]. Incorporating polyethylene glycol chains into one of the chelating groups, a teraphthalamide (TAM), through a para amide had the expected effect of increasing the water solubility of the complex [51]. Long polyethylene glycol chains containing 44 – 123 oxygen atoms had the added effect of displacing one of the inner-sphere water molecules on the Gd$^{3+}$ rendering a $q = 1$ complex while a shorter PEG chain having 3 oxygen atoms did not displace either of the two inner-sphere water molecules and these waters were found to exchange at an optimal rate via an associative mechanism ($\Delta V^\ddagger = -5$ cm$^3$mol$^{-1}$) [52]. By incorporating both HOPO and TAM chelating units into the ligand it was also discovered that the stability of the complex also increased [58]. This was attributed to the substantial increase in basi-city observed when these chelators are employed together as compared to ligands possessing just one or the other. Thus, the complex GdTREN-bis-(6-Me-HOPO)-TAM-TRI exhibits both a favorable stability and near optimal water exchange required for the basis of a targeted contrast agent.

3. RESPONSIVE MR CONTRAST AGENTS

Gd$^{3+}$ complexes having two inner-sphere water molecules ($q = 2$) are generally less stable than $q = 1$ complexes and tend to form ternary complexes with a large number of endogenous species such as, carbonate and bicarbonate, lactate, acetate, phosphate, citrate, amino acids and certain residues in proteins. These endogenous ligands displace either one or both of the water molecules from the inner coordination sphere and, in so doing, diminish the relaxivity of the agent. Even more importantly, it is the inner-sphere component that is compromised and so lengthening $\tau_R$ will have very little effect upon the overall relaxivity of a $q = 2$ complex once a ternary complex has formed. However, this “disadvantage” is potentially very helpful when designing a contrast agent operates in response to the presence of one of these secondary ligands.

Blocking and unblocking of the water coordination sites by endogenous ligands forms the basis of detecting such anions by MRI. The inner-sphere relaxivity of an agent will be “turned off” when the secondary ligand binds and “turned on” again if the water then displaces that ligand. An example of this was provided by Parker and co-workers with the $q = 2$, tri-amide complex, GdDO3ALA [60,61]. The relaxivity of this agent in the presence of carbonate was found to be pH dependent. At high pH, water is displaced from Gd$^{3+}$ by carbonate and the relaxivity is typical of a $q = 0$ complex (~2 mM$^{-1}$s$^{-1}$, 20 MHz, 25°C). As the pH is adjusted toward the acid range, the carbonate/bicarbonate/carbonic acid equilibrium shifts away from CO$$_3^{2-}$$ and HCO$$_3^{-}$$ (both good bidentate ligands for Gd$^{3+}$) toward H$$_2$$CO$$_3$$ and CO$$_2$$ (very weak ligands for Gd$^{3+}$) and the complex becomes progressively more hydrated thereby “switching on” the relaxivity (Fig. (6)). The maximum relaxivity of 7.1 mM$^{-1}$s$^{-1}$ observed at pH 6 and below represents a 255% enhancement from the $q = 0$ complex to the $q = 2$ complex.

Some ternary complexes formed between $q = 2$ Gd$^{3+}$ complexes and secondary ligands are quite stable. The crystal structure of the YbDO3Ph$^{3+}$-lactate ternary complex clearly shows that lactate binds to the Yb$^{3+}$ in a bidentate fashion and in doing so displaces both water
molecules from the metal center (Fig. (7)) [62]. Not all $q = 2$ lanthanide complexes are found to adopt this type of ternary complex. One of the advantages cited in favor of GdPCTA and GdTREN-3, 2-Me-HOPO as $q = 2$ complexes is that they appear to be immune to the effects of endogenous anions and do not suffer the drop relaxivity observed for DO3A derived complexes. It is thought that GdTREN-3, 2-Me-HOPO does not form ternary complexes because the bound waters in this $q = 2$ complex are positioned trans to each other, thereby precluding formation of ternary complexes with bidentate ligands such as lactate. However, it is less clear why GdPCTA fails to form ternary complexes other than the complex is overall charge neutral (compared with the triplositive charge of YbDO3Ph$^{3+}$). Thus, although $q = 2$ complexes may be an important feature for high relaxivity, targeted agents, the rational design of such agents is still rather hit-or-miss.

The process of capping a Gd$^{3+}$ complex to prevent access of water to the inner coordination sphere has been employed in a number of model responsive agent systems. For example, this was the approach taken in the first reported example of a “smart” agent by Meade and co-workers in 1997 [63]. The Gd$^{3+}$ complex of 1-(2-β-galactopyranosylethoxy)-4,7,10-tris(acetic acid)-1,4,7,10-tetraazacyclododecane (EGad) prevents water access to the coordination sphere of Gd$^{3+}$ by a galactose moiety blocking the apical position otherwise occupied by water. The hydration state of the Tb$^{3+}$ analog of EGad determined by luminescence measurements [64,65], was $q = 0.7$. However, when contributions from outer sphere and other proximate OH oscillators associated with the sugar moiety are taken into consideration the complex is consistent with a $q = 0$ complex [66]. Upon exposure of EGad to β-galactosidase, the galactose moiety is cleaved from the complex resulting in the unblocking of the apical coordination site (Fig. (8)) and enabling water coordination. Indeed, the terbium complex has a measured $q$ value of 1.2 water molecules confirming that the enzyme is able to modulate the hydration state of the complex. The authors reported a 20% decrease in the measured $T_1$ values upon exposure to the enzyme, consistent with the “switching on” of inner sphere relaxivity. It should be pointed out however, that the outer-sphere relaxivity (~50% of the total) is always “on” so even the inactive form of such smart agents are not silent. Nevertheless images of a phantom system with the agent in the presence and absence of β-galactosidase show a clear contrast enhancement as a result of the enzymatic cleavage (Fig. (8)). The utility of this agent has been demonstrated in vivo after microinjection of the “smart agent” into Xenopus embryos [67].

This same blocking strategy was also used to design of a Ca$^{2+}$-sensitive MR agent [68,69]. This agent uses a heptadentate DO3A substructure to chelate Gd$^{3+}$ thereby leaving two coordination sites vacant for ligation by water. Two of these units were attached to a 1,2-bis(o-amino-phenoxy)ethane-N, N', N'-tetraacetic acid (BAPTA) derivative via propyloxy linkers to allow the carboxylates of the BAPTA to coordinate the vacant sites on Gd$^{3+}$ as shown (Fig. (9)).

In the absence of Ca$^{2+}$, the carboxylates of the central BAPTA unit bind to the water sites on the two appended GdDO3A moieties thereby excluding water molecules from each Gd$^{3+}$ center. Upon exposure to Ca$^{2+}$, these same carboxylates are required to bind Ca$^{2+}$ into the central BOPTA moiety and are released from each GdDO3A unit thereby exposing Gd$^{3+}$ to water and facilitating inner-sphere relaxation. Ca$^{2+}$ binding to GdDOPTA occurs in the micromolar range and gives rise to an increase in relaxivity from 3.26 to 5.76 mM$^{-1}$s$^{-1}$ (500 MHz, 25°C), an enhancement of some 80%.

A second example of changes in hydration of Gd$^{3+}$ upon addition of a second metal ion is given by the bis-amide system, GdDTPA-BPYED (Fig. (10)). This $q = 1$ complex has a relaxivity of 6.1 mM$^{-1}$s$^{-1}$ (300 MHz, 25°C) in the absence of Zn$^{2+}$ [70]. Upon exposure to Zn$^{2+}$, the appended pyridyl ligands form a tetrahedral complex with Zn$^{2+}$ and the relaxivity
of the complex drops to 4.0 mM\(^{-1}\)s\(^{-1}\). As this relaxivity value is rather high for the \(q = 0\) complex shown in Figure 10, it is more likely that the Gd\(^{3+}\) remains a \(q = 1\) complex after formation of the 1:1 Zn\(^{2+}\) complex but that exchange of the bound water with bulk water slowed by the “capping” Zn\(^{2+}\) complex. If correct, this model introduces a new twist on responsive agent design in demonstrating that it makes little difference whether there is a coordinated water molecule or not. If the exchange rate of a coordinated water molecule can be modulated by the chemistry, then even a bound water may contribute little to inner-sphere relaxivity. This particular Zn\(^{2+}\) system suffers from the fact that addition of even more Zn\(^{2+}\) results in formation of the M\(_2\)L type complex which re-exposes the Gd\(^{3+}\)-bound water molecule to bulk solvent (Fig. (10)) and a return of relaxivity to its original value (6.1 mM\(^{-1}\)s\(^{-1}\)) in the presence of two equivalents of Zn\(^{2+}\).

The examples given above are not the only way in which relaxivity may be modulated in response to a change in chemistry. We have already seen how relaxivity may be improved by optimizing the rate of water exchange, an effect that would be accentuated if the complex were rotating slowly (long \(\tau_R\)). Thus, modulation of water exchange between fast and slow régimes could translate into a substantial modulation of relaxivity. Although designing complexes with optimal water molecule exchange has proven difficult, it should be pointed out that it is not necessary for the entire water molecule to exchange with bulk solvent. Rather, exchange of the bound water protons with bulk water protons would produce the same desired effect and this may prove easier for the experimentalist to manipulate. Thus, \(\tau_M\) should be considered as the sum of the water molecule exchange and prototopic exchange processes.

\[
\frac{1}{\tau_M} = \frac{1}{\tau_M^w} + \frac{2}{\tau_M^p}
\]  
(Eqn. 4)

where \(\tau_M^w\) is the residence lifetime of the water molecule and \(\tau_M^p\) is the residence lifetime of a proton on the bound water molecule.

We have recently demonstrated the potential advantages of this approach as a basis for responsive MR agents [71,72]. As a first example, the pH sensitive agent GdDOTA-4AmP\(^5^-\) has a methylene phosphonate substituent on each amide and predominantly adopts a SA geometry in solution (>99%). The water residence lifetime \(\tau_M^w\) in the dysprosium complex measured by \(^{17}\)O NMR was 21 µs and insensitive to pH. In contrast, the relaxivity pH profile of GdDOTA-4AmP\(^{5^-}\) displays a strongly pH dependent profile, (Fig. (11)) with a steep increase in relaxivity observed as the solution becomes more acidic (\(r_1\) changes from 3.8 mM\(^{-1}\)s\(^{-1}\) at pH 9.5 to 9.8 mM\(^{-1}\)s\(^{-1}\) at pH ~6 when measured at 20 MHz, 25°C). This provided the first example of a complex where neither the inner-sphere coordination number (\(q = 1\) at all pH values) nor the Gd\(^{3+}\)-bound water molecule lifetime is altered while the relaxivity increases by 260%. However, the four appended phosphonate groups in this complex were shown to have protonation constants of 6.0, 6.6, 7.3 and 8.7, values that coincide with the region of enhanced relaxivity. This indicates that upon protonation, the phosphonates act as highly effective catalysts of prototopic exchange of the protons on the coordinated water. Interestingly, the relaxivity near pH 6 (9.8 mM\(^{-1}\)s\(^{-1}\)) is near that expected for a low molecular weight Gd\(^{3+}\) complex with an optimized water molecule exchange rate. This demonstrates that it may be easier to modulate proton exchange over perhaps several orders of magnitude than altering exchange of the complete water molecule. Analogues that exhibit faster water molecule exchange such as GdDOTA-2AmP\(^3^-\) and GdDOTA-1AmP\(^2^-\) display pH independent relaxivities so slow water molecule exchange appears to be a prerequisite for this sort of responsive agent. It should be noted that the minimum relaxivity observed for this complex near pH 9.5 (3.8
mM$^{-1}$s$^{-1}$) is somewhat higher than the values typically observed for tetraamide complexes without the appended phosphonate groups, indicating that even fully deprotonated phosphonates catalyze prototopic exchange to some limited degree.

The pH sensitivity of GdDOTA-4AmP$^{5-}$ has been successfully employed to obtain a pH map of mouse kidney *in vivo* by MRI (Fig. (12)) [73]. Given that the concentration of the agent in tissue must be known to correlate the observed relaxation rates (image intensity) with pH and that the biodistribution of the agent is not necessarily uniform in tissues such as kidney, the pH map shown (Fig. (12)) was not as simple to generate as one may first think. To correct for these unknowns, mice were first injected with GdDOTP$^{5-}$ and imaged as a function of time. After complete washout of the first agent an equivalent amount GdDOTA-4AmP$^{5-}$ was injected and images were collected with the same temporal resolution. Given that GdDOTP$^{5-}$ has the same overall charge and similar size as GdDOTA-4AmP$^{5-}$, the pharmaco-kinetics of the two agents was assumed equivalent and any differences in image intensity at any time-point during wash-out were assumed to reflect a true alteration in tissue pH. This procedure is certainly not optimal for human studies but the experiment does illustrate that responsive agents such as this can indeed provide important physiological information *in vivo*.

4. THE DETECTION LIMIT OF Gd$^{3+}$-BASED MR AGENTS

For a targeted or responsive contrast agent to be effective it must be able to increase image contrast to such an extent that the increase can be discriminated from neighboring regions that are not enhanced. So before selecting a biological target or an *in vivo* analyte, it is important to appreciate the lower detection limits of MR contrast agents in tissue. Ahrens et al [74], addressed this question using tissue contrast model (TCM) that considers two regions, A and B, wherein region A contains the target or analyte of interest and region B does not. By experimentation, the minimum contrast-to-noise ratio between the two regions required to visualize a difference in MR contrast was established as five. From this, a set of equations were derived that express the minimum required concentration of the contrast agent in region A in terms of $k/N$ for a given imaging pulse sequence, where N is the statistical image noise and $k$ a functional constant that is related to the image intensity. For a spin-echo pulse sequence, $k/N = (I_b/N)/(1-exp(-T_R/T_{1b}))$ where $I$ is the image intensity and $T_R = t + T_E$ (the delay time and the echo time of the pulse sequence). The signal-to-noise ratio ($I_b/N$) was determined experimentally and found to lie between 13 and 15. The equations presented describe two limiting cases, one where the relaxivity of the agent remains the same in the two regions but the concentration varies (eqn. 5) and another where the concentration of the agent is the same in the two regions but the agent has different relaxivities in the two regions (eqn. 6).

$$\left[ M \right]_a \neq \left[ M \right]_b, R_a\neq R_b = R$$

$$\left[ M \right]_a = \frac{1}{T_{1a}} + \frac{\left[ M \right]_b}{T}$$  \hspace{1cm} (Eqn. 5)

$$\left[ M \right]_a = \left[ M \right]_b = \left[ M \right], R_a \neq R_b$$

$$\left[ M \right]_a = \frac{1}{T_{1a}(R_a-R_b)}$$  \hspace{1cm} (Eqn. 6)

$$\Gamma_{kew} = f \left( \frac{5N}{k} e^{-f'} \right)^{-1}$$  \hspace{1cm} (Eqn. 7)
The equations presented here are for a spin-echo pulse sequence (others were also presented for an inversion-recovery spin-echo pulse sequence). Equation 7 and Equation 8 allow the value of $\Gamma$ to be determined for a given value of $(k/N)$ found for an image acquired using a spin-echo pulse sequence. $T_{01}$ is the longitudinal relaxation time of the tissue in the absence of contrast media, $R$ is the longitudinal relaxivity where $R_a$ is the relaxivity in region $a$ and $R_b$ is the relaxivity in region $b$. $[M]_a$ is the concentration of agent in region $a$ and $[M]_b$ the concentration of agent in region $b$. GdHP-DO3A was microinjected into *Xenopus laevis* embryos at different concentrations, the embryos were imaged and the results compared with those predicted by equation 5 (a relaxivity value of $4.1 \text{ mM}^{-1}\text{s}^{-1}$ was used for GdHP-DO3A in tissue). Good agreement between the injected dose and visibility of differences in images contrast was observed when compared to that predicted by equation 5.

Unfortunately, equation 5 does not fully describe the prevailing situation for a targeted contrast agent nor does equation 6 describe adequately that for a responsive agent. Consider a low molecular weight targeted agent whose excess is quickly excreted after injection. This leaves a situation in which some of the agent will be present in both region $A$ and $B$, although ideally only traces would be present in region $B$. Also, because the agent binds to its target, it is expected that its rotation will be slowed and its relaxivity will be enhanced. Similar problems exist in the case of a responsive agent, which, although relaxivity increases on going from $B$ to $A$, may not have a uniform concentration of the agent over the two regions. So we need to be able to assess what is the lowest concentration of the agent in region $A$, $[M]_a$, at which contrast may be observed as a function of $[M]_b$ and the difference in relaxivity between the two regions. In this way an appreciation of the lower detection limit of a given system may be achieved. Equation 9 was developed as a part of this study but expresses the detection limit in terms of our desired parameters.

\[
M_a = \frac{1}{\Gamma \left(1 + T_{01} R_b [M]_b \right) - 1} \frac{T_{01}}{R_a}
\] (Eqn. 9)

Using Eqn 9 in conjunction with Eqn 7 and Eqn 8 we can model the lower detection limits for a given system. Take for example a hypothetical contrast agent that in region $B$ possesses a relaxivity typical for a low molecular chelate ($R_b = 4.5 \text{ mM}^{-1}\text{s}^{-1}$). When the agent moves into region $A$ it binds to a target and the relaxivity is enhanced. By using the experimentally determined parameters determined for the *Xenopus* system [74] using a spin-echo pulse sequence, $I_p/N = 14$, $T_R = 1.28 \text{ s}$ and $T_{01} = 1.3 \text{ s}$, we can calculate how little of the agent is required to provide image contrast for an array of $R_a$ and $[M]_b$ values (Fig. (13)). As can be seen the lower detection limit improves with increasing relaxivity and the effect of relaxivity is most important when the concentration of the agent in region $B$ is higher. This is analogous to the situation encountered when a responsive contrast agent is employed. Assuming that no anaylate is present in region $B$ the relaxivity of the agent in region $B$ remains at $4.5 \text{ mM}^{-1}\text{s}^{-1}$. However, a large proportion of the administered dose (between 200 and 500 $\mu\text{molkg}^{-1}$) is expected to be present in region $B$. From Fig. (13) it can be seen that higher concentrations of the agent in region $B$ require that the agent achieve high relaxivities in region $A$ if a lower detection limit is to be realized. Even at relaxivity values in excess of $70 \text{ mM}^{-1}\text{s}^{-1}$ it may only be possible to detect the agent at concentrations above 30 $\mu\text{M}$. A similar situation might result from a targeted agent which possessed only...
poor binding affinity for its target. However, it is implicit in this model that the binding of a targeted agent is strong and that all the agent in region A is bound. Under these circumstance substantial clearance of the agent from region B, in which no targets are present, would be expected to reduce the concentration of the agent in region B. Now the detection limits improve substantially while the dependence of the detection limit on the relaxivity of the agent diminishes. The model suggests that when the concentration of the agent in region B becomes very low (~ 10 µM) even modest relaxivity enhancements upon binding (~ 40 mM$^{-1}$s$^{-1}$) would enable detection limits below 5 µM. In fact, in a best-case scenario, the TCM predicts that it should be possible to image any in vivo target to which it is possible to deliver greater than 1 × 10$^{-9}$ M of contrast agent. Of course these detection limits apply only to a system that fulfills the considerable constraints employed in this modeling exercise. Not least of which is that the contrast agent should be able to change its relaxivity from a modest 4.5 mM$^{-1}$s$^{-1}$ to an impressive 90 mM$^{-1}$s$^{-1}$ or better.

Since a twenty-fold increase in relaxivity is a difficult challenge, let’s examine potential targeted imaging agents that have been reported and what detection limits may apply to those. In our laboratory we have developed an agent (Gd-G80BP) that binds specifically to the transcription repressor protein, GAL80 [75]. Upon binding to GAL80, the relaxivity of Gd-G80BP increases 440% from 8.3 mM$^{-1}$s$^{-1}$ (20 MHz, 25°C, pH 7.4) to 44.8 mM$^{-1}$s$^{-1}$.

In phantom samples using a 1.5T clinical scanner, 14 µM Gd-G80BP in the presence of GAL80 was easily distinguished from a sample containing 14 µM Gd-G80BP in the presence of HSA as a control. Using the model presented above, the calculated lower detection limit was found to be even lower, just 3.5 µM. Significantly, the Gd-G80BP•Gal80 binding constant is high (5 × 10$^{5}$ M$^{-1}$), about 20 times stronger than the binding constant found for MS-325 binding to HSA. This high binding affinity assists in the detection of the Gd-G80BP•Gal80 adduct by MRI since at the concentrations employed in the experiment around 59% of the agent is bound to the protein. However, this in turn suggests that our calculated lower limit of detection errs on the low side. For Gd-G80BP this factor does not have a serious detrimental effect on the model, but consider the effect of the binding constant of MS-325. Since $K_a = 1.1 \times 10^4$ M$^{-1}$, at the concentrations explored with Gd-G80BP only 6% of MS-325 would have bound to its target HSA. With so little of the chelate in the high relaxivity bound form it is unlikely that MS-325 could be detected at anywhere near the concentrations suggest by the model. Fortunately for the use of MS-325, HSA is present in plasma at concentrations of 0.6 × 10$^{-3}$ M and at this level some 70% of the agent is in the high relaxivity bound form. Nonetheless, this clearly demonstrates that, in addition to good relaxivities, it is necessary to have a strong chelate-target binding interaction for a successful targeted agent.

The abundance of HSA in plasma is one of the features that make it an attractive target for MR contrast media. However, a second consideration also renders HSA a convenient target, the ease with which the contrast agent may be delivered to its target. Since contrast agents are administered via intravenous injection, in this case administration of the contrast agent delivers the agent directly to its target. This is not the case for many other potential targets that would rely upon the vasculature to deliver the agent to the tissue of interest. As one example, consider a cell receptor over expressed on the surface of a tumor where poor vascularisation of the tumor may hamper distribution of a targeted agent to a large proportion of the receptors. Nunn et al. [76], have suggested that receptors, expressed on the cell surfaces at concentrations of 10$^{-6}$–10$^{-10}$ M, represent viable targets for MRI agents and this conclusion is supported by the results obtained from the TCM model.

A range of receptor targeting molecules including somatostatin, octreotide, octreotate, vapreotide, lanreotide, bombesin and α-melanocyte have been identified [76]. These compounds have successfully been conjugated to ligands, which then allow a radionuclide to
be targeted to the receptor. Such conjugates have found application in radiotherapy as well as PET and SPECT imaging although more complete discussions of this topic can be found in other review articles [2,77,78]. If the receptor that bound one of these targeting molecules were present in high enough concentrations to allow it to be detected by MRI then such an approach to targeted contrast agents could be applied to MRI as well. This idea has previously been considered by Nunn et al. who estimated that the concentration of such receptors in tissue typically lies between 10^{-6} and 10^{-10} M [76]. So although parts of this concentration range overlap the lower detection limits predicted by the TCM model to assume that this means these receptors represent viable targets begs a very important question. Namely, does the concentration of the receptor represent the concentration of the contrast agent in that region of tissue? This is a particularly important consideration in a case where the target receptor is likely to be located in a poorly vascularised region, for example a tumor. The authors considered the case of data published by Forsell-Aronsson and co-workers [79] for the $^{111}$In chelate of DTPA-D-Phe-1-Octreotide. This chelate was administered to a number of patients with three types of tumor. Tumor samples were taken from a patient with medullary thyroid carcinoma 24 hours after injection. The levels of the chelate present in the tumor were determined by measuring the radioactive emissions of the $^{111}$In. By reexamining this data Nunn et al. were able to estimate that around 2% of the administered dose was present per kilogram of tumor tissue and this in tumors smaller than 5 g. This means that the concentration of agent in the tumor was, at best, only $2.8 \times 10^{-11}$ M. Only about one thousandth of the receptors were bound due to the poor vascularisation of the tumor. Not only does this render the use of this receptor as a tumor targeting device for MR contrast agents impractical but it also serves to highlight the importance of a viable delivery system in achieving target selective contrast. The body must be capable of delivering to the site of the target. In the case of poorly vascularised regions, and most particularly tumors, the chosen target will have to be present in far higher concentrations than it would in other areas.

The capacity of HSA to bind hydrophobic groups of a wide variety of shapes and sizes is a further reason its popularity as an imaging agent target. A wide range of HSA binding contrast agents have been developed. Complexes bearing benzyloxymethyl substituents such as GdBOPTA$^{2-}$, GdDTPA-EOB$^{2-}$ and GdDOTA(BOM)$_n^{-}$ (n = 1, 2 or 3) have also been found to bind to HSA [80–83]. Benzyloxymethyl groups have been found to bind to two sites on HSA, the IIA and IIIA sites. The binding of benzyloxymethyl groups to HSA is not particularly strong with $K_a \approx 10^2$ M$^{-1}$ for GdDOTA (BOM)$_1^{-}$. Increasing the number of benzyloxymethyl groups in the complex increases the binding affinity of the complex to HSA by a factor of approximately 50 per benzyloxy-methyl substituent. This increase in binding strength is reflected in the relaxation enhancements obtained in the presence of HSA [84]. GdDOTA(BOM)$_3^{-}$ was found to have the highest relativity, 53.2 mM$^{-1}$s$^{-1}$ (20 MHz, 25°C, pH 7.4) in a 3 mM HSA solution. It is important to note that the presence of aromatic rings in the ligand improves uptake of the complex into the liver, so these agents act as liver specific agent as well as blood pool agents. GdBOPTA$^{2-}$ is the first clinically approved liver specific/blood pool agent that takes advantage of this receptor induced relaxivity enhancement.

Substantial as these relaxivities are, it is important to bear in mind that these values are obtained using chelates that have not been optimized for water exchange. The potential is, therefore, that these relaxivities could be further enhanced by a considerable margin. This is best exemplified by the complex GdTREN-HOPO-TAM-PEG-5000 [51]. Long ethylene glycol chains are known to have affinity, all be it rather weak, for the hydrophobic binding sites on HSA. As we have already seen GdTREN-HOPO-TAM-PEG-5000 is a q = 1 complex with a water residence lifetime that lies well within the range calculated to offer the highest relaxivities. The already respectable relaxivity of the complex (9.1 mM$^{-1}$s$^{-1}$ at 20
MHz and 25 °C) is enhanced 713 % to 74 mM−1s−1, remarkably high given that the binding constant between the complex and HSA is weak at only 1.8 × 10^2 M−1. The potential for even higher relaxivities is clearly present when water exchange is optimal and the binding affinity improved with these complexes.

The agent MP2269 employs the non-aromatic lipophilic moiety, 4-pentylbicyclo[2.2.2]octane-1-carboxylate, linked to a GdDTPA unit through two aspartate residues to bind to HSA [85]. Consequently the water residence lifetime is very similar to that of GdDTPA, τM = 238 ns vs 303 ns for GdDTPA. The slightly higher relaxivity of MP2269, 6.2 mM−1s−1 (20 MHz, 35°C) is ascribed to the longer rotational correlation time of the larger complex. It was found that upon binding to HSA the relaxivity is enhanced 200% to 18.0 mM−1s−1 (20 MHz, 35°C). Given that MP2269 is not operating under optimal water exchange conditions this comparatively modest relaxivity enhancement is not altogether surprising. But what if the interaction of the complex with the protein is in some way rendering water exchange more difficult and slowing water exchange? Such a situation would have a serious effect upon the observed relaxivity. To investigate this possibility, Merbach and co-workers examined the rate of water exchange under conditions where 60% of the complex would be bound to HSA. Direct assessment of the water exchange rate in the bound form of the complex was not possible because of the large amount of unbound chelate present during the experiment. Nonetheless, by modeling the variable temperature 17O NMR results the authors were able to conclude that the binding of MP2269 to HSA did not substantially affect the rate of water exchange.

Of course it would be desirable to target other proteins in addition to HSA. McMurray and co-workers developed a means of targeting the enzyme thrombin-activatable fibrinolysis inhibitor (TAFI) while still using HSA as the mediator of relaxivity enhancement [86]. TAFI is a human carboxy-peptidase B that inhibits clot degradation by cleaving C-terminal lysine residues from the surface of fibrin binding sites. The enzyme has been implicated in thrombotic diseases. The authors designed two contrast agents in which a hydrophobic targeting vector was masked by the presence of three lysine residues. Upon exposure to TAFI, the lysine residues are cleaved from the contrast agent and the targeting capability of the agent is then realized. The hydrophobic group now targets serum albumin leading to a relaxivity enhancement that can only be observed after the chelate has been exposed to TAFI [86]. In the presence of 4.5% HSA and the enzyme TAFI the relaxivity values of 24.5 and 26.5 mM−1s−1 (37 °C 20 MHz, pH 7.4) were measured for GdDTPA-BIPEN and GdDTPA-DIPOH forms, respectively. This represents an enhancement of 121% and 170% over the blocked parent compounds.

The active site of an enzyme also makes an attractive target if a suitable targeting vector is known. One recent example is given by the work of Anelli, et al. [87], who designed a sulfonamide derivative as a specific inhibitor of the zinc-containing enzyme, carbonic anhydrase. GdDTPA-MSAD. Sulfonamides are known to have a high affinity for the zinc center of carbonic anhydrases and the enzyme was found to bind the complex GdDTPA-MSAD well, Kₐ = 1.5 × 10^4 M−1. The increase in relaxivity upon binding from 3.5 mM−1s−1 to 25 mM−1s−1 represents an enhancement of 610 % [87]. According to the TCM model it might be possible to detect this complex GdDTPA-MSAD at levels as low as 2 µM. However, its binding constant with carbonic anhydrase is little better than that of MS-325 with HSA, so very little of the complex will be bound at such low concentrations. It is therefore unlikely that the complex can be detected at anything like those levels. Nonetheless, this complex illustrates that a wide range of enzyme-specific recognition agents might be possible by taking advantage of known substrate/inhibitor constants.

However, the following four considerations must be taken into account if the low levels of detection necessary for imaging a target are to be met:
• The target must be present in vivo at concentrations high enough to be imaged (> 1 µM),

• The target must be in a region where the agent can be delivered in sufficient quantities,

• The affinity between the agent and the target must be high enough to ensure good binding at low concentrations (> 1 × 10^{-5} M),

• The water residence lifetime on the complex must be optimized to ensure good relaxivity enhancement upon binding to the target (10 – 40 ns)

5. PARACEST AGENTS: A NEW CONCEPT IN IMAGING AGENTS

Recently, Balaban and coworkers demonstrated that low molecular weight compounds with slowly exchanging –NH or –OH protons may also be used to alter image contrast via chemical exchange saturation transfer (CEST) of pre-saturated spins to bulk water [88,89]. This technique requires appropriate exchange kinetics between the two exchanging sites, relatively long relaxation times, and of course a chemical shift difference between the protons undergoing exchange. One advantage of molecules containing exchanging –NH and –OH protons is that it is easy to amplify the CEST effect considerably by using macromolecules with a large number of chemically equivalent exchanging sites [90]. One disadvantage is that the chemical shift difference between exchanging –NH and -OH protons in diamagnetic molecules and bulk water is typically no larger than 5 ppm. This makes it difficult to apply the CEST effect in tissues where the bulk water can have a very broad base-line resonance that can receive RF from the saturating pulse used to initiate the CEST effect. Nevertheless, Van Zijl and coworkers have demonstrated that the amide protons of intracellular proteins may be used in a CEST experiment to image tissue pH [91] and brain tumors [92]. We and others have demonstrated that the CEST effect also works in certain paramagnetic lanthanide complexes where exchange between a Ln^{3+}-bound water molecule and bulk water is surprisingly slow. The advantage of such paramagnetic CEST agents is that the bound water resonance can be found shifted much further away from the bulk water resonance, in some complexes up to as much as 800 ppm [93–102]. This reduces the possibility of inadvertently saturating the broad bulk water resonance of tissue and allows the use of much faster exchanging systems. Such paramagnetic CEST agents, often referred to as PARACEST agents, offer a novel way to produce MR image contrast that is highly dependent upon the chemical exchange characteristics of lanthanide complexes and less dependent upon their relaxation characteristics [102].

The magnitude of the PARACEST effect, adequately described by the Bloch equations modified for chemical exchange, depends upon many factors including concentration of the agent, the bound water lifetime, the hyperfine shift, the T₁/T₂ values, the frequency offset and strength of the applied RF irradiation [102]. For a two pool exchange system, the net equilibrium magnetization of bulk water (Mₐ₀) after a long presaturation pulse at the exchanging site is related to concentration of PARACEST agent by equation 10 [103]:

\[
\frac{M_{q}}{M_{q}^{0}} = \frac{\tau_{a}}{\tau + T_{1a}} = \left(1 + \frac{Cq T_{1a}}{55.5 \tau_{st}}\right)^{-1}
\]  
(Eqn. 10)

where C is the concentration of agent, q is the number of bound inner-sphere water molecules in the Ln^{3+} complex, τ_M is the lifetime of the bound water, and T_{1a} is the spin-lattice relaxation time of bulk water. This relationship allows one to predict the lower detection concentration limit for any agent with a known bound water lifetime (τ_M) and T_{1a} (bulk water). Fig. (15) shows a plot of net magnetization versus concentration of a
hypothetical agent with a τ_M of 3 µs and a bulk water T_1a = 1 s. This relationship indicates that the bulk water signal intensity can be reduced by ~37% with ~100 µM agent and by ~5% with as little as ~10 µM agent. This estimate is well below the detection limit of a low molecular weight Gd^{3+}-based contrast agent with a typical relaxivity (r_1) of 4–5 mM^{-1}s^{-1} but may or may not approach the sensitivity of an optimized Gd^{3+}-based T_1 agent [74]. These conservative estimates suggest at least that PARACEST agents have the potential of being as sensitive as or even more sensitive than Gd^{3+}-based T_1 agents, assuming that a frequency-selective presaturation sequence can be implemented on the clinical scanner that fully saturates the bound water resonance within acceptable SAR limits. Just as one can increase the relaxivity of Gd^{3+}-based agents substantially by conjugation to a polymer or formation of an aggregate, similar modifications of PARACEST agents could be done and this could easily extend the lower detection limit of a PARACEST agent into the sub-µM range. This was recently demonstrated for a sample of poly-arginine to which TmDOTP^5\(^-\) had been added to shift the exchanging -NH arginine protons well away from their normal diamagnetic position [100].

Since CEST efficiency is dependent on has a multitude of experimental variables (bound water exchange rate, proton exchange rate, chemical shift separation, T_1/T_2 values) that could in principle be modulated to produce a biologically responsive MR imaging agent. This is best illustrated by an example. It is well-known that boronic acids can bind of new saccharide sensors [104,105]. This property has previously been used to target Gd^{3+} chelates to glycosylated proteins to improve the relaxivity [106,107]. With this background, we set out to build a PARACEST agent for mapping tissue glucose (see structure in Fig. (16)) [101]. A Eu^{3+}-bound water peak, usually observed between 50–55 ppm in complexes such as this, is not observed in the absence of glucose but is observed upon addition of excess glucose. This indicates that binding of glucose to this EuDOTA-4AmBBA does indeed slow water exchange in this system. A phantom consisting of four plastic tubes (ID 4 mm) each containing 10 mM EuDOTA-4AmBBA and different amounts of glucose at pH 7.1 was prepared. MR images of these phantoms collected at 4.7T show clear differences in image intensities between the four samples (Fig. (16)). This is an exciting result because it illustrates that one should be able quantify glucose at physiologically relevant concentrations using a standard clinical imaging MR system and this unique PARACEST agent. It is important to emphasize that these images were collected not by direct detection of glucose (typically 5 mM in blood) but rather via the bulk water signal (55 M) as normally detected by MRI. This is an important advance for the field of molecular imaging because it demonstrates for the first time that one should be able to design PARACEST imaging agents for monitoring a variety of important metabolites in tissue. One can envision applications of such EuDOTA-4AmBBA complexes for imaging the distribution of glucose in livers of diabetic patients in vivo (for overproduction of glucose), for monitoring glucose consumption in tumors (analogous to FDG in PET imaging), and for detecting metabolic activation of the brain during functional stimulation (fMRI).

6. THE POTENTIAL OF MR AGENTS FOR IMAGING β-CELL FUNCTION

Pancreatic islets are complex multi-celled highly vascularized organs capable of rapidly sensing changes in blood glucose. An increase in blood glucose levels above the normal concentration of 5.5 mM after a meal stimulates the release of insulin from β-cells contained within islets of the pancreas. Insulin release is stimulated through multiple intracellular signals related to metabolism of glucose [108–110]. Glucose enters the β-cell through facilitated glucose transporters, in rat islets this is primarily through the GLUT-2 receptor. The glucokinase reaction then converts glucose to glucose-6-phosphate, which enters the glycolytic pathway ultimately producing two moles of pyruvate and some ATP. Most pyruvate enters the mitochondria where it is converted to acetyl-CoA or undergoes
carboxylation to oxaloacetate (anaplerosis). Complete oxidation of acetyl-CoA in the TCA cycle produces an excess of reducing equivalents and ultimately more ATP by electron transport and oxidative phosphorylation. Metabolic pathways that generate ATP in the β-cell appear to be fundamental to insulin release because a rise in the ratio of ATP to ADP has been shown to inhibit potassium channels that are sensitive to ATP (K<sub>ATP</sub>) and stimulate voltage-gated calcium channels [111]. This results in an influx of Ca<sup>2+</sup> into the β-cell which causes the activation of certain protein kinases and other enzymes, ultimately leading to exocytosis of insulin from preformed secretory granules. While regulation of K<sub>ATP</sub> channels is undoubtedly an important feature of the control of insulin secretion, other factors are also involved. Various laboratories have shown that glucose stimulated insulin secretion (GSIS) occurs even when the K<sub>ATP</sub> channels are taken out of play by pharmacologic agents or the application of very high concentrations of potassium to the cell [112,113]. However, the biochemical mechanism of this so-called “K<sub>ATP</sub> channel-independent” pathway of glucose-sensing is not well understood. Anaplerosis has long been considered important in this process but a link between anaplerosis and insulin secretion has not been identified. We have recently shown that pathways involving carboxylation/decarboxylation of pyruvate (pyruvate cycling) are quite active in the β-cell. Stimulation of pyruvate cycling pathways with pharmacological agents enhances GSIS while inhibition of these pyruvate cycling pathways using an inhibitor of pyruvate carboxylase reduces GSIS [114]. However, the metabolic connection between pyruvate cycling and insulin secretion remains unknown. This understanding of β-cell function may be provide several options to consider in the design of new agents for detecting β-cell function by MRI. As the influx of Ca<sup>2+</sup> into β-cells precedes insulin secretion, one approach could be the use of a surrogate paramagnetic ion as a mimic for Ca<sup>2+</sup>. Although Mn<sub>2+</sub> is a first row transition metal ion and has a much smaller ionic diameter than Ca<sup>2+</sup>, it has been shown that Mn<sup>2+</sup> does accumulate in β-cells in proportion to the glucose concentration presented to the cells, presumably via Ca<sup>2+</sup> channels [115]. Images collected of isolated pancreatic islets in the presence of an ultra-high concentration of MnCl<sub>2</sub> (25 mM) at two different glucose concentrations (5 and 20 mM) show that a clear contrast enhancement is obtained when the stimulatory levels of glucose are higher (Fig. (17)). This demonstrates one possible approach to imaging Ca<sup>2+</sup> influx into β-cells. Chemically speaking, the free Gd<sup>3+</sup> aqauion represents a much better Ca<sup>2+</sup> surrogate for this experiment but gadolinium is considerably more toxic than Mn<sup>2+</sup>. Levels of Mn<sup>2+</sup> as high as 20 mM will almost certainly not be tolerated in vivo, however, these results suggest that a suitable alternative – perhaps a cationic complex of Mn<sup>2+</sup> that is transported by calcium channels – could be employed to detect calcium channel activity in β-cells.

It is also known that β-cells package insulin into granules for storage in the form of a Zn<sup>2+</sup> complex. Glucose stimulated release of insulin then results in a concomitant release of Zn<sup>2+</sup> ions from the β-cell and this release may offer another way to measure β-cell function. We have already discussed a zinc-responsive MRI agent [70] so, in principle, imaging changes in zinc concentration should at least be feasible. This agent has two drawbacks however: excess zinc quenches the responsiveness of the agent and the relaxivity of the agent is just too low for detection. Even so, a higher relaxivity agent may not render imaging of Zn<sup>2+</sup> release feasible given that the concentrations of zinc ions are only around 10<sup>−7</sup> – 10<sup>−6</sup> M [116]. Such low concentrations are probably beyond the detection capabilities of MRI and would require a more sensitive detection technique such as fluorescence [116,117]. Likewise, the expulsion of protons from β-cells that is known to accompany insulin secretion is likely to cause only a small change in pH [118]. If however a pH responsive agent such as GdDOTA-4AmP<sub>5-</sub> [71,73], could be conjugated to a macromolecule to improve its relaxivity, it may be possible to detect the pH changes in the vicinity of β-cells as an indicator of insulin secretion.
The potentially high sensitivity of PARACEST agents may eventually render this class of agent more effective as a responsive agent than conventional agents. After all, the lower detection limits for conventional imaging agents predicted by the tissue contrast model were not particularly low when compared to the concentration of many of the analytes of interest in imaging beta-cell function. The use of a PARACEST agent such as the EuDOTA-4AmBBA complex used to sense glucose (Fig. 16) might be the key to functional imaging [101]. It may be possible to design new agents that report one of the analytes discussed above. Alternatively, EuDOTA-4AmBBA could be used to determine the actual glucose concentrations within the pancreas. This could potentially provide vital information concerning the state of blood flow in the normally highly vascularized pancreatic-islets. Such information could provide valuable insights into the loss of beta-cell sensitivity to increases in glucose levels during the progression of diabetes.

Although it is intellectually stimulating to think about responsive MR agents that might report on some aspect of beta-cell function, beta-cell mass may be a more important goal. Here, a targeted MRI agent would be required. Identification of a receptor or binding sites unique to beta-cells, such as the GLUT-2 transporter may allow for the development of a suitable targeting vector through phage display techniques [75, 119], or some other approach. By taking the relaxivity values obtained for the agent Gd-G80BP (Rg = 8.3 mM⁻¹ s⁻¹, Ra = 44.8 mM⁻¹ s⁻¹) [75] and assuming that a binding constant of 10⁻⁷ M could be achieved, the tissue contrast model [74] could then be applied to estimate the minimum amount of targeted agent that could potentially be detected by MRI. As pancreatic islets are highly vascularized, it is reasonable to assume that delivery of a targeted agent to beta-cell binding sites will not be a problem. Assuming good clearance of the agent from adjacent sites such that [M]b ≤ 10 µM then a lower detection limit of 3.4 µM for the Gd-G80BP described above is estimated by the TCM model. Pancreatic-islets are about 300 µm in diameter and beta-cells make up 60% or so of this volume. With a diameter of just 8 µm there are around 31000 beta-cells per islet. So if one further assumes that a typical voxel resolution of 1 mm³ can be achieved, one could expect to find about 71 islets or 2.23 × 10⁶ beta-cells per voxel. An estimated detection limit of 3.4 µM in the voxel would require 2 × 10¹² molecules of the contrast agent or 9.2 × 10⁵ binding sites per beta-cell. Although the number of GLUT-2 receptors per beta-cell is unknown and would likely vary with physiology, this estimate is at least within the upper concentration range for receptors in other cell types [76]. These detection limits could of course be improved considerably by using low molecular weight multimers of targeted Gd-agents (100-fold should be easily achievable) and by further increasing the relaxivity of the targeting moiety (~2-fold).

Acknowledgments

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Fig. (1).
Theoretical calculations of relaxivity as a function of the rotational correlation time ($\tau_R$) and the water residence lifetime ($\tau_M$). The upper chart shows the relaxivity obtained if the electronic relaxation time is optimal ($\tau_{S0} = 1$ ns), the lower chart when a shorter time is employed ($\tau_{S0} = 0.12$ ns).
Fig. (2). 
The effect of increasing the number of acetates in a complex is clearly seen from the temperature dependence of the $^{17}$O transverse relaxation rates of GdDOTA-4AmP$^{5-}$, GdDOTA-2AmP$^{3-}$ and GdDOTA-1AmP$^{2-}$. 

$\blacktriangleleft$ GdDOTA-4AmP$^{5-}$: $R^1 = R^2 = \text{CH}_2\text{CONHCH}_2\text{PO}_3^{2-}$ 

$\square$ GdDOTA-2AmP$^{3-}$: $R^1 = \text{CH}_2\text{CO}_2^-$ 
$R^2 = \text{CH}_2\text{CONHCH}_2\text{PO}_3^{2-}$ 

$\bullet$ GdDOTA-1AmP$^{2-}$: $R^1 = R^2 = \text{CH}_2\text{CO}_2^-$
Fig. (3).
The structures of the four stereoisomeric coordination geometries of LnDOTA$^-$ complexes and the processes by which they interconvert.
Fig. (4).
The Extended spectral width $^1$H NMR spectra of the two isomers $S(\text{RRRR})$ (a) and $S(\text{SSSS})$ (b) NO$_2$BnDOTMA that adopt SAP and TSAP coordination geometries, respectively. The greater chemical shifts observed in the SAP isomer is characteristic of the more compact structure.
Fig. (5).
Schematic representations of the SAP and TSAP isomers in profile. The effect of the smaller N-C-C-O torsion angle in pushing the acetate oxygens towards the water coordination site can easily be seen.
Fig. (6).
pH dependence of the relaxivity of GdDO3ALA in the presence of carbonate, showing how relaxivity may be quenched by formation of ternary complexes.
Fig. (7).
The crystal structure of YbDO3Ph$^{3+}$ with lactate chelated to the metal center.
Fig. (8). The cleavage of the galactose moiety in EGad allows water access to the gadolinium(III) ion.
The binding of calcium(II) by GdDOPTA results in a change of hydration state at the gadolinium(III) centers “switching on” the inner sphere relaxivity.
Fig. (10).
The binding modes of GdDTPA-BPYED with zinc(II) changes according the zinc concentration.
Fig. (11).
The relaxivity of GdDOTA4AmP$^{5-}$ shows a marked pH dependence.
Fig. (12).
A pH map of mice kidneys obtained using GdDOTA4 AmP$^{5-}$.
Fig. (13).
The lower detection limit of a contrast agent predicted from the tissue contrast model (TCM) [74]. The imaging field is 500 MHz and the signal to noise ratio has been fixed at 14. It has been assumed that the relaxivity of the agent in region B would remain at a constant 4.5 mM⁻¹s⁻¹. The detection limit is plotted as a function of the relaxivity of the agent in region A for a number of concentration values of the contrast agent in region B.
Fig. (14).
TAFI cleaves the lysine residues from GdDTPA-BIPHEN-TRIK unmasking the biphenyl group which is then able to bind to HSA enhancing relaxivity.
Fig. (15).
Plot of the fractional decrease in bulk water signal intensity expected for a PARACEST agent having a bound water lifetime ($\tau_M$) of 3 µs and a bound water ($^1$H) paramagnetic shift of 500 ppm (Eqn. 10).
Fig. (16).
CEST images of phantoms containing 10 mM EuDOTA-4AmBBA plus either 0, 5, 10 or 20 mM glucose, which was obtained by subtraction the image by saturating at 50 ppm from that at 30 ppm [101]
Fig. (17).
Mn$^{2+}$-enhanced T$_1$-weighted contrast of rat pancreatic islets. Two capillary tubes containing pancreatic-islets were incubated for 30 minutes in the presence of 25 mM MnCl$_2$. The tubes were incubated in 5 mM glucose (left) and 20 mM glucose (right). The image was acquired at 500 MHz with TR = 400 ms, TE = 7.2 ms, slice thickness = 100 mm, field-of-view = 4.8 mm × 2.4 mm, acquisition matrix = 128 × 64, and number of averages = 64. Reproduced with permission from reference [115].
Chart 1.
The structures of contrast agents currently approved for clinical use and MS-325, an agent under development.
Chart 2.  
The structures of some gadolinium chelates that exhibit more rapid water exchange rates.
Chart 3.
Table 1
The Water Exchange Rates, Relaxivities and Thermodynamic Stability Constants of the Four Longest Serving Contrast Agents

<table>
<thead>
<tr>
<th>Complex</th>
<th>τ_M^298/μs</th>
<th>R_1(a)/mM^−1s^−1</th>
<th>log K_GdL</th>
</tr>
</thead>
<tbody>
<tr>
<td>GdDTPA^2−</td>
<td>303(b)</td>
<td>4.3(c)</td>
<td>22.5(d)</td>
</tr>
<tr>
<td>GdDOTA^-</td>
<td>244(b)</td>
<td>4.2(e)</td>
<td>24.7(e)</td>
</tr>
<tr>
<td>GdHP-DO3A</td>
<td>-</td>
<td>3.7(f,g)</td>
<td>23.8(h)</td>
</tr>
<tr>
<td>GdDTPA-BMA</td>
<td>2222(b)</td>
<td>4.4(b)</td>
<td>16.9(i)</td>
</tr>
</tbody>
</table>

a) Measured at 20 MHz and 25 °C
b) reference [17]
c) reference [18]
d) reference [19]
e) reference [20]
f) Measured at 20 MHz and 40 °C
g) reference [21]
h) reference [22]
i) reference [23]
Table 2
The Effect of Increasing the Number of Acetates in a Complex Upon the Water Residence Lifetimes. The $\tau_{M298}$ Values were Determined by Fitting the Data Shown in Fig. 2 to Theory

<table>
<thead>
<tr>
<th>Complex</th>
<th># of Acetates</th>
<th>$\tau_{M298}/\mu$s</th>
</tr>
</thead>
<tbody>
<tr>
<td>GdDOTA-4AmP$^+$</td>
<td>0</td>
<td>26</td>
</tr>
<tr>
<td>GdDOTA-2AmP$^{1+}$</td>
<td>2</td>
<td>6.2</td>
</tr>
<tr>
<td>GdDOTA-1AmP$^{2+}$</td>
<td>3</td>
<td>1.3</td>
</tr>
<tr>
<td>GdDOTA$^-$</td>
<td>4</td>
<td>0.24</td>
</tr>
</tbody>
</table>
Table 3
The Water Residence Lifetimes and Stability Constants of a Number of Gadolinium Chelates that Exhibit Rapid Water Exchange Rates

<table>
<thead>
<tr>
<th>Complex</th>
<th>q</th>
<th>$\tau_{M}^{298/\text{ns}}$</th>
<th>log $K_{\text{Gd}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>GdDTTA-prop$^{2-}$</td>
<td>1</td>
<td>32.3(a)</td>
<td>16.7(b)</td>
</tr>
<tr>
<td>GdEPTPA$^{2-}$</td>
<td>1</td>
<td>3.0(a)</td>
<td>22.8(a)</td>
</tr>
<tr>
<td>Gd(NO$_2$BnEPTPA)$^{2-}$</td>
<td>1</td>
<td>6.7(a)</td>
<td>19.2(a)</td>
</tr>
<tr>
<td>GdTRITA$^{-}$</td>
<td>1</td>
<td>3.9(c)</td>
<td>19.2(d)</td>
</tr>
<tr>
<td>Gd(NO$_2$BnTRITA)$^{-}$</td>
<td>1</td>
<td>8.3(c)</td>
<td>-</td>
</tr>
<tr>
<td>GdDO3A</td>
<td>2</td>
<td>166(e)</td>
<td>21.1(f)</td>
</tr>
<tr>
<td>GdDO3MA</td>
<td>2</td>
<td>25.3(g)</td>
<td></td>
</tr>
<tr>
<td>GdPCTA</td>
<td>2</td>
<td>71(c)</td>
<td>21.0(h)</td>
</tr>
<tr>
<td>GdPCP2A</td>
<td>2</td>
<td>60(h)</td>
<td>23.4(h)</td>
</tr>
<tr>
<td>GdTREN-3, 2-Me-HOPO</td>
<td>2</td>
<td>-</td>
<td>20.3(i)</td>
</tr>
<tr>
<td>GdTREN-bis-(6-Me-HOPO)-TAM-PEG-2000</td>
<td>1</td>
<td>19(j)</td>
<td>-</td>
</tr>
<tr>
<td>GdTREN-bis-(6-Me-HOPO)-TAM-PEG-5000</td>
<td>1</td>
<td>31(j)</td>
<td>-</td>
</tr>
<tr>
<td>GdTREN-bis-(6-Me-HOPO)-TAM-TRI</td>
<td>2</td>
<td>19(k)</td>
<td>24.9(k)</td>
</tr>
</tbody>
</table>

a) reference [43]
b) reference [46]
c) reference [41]
d) reference [47]
e) reference [25]
f) reference [48]
g) reference [49]
h) reference [26]
i) reference [50]
j) reference [51]
k) reference [52]