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# A PARACEST MRI Contrast Agent To Detect Enzyme Activity

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Many recent reports have demonstrated the advantages of *para*magnetic *c*hemical *e*xchange saturation *t*ransfer (PARACEST) for providing molecular-scale information using magnetic resonance imaging (MRI).[1,2] Some types of PARACEST agents are considered to be "smart", because they alertly respond to changes in their molecular environments.[3] Examples include PARACEST agents that measure tissue pH,[4,5] temperature,[6] and concentrations of glucose,[7,8] lactate,[9] arginine,[10] or zinc.[11] However, the modest sensitivity of PARACEST agents, often requiring a minimum concentration of 1−10 mM for adequate detection, has limited the applicability of this approach to detect endogenous molecular targets that exist only at relatively high concentrations within tissues. Furthermore, intermolecular associations may lack high specificity, which may lead to problems with interpretation of MR images.

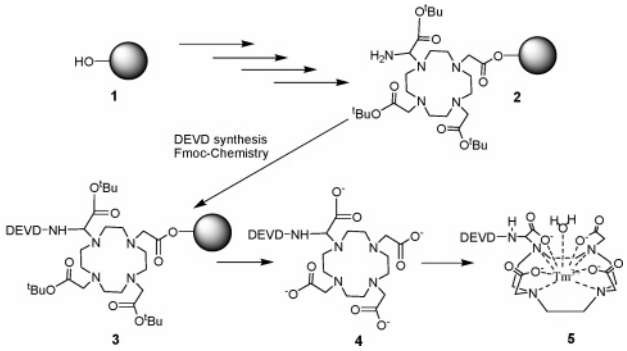
To address these limitations, we hypothesized that enzymatic catalysis may be exploited to change the chemical structure of a high concentration of PARACEST agents and cause a detectable change in the PARACEST effect. Chemical changes caused by enzymatic catalysis are often highly specific, and rapid catalysis may allow for indirect detection of a relatively low concentration of the enzyme. Enzymatic conversion of an amide to an amine may change PARACEST by accelerating the chemical exchange rate between this amide/amine group and water from ∼300 to ∼3000 $s^{-1}$.[2,12] Also, the MR chemical shift frequency of the amide and amine will be significantly different, especially if these functional groups are proximal to a paramagnetic lanthanide ion. This chemical shift change may be especially advantageous for detection, because MR methods are very sensitive to changes in MR frequencies.

To demonstrate our approach, we have designed a "smart" PARACEST MRI contrast agent that can detect an active caspase-3 enzyme. This enzyme is known as an "executioner" in the metabolic death cascade during cell apoptosis, and therefore serves as a critical early biomarker for evaluating apoptosis-promoting antitumor therapies.[13] Among the identified substrates of caspase-3, DEVD (Asp-Glu-Val-Asp) is efficiently and selectively cleaved by caspase-3 and has been incorporated in fluorescence dyes, such as DEVD-AMC, for detecting caspase-3 activity.[14,15] Our PARACEST MRI contrast agent uses a similar motif by replacing AMC with 1,4,7,-10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA).

To synthesize DEVD-DOTA amide **4**, a polymer support preloaded with a DOTA derivative **2** was developed by surface modification of Wang resin **1** (Scheme 1). Standard Fmoc solid-phase peptide synthesis methods were then used to "grow" the DEVD peptide chain onto the amino group of **2**. Following the synthesis, the acquired compound **4** was characterized with MALDI-MS (*m*/*z* 885.80 [M + H]). Thullium was chelated with **4** to prepare the final compound **5** (*m*/*z* 1088.74 $[M + Na]^+$).

To show the practical application of **5** to detect the activity of caspase-3, the PARACEST spectrum of **5** was recorded by applying selective saturation in 1 ppm increments from +100 to −100 ppm (Figure 1A). A PARACEST effect was detected at −51 ppm, which

***Scheme 1.*** Synthesis of DEVD-(Tm-DOTA) Using Fmoc Chemistry




was assigned to the amide most proximal to the lanthanide ion in **5** on the basis of identical results obtained from a similar compound, $Tm^{3+}$-DOTAMGly.[4] After 48 nM of caspase-3 was added and the mixture was incubated at 37 °C and pH 7.4 for 1 h, the PARACEST effect at −51 ppm was dramatically decreased, and an asymmetrical shape in the PARACEST spectrum was observed near water (0 ppm). This asymmetry was analyzed by spectral deconvolution to show a PARACEST effect at +8 ppm. Considering that the PARACEST spectrum of **2** also shows an identical PARACEST

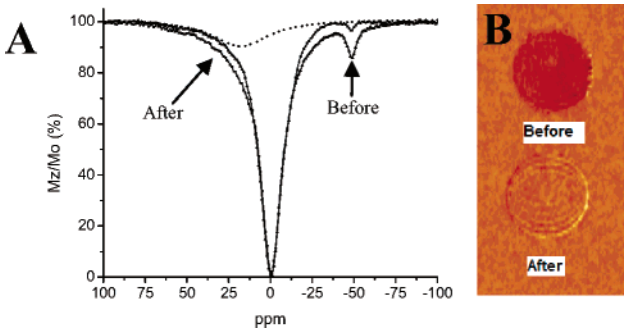



***Figure 1.*** PARACEST spectra (A) and MR parametric map (B) of DEVD-(Tm-DOTA) amide before and after addition of caspase-3. PARACEST spectra were acquired using a Varian Inova 600 MHz NMR spectrometer with a concentration of 25 mM at 37 °C and pH 7.4, with a continuous-wave saturation pulse applied at 31 $\mu$T for 4 s. The deconvoluted PARACEST spectrum of the product after reaction, showing a PARACEST effect at +8 ppm, is also shown. MR images were acquired at 37 °C and pH 7.4, with a Bruker Biopsin 9.4 T MR scanner. A MSME $T_1$ method was used with $T_R/T_E$ = 1623/10.9 ms and a train of Gaussian-shaped saturation pulses applied at 50 $\mu$T for 1.106 s, and with saturation offsets at −51 and +51 ppm. The parametric map was obtained by subtracting the MR image with a saturation offset at −51 ppm from the MR image with saturation offset at +51 ppm. This parametric map shows a 14.5% decrease in water MR signal before the enzymatic reaction, and no significant change in water MR signal after reaction. The magnitude of the scale of the original MR images was used as the scale for this parametric map to properly represent the practical difference in MRI contrast obtained with different saturation offsets.



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peak at +8 ppm, this effect further confirms that caspase-3 has converted the DOTA-amide of **5** to the DOTA-amine of **2**.

To further demonstrate the utility of **5**, a MR image with selective saturation at −51 ppm was acquired with **5** before and after reaction with caspase-3 (Figure 1B). A MR image with selective saturation at +51 ppm was also acquired as a control to account for direct saturation of water. The difference between these images showed a 14.5% decrease in water MR signal before the enzymatic reaction due to the PARACEST effect, and no significant change in water MR signal after reaction.

To determine the sensitivity of detecting **5** under physiological conditions, the PARACEST effect of the agent was correlated with concentrations using modified Bloch equations.[2] After validating a linear relationship between concentration and $T_1$ relaxation under selective saturation conditions and confirming that the selective saturation pulse was sufficiently long to achieve steady-state conditions, we further modified this theory to obtain a linear relationship that correlates concentration to the PARACEST effect (see Supporting Information). These results indicate that 5.2 mM of the agent can be detected by saturating the amide MR frequency to generate a 5% change in water MR signal. Therefore, **5** may be used to indirectly detect caspase-3 enzyme concentrations as low as 5−50 nM, although the minimum detection threshold will depend on the enzyme's catalytic efficiency, other environmental conditions, and the performance of the MR scanner.[16]

As an example of the effect of environmental conditions, temperature can influence chemical exchange rates and therefore can modulate the PARACEST effect.[12] However, changes in temperature caused only a moderate change to the PARACEST effect of **5** when the temperature was changed from 25 to 45 °C (Supporting Information). This behavior can be explained by a complicated effect of temperature on water $T_1$ and $\Delta\omega$, in addition to the proton chemical exchange rate. More importantly, the effect of temperature is negligible throughout the relatively invariant range of temperatures under physiological conditions.

pH can also influence the PARACEST effect, as proton chemical exchange between water and amides is catalyzed by hydroxide ions.[13] The amide proton showed increasingly greater PARACEST with increasing pH, reaching the greatest effect near pH 8 (Supporting Information). The proton chemical exchange rate between an amide and water is relatively slow on the MR time scale, which is characterized by the chemical shift difference between the amide and water. Therefore, an increasing hydroxide ion concentration accelerates this rate to improve the PARACEST effect. pH also influenced the PARACEST effect of the amine, which further confirmed that these two PARACEST effects do not arise from metal-bound water, which does not exhibit a pH-dependent PARACEST effect.[4]

This work is inspired by the development of relaxivity-based MRI contrast agents that undergo a permanent structural change through enzymatic catalysis that causes a change in contrast within relaxation-weighted MR images.[17,18] The absolute sensitivity of relaxivity-based MR agents has been shown to be 1−2 orders of magnitude better than the sensitivities of PARACEST agents.[2,16] However, the ability to selectively detect PARACEST agents may provide additional advantages. For example, an enzymatically inert PARACEST agent with a unique saturation frequency may be directly linked to **5** to account for variances in concentration. This advantage is critical for validating caspase-3 activity detection during in vivo biomedical applications.

To summarize, DEVD-(Tm-DOTA) amide **5** shows PARACEST with good sensitivity at physiological pH and temperature, indicating that this MRI contrast agent may be used for in vivo molecular imaging. The detection of catalytic activity of caspase-3, rather than the presence of caspase-3, has many important advantages for molecular imaging. A relatively low concentration of enzymes with rapid catalytic activities can quickly convert a high concentration of MRI contrast agents for detection using PARACEST MR methods. Similar to many other important enzyme biomarkers, caspase-3 is constitutively expressed as an inactive proenzyme, so that detecting enzyme activity avoids detection of the inactive form. Specificities for different substrates are relatively good for different members of the caspase enzyme family, so that detecting enzyme activity can exploit substrate specificity. Finally, a variety of important enzyme biomarkers can catalyze the conversion of amines, amides, and other functional groups that exchange protons with water. Therefore, this concept of a "smart" PARACEST MRI contrast agent that detects enzyme activity constitutes a fundamentally new type of molecular imaging agent that may have broad applicability for assessing enzyme biomarkers in many biological processes and disease pathologies.

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**Supporting Information Available:** Detailed experimental procedures and complete ref 14. This material is available free of charge via the Internet at http://pubs.acs.org.

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