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A CatalyCEST MRI Contrast Agent That Detects the Enzyme-Catalyzed Creation of a Covalent Bond

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Supporting Information

ABSTRACT: CatalyCEST MRI can detect enzyme activity by employing contrast agents that are detected through chemical exchange saturation transfer (CEST). A CEST agent, Tm-DO3A-cadaverine, has been designed to detect the catalytic activity of transglutaminase (TGase), which creates a covalent bond between the agent and the side chain of a glutamine amino acid residue. CEST appeared at −9.2 ppm after TGase conjugated Tm-DO3A-cadaverine to albumin, which also caused a decrease in CEST from albumin at +4.6 ppm. Studies with model peptides revealed similar appearances and decreases in detectable CEST effects following TGase-catalyzed conjugation of the contrast agent and peptide. The MR frequencies and amplitudes of these CEST effects were dependent on the peptide sequence, which demonstrated the sensitivity of CEST agents to ligand conformations that may be exploited to create more responsive molecular imaging agents. The chemical exchange rates of the substrates and conjugated products were measured by fitting modified Bloch equations to CEST spectra, which demonstrated that changes in exchange rates can also be used to detect the formation of a covalent bond by catalyCEST MRI.

Chemical exchange saturation transfer (CEST) is a MRI contrast mechanism that reduces the detectable water signal after selective saturation of an exchangeable proton of a MRI contrast agent (Figure 1A).[1] CEST agents have been designed that detect the catalytic activity of enzymes, termed "catalyCEST MRI", including CEST agents that are catalyzed by protease,[2−4] galactosidase,[5,6] esterase,[7] and deaminase[8] enzymes. In these examples, the enzyme-catalyzed cleavage of a covalent bond within the agent caused a change in CEST produced by the agent. CEST agents that can only be cleaved by enzymes limits catalyCEST to the detection of a subset of enzyme activities. CEST agents that create a covalent bond through enzyme catalysis are needed to expand this technology.

We have designed a CEST agent to detect transglutaminase (TGase) catalysis. The entire family of TGases catalyzes the formation of an amide bond between the side chains of lysine and glutamine amino acids, which cross-links the extracellular matrix in tissues, especially in tumors.[9] Previous reports have shown that a fluorescent imaging agent with a generic aliphatic amine ligand can be conjugated to a glutamine side chain via TGase activity.[10] We hypothesized that replacing a fluorescent

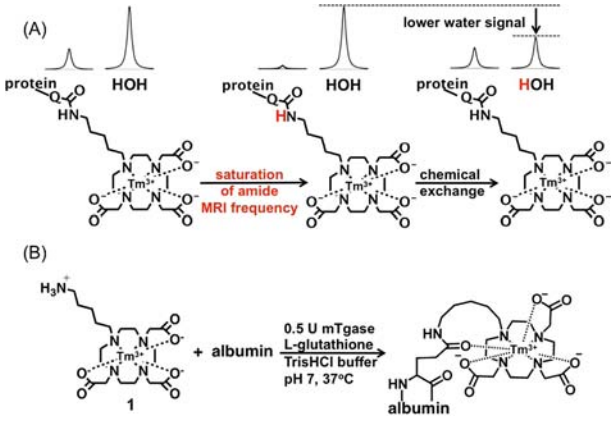

Figure 1. CatalyCEST MRI. (A) MR saturation of the amide proton of {Tm-DO3A-cadaverine}-protein (shown in red), followed by chemical exchange with water, transfers the MR saturation to water. (B) Conjugating the CEST agent to a protein's glutamine side chain by TGase converts an amine to an amide that generates CEST.

agent with Tm-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid (Tm-DO3A) would create a paramagnetic CEST agent that can be similarly conjugated to glutamine via TGase (Figure 1B). Tm-DO3A-cadaverine **1** was synthesized by alkylating DO3A with a cadaverine derivative and using the product to chelate Tm(III) (Schemes S1 and S2).

To show the practical application of **1** to detect the catalytic activity of TGase, 25 mM **1** was added to 0.75 mM bovine serum albumin and 10 mM glutathione in Tris-HCl buffer at pH 7 (glutathione was included to maintain a reducing environment that is required for TGase activity). A CEST spectrum was recorded using a 600 MHz NMR spectrometer by applying selective radio frequency saturation from 30 to −30 ppm in 1 ppm increments (Figure 2A). A function of Lorentzian line shapes was fit to the CEST spectrum to accurately measure the chemical shifts that generated maximum CEST (Figure 2B).[11] A CEST effect was detected at +4.6 ppm, which was assigned to the diamagnetic amide and amine protons of albumin. After this sample was incubated with 0.5 unit (0.327 μM) of microbial TGase from *S. mobaraensis* at 37 °C for 24 h, the CEST effect at +4.6 ppm dramatically decreased, and a new CEST effect appeared at −9.2 ppm. This reaction was repeated three times to ensure that the appearance

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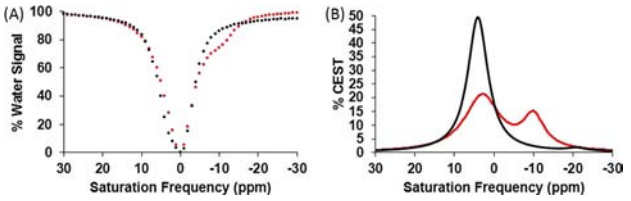

Figure 2. CEST before and after TGase catalysis. (A) The CEST spectrum and (B) Lorentzian line shape spectrum of a mixture of albumin, Tm-DO3A-cadaverine and L-glutathione before (black) and after (red) incubation with TGase showed a decrease in CEST at +4.6 ppm and the appearance of CEST at −9.2 ppm following the creation of a covalent bond by TGase catalysis.

of the CEST effect at −9.2 ppm was reproducible. This change was attributed to the conjugation of **1** to the glutamine side chains of albumin, causing a decrease in diamagnetic amines and an increase in paramagnetic amides within the sample. More specifically, the newly formed amide was expected to form a dative bond with the Tm(III) ion, causing the amide proton to experience a hyperfine contact shift from the paramagnetic Tm(III) ion and resonate at a unique value of −9.2 ppm, as shown in similar reports of CEST with Tm(III) chelates.[5,12]

The CEST spectrum of **1** alone showed a weak CEST effect at −5.2 ppm (Figure 3). This paramagnetic CEST effect was

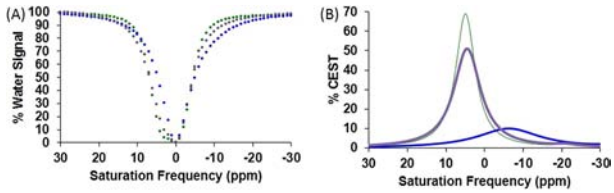

Figure 3. CEST of individual reactants. (A) The CEST spectrum and (B) Lorentzian line shape spectrum of an individual sample of albumin showed CEST at +5.6 ppm (purple circles and purple line), L-glutathione showed CEST at +5.4 ppm (green circles and green line), and Tm-DO3A-cadaverine showed CEST at −5.2 ppm (blue circles and blue line).

attributed to a non-covalent supra-molecular adduct between the aliphatic amines and the carboxylate groups of DO3A. Similar non-covalent interactions have been observed with lanthanide chelates.[13] This observation demonstrated the sensitivity of CEST agents to ligand interactions that may be further exploited to create responsive molecular imaging agents. As expected, the CEST spectra of albumin or glutathione alone showed a CEST effect at +4.6 ppm.[14,15] These control samples were only analyzed once because no reaction was involved.

To further validate the creation of a covalent bond between **1** and a side chain of glutamine, CEST spectra were recorded before and after the addition of 0.5 unit of TGase to a solution of 25 mM **1**, 25 mM GQR peptide, and 10 mM glutathione in the same buffer (Figure 4A). Before addition of TGase, a weak CEST effect was detected at −9.0 ppm from the supra-molecular adduct of **1**. After addition of TGase, the CEST effect shifted to −10.8 ppm and dramatically increased, and a new CEST effect was observed at +5.8 ppm. Similar results were obtained before and after conjugating **1** with a QR peptide using TGase (Figure 4B). These reactions with the two peptides were reapeated three times to ensure that the changes in CEST effects were reproducible. As with the reaction of **1**

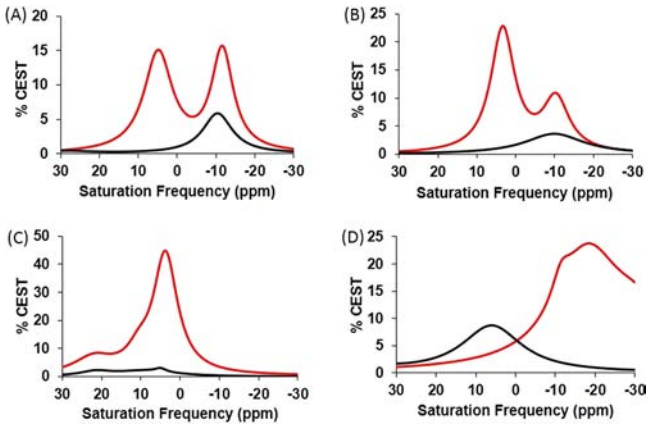

Figure 4. Lorentzian line shape spectra of peptides and Tm-DO3A-cadaverine. (A) GQR, (B) QR, (C) ZQG, and (D) Boc-Gln-OH before (black) and after TGase catalysis (red).

and albumin, the CEST effect at −10.8 ppm was assigned to the paramagnetic amide created by TGase activity. The appearance of CEST at +5.8 ppm after conjugation to GQR or QR was attributed to a change in the chemical exchange rate of the QR amide proton or the guanidinium group of arginine after conjugation to **1**. CEST at +5.8 ppm was unexpected, and further demonstrated the sensitivity of CEST agents to ligand conformations that cause changes in chemical exchange rates, which may be exploited to create additional responsive molecular imaging agents.

We investigated the TGase-catalyzed creation of amide bonds between **1** and other peptides. The conjugation of **1** and CBZ-protected QG generated a three CEST effects at +4.6, +9.8, and +22.5 ppm (Figure 4C). These positive CEST effects were attributed to heterogeneous ligand conformations of the hydrophobic CBZ-QG peptide. The conjugation of **1** and Boc-protected glutamine converted a CEST effect at +7.2 ppm to a broad CEST effect between −10 and −20 ppm (Figure 4D). This result was attributed to the hydrophobicity of Boc-Gln-OH, which may cause conformational heterogeneity that would create a broad CEST effect. These studies were performed once for the ZQG system and twice for Boc-Gln-OH. These results demonstrate that a full CEST spectrum should be acquired during in vivo studies to ensure the detection of CEST effects that arise from **1** conjugated to hydrophilic or hydrophobic proteins.

A responsive CEST effect can also arise from changes in chemical exchange rates. The Bloch-McConnell equations modified for chemical exchange were fit to CEST spectra to measure chemical exchange rates (Table S1).[16,17] The average chemical exchange rate of **1** that generated weak CEST at −12.7 ppm was determined to be 2533 Hz. This rate is slower than expected for an amine group at 37 °C and pH 7.0, which suggested that the aliphatic amine had an intra- or intermolecular interaction that reduced the exchange rate.[18] After **1** was conjugated to albumin, the strong CEST at −11.6 ppm had an average chemical exchange rate of 1,260 Hz. This rate is much faster than expected for a diamagnetic amide group at 37 °C and pH 7.0, which suggested that the amide of the product was proximal to the lanthanide ion.[14] Furthermore, this measured change in exchange rate from 2533 Hz before TGase catalysis to 1,260 Hz after TGase catalysis provided an additional method for detecting the creation of a covalent bond by TGase enzyme activity.

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The chemical exchange rates of **1** conjugated to CBZ-protected QG were determined to be 3323 Hz at 22.5 ppm and 6739 Hz at 9.8 ppm. These chemical exchange rates are faster than expected for diamagnetic amide and carbamate groups, which suggests that the amide or carbamate protons in this product are proximal to the lanthanide ion.

Detecting TGase catalysis in biological systems has been accomplished by using a T1 relaxation agent attached to a short peptide.[19,20] The glutamine side chain of the peptide was designed to link to lysine in the tumor ECM, which caused a change in T1 relaxation in the tumor. However, the surrounding tissue also experienced a change in T1, suggesting that non-covalent retention may compromise the specificity of the results. Our approach avoids this issue because CEST from non-covalent retention of **1** with proteins is absent at approximately −10 ppm and appears only after TGase creates a covalent bond between **1** and the glutamines in a protein.

Exogenous CEST agents have been detected during in vivo studies.[21−23] However, rapid pharmacokinetic washout of the agent can complicate in vivo detection of CEST MRI. In addition, rapid pharmacokinetic washout can also limit the development of in vivo CEST MRI methods with exogenous contrast agents, because the concentration of the exogenous agent in the in vivo tissue may continuously change while optimizing CEST MRI methods. The eventual use of **1** during in vivo studies has the potential advantage of obviating the need to account for pharmacokinetic washout from the tissue when the agent is covalently bound to the extracellular matrix by TGase catalysis. In addition, the covalent attachment of **1** to the extracellular matrix may facilitate the accumulation of sufficient concentration of the agent for detection with CEST MRI. However, the extracellular matrix within tissues generates endogenous magnetization transfer effects that compete with CEST, which will require high accumulation of the agent within in vivo tissues to generate sufficient detection with CEST MRI.[24]

In summary, this report demonstrates that a catalyCEST MRI contrast agent can detect the creation of a covalent bond by enzyme catalysis. Changes in chemical exchange rate measurements can also provide evidence for enzyme catalysis. Ligand conformations can have a profound effect on CEST.

■ ASSOCIATED CONTENT

Supporting Information

Synthesis methods, characterization of synthesis products, CEST MR methods, CEST spectra, and the fittings of Lorentzian line shapes and Bloch equations to CEST spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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