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Detection of Alkaline Phosphatase Enzyme Activity with a Cataly-CEST MRI Biosensor

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ABSTRACT: Responsive CEST MRI biosensors offer good sensitivity and excellent specificity for detection of biomarkers with great potential for clinical translation. We report the application of fosfosal, a phosphorylated form of salicylic acid, for the detection of alkaline phosphatase (AP) enzyme. We detected conversion of fosfosal to salicylic acid in the presence of the enzyme by CEST MRI. Importantly the technique was able to detect AP enzyme expressed in cells in the presence of other cell components, which improves specificity. Various isoforms of the enzyme showed different Michaelis-Menten kinetics and yet these kinetics studies indicated very efficient catalytic rates. Our results with the fosfosal biosensor encourage further *in vivo* studies.

Keywords: CEST MRI, CATALYCEST, Alkaline Phosphatase, Molecular Imaging, Michaelis-Menten Kinetics

Alkaline phosphatases (AP) are a large family of enzymes that remove the phosphate group from various types of biomolecules including proteins, carbohydrates, and nucleic acids. This class of enzymes shows optimized catalytic efficiency in basic conditions. The enzyme is routinely used for biological applications such as antibody conjugates and gene expression. Furthermore, unregulated activities of subtypes of the enzyme have been reported in various disease states including osteogenic sarcoma, primary sclerosing cholangitis, and metabolic syndrome.¹

Chemical exchange saturation transfer (CEST) MRI is an imaging method that can detect biomarkers with good specificity while retaining the excellent spatial resolution and depth of view offered by MRI.² In this technique, selective radio frequency saturation pulses are applied at the MR frequency of a proton on the agent, which creates a pool of saturated protons. The exchange of saturated protons between the contrast agent and water molecules surrounding the contrast agent can transfer saturation to the water molecules, which results in the loss of a portion of MR signals from the bulk water (Figure 1). This loss of the bulk water signal caused by CEST is typically demonstrated by plotting the MR signal amplitude of bulk water versus a range of saturation frequencies, known as a CEST spectrum (Figure 2).³

Diamagnetic contrast agents are promising biosensors for the detection of biological events with CEST MRI as shown by many recent examples.⁴ A large chemical shift facilitates

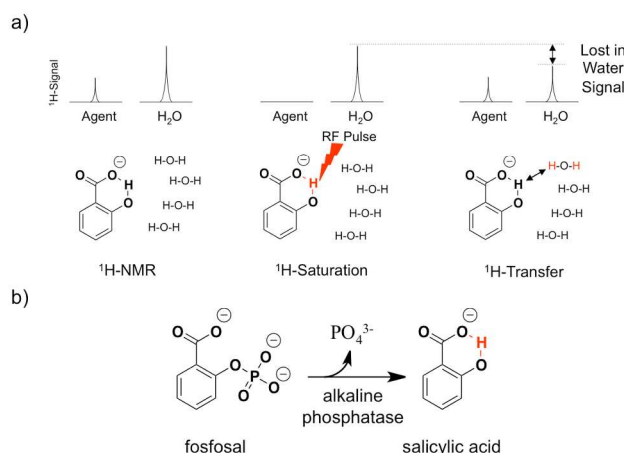


Figure 1. (a) The CEST mechanism (b) Alkaline phosphatase hydrolyzes the phosphoester bond of fosfosal to release the phosphate group and salicylic acid. A hydroxyl proton (in red) of the salicylic acid can generate a CEST signal.

the selective detection of CEST biosensors relative to detecting CEST signals from endogenous biomolecules. Salicylic acid (SA) and its derivatives are a promising class of diamagnetic CEST biosensors that have a hydroxyl proton “trapped” by hydrogen bonding in a 6-member ring state, which allows SA to produce a detectable CEST signal relative to image noise (Figure 1b).⁵ The ring current places the hydroxyl proton in a deshielding region that induces a large chemical shift of 7–12 ppm (relative to water that is defined to be 0 ppm during MRI studies). SA and its derivatives have a good toxicity pro-

file even in concentrations as high as 500 mM, which makes these biosensors to be superb candidates for clinical translation.⁶ Cells tolerate relatively high doses of SA, which increase the level of sensitivity of the technique.

CatalyCEST MRI is a unique variation of CEST MRI methodology that detects an appearance or disappearance of a CEST signal after an enzyme catalyzes a change in the chemical structure of a CEST biosensor. One or more functional groups, such as amines, amides, and/or hydroxyl groups, are often incorporated into CEST biosensors, which can be chemically modified via enzyme catalysis.⁷ Novel catalyCEST biosensors based on SA derivatives have been reported recently that detect sulfatase, esterase, and cathepsin B enzymes.⁸

We sought to detect AP activity using catalyCEST MRI with a biosensor that is potentially translatable to clinical applications. Previous reports showed that nitrophenyl phosphate and other phosphorylated phenyl-based compounds are fluorogenic or bioluminogenic substrates for AP enzymes.⁹ Our search for a SA based compound with a phosphate group suggested fosfosal as a new catalyCEST biosensor for detecting AP enzymes. Fosfosal was introduced in the early 1980s as an analgesic that showed improved pharmacokinetic properties and biocompatibility at 1500 mg/kg (approximately 6.8 mM *in vivo*), suggesting potential for eventual clinical translation.¹⁰ We hypothesized that a CEST signal would not be observed from fosfosal as there is no exchangeable proton on this molecule that has an appropriate chemical exchange rate to generate a strong CEST signal. In contrast, we hypothesized that the product of the reaction of fosfosal and AP enzyme should produce a CEST signal with a chemical shift of approximately 9 ppm as previously reported for SA (Figure 1b).

To test our hypothesis, calf intestinal alkaline phosphatase (CIAP), an AP enzyme commonly used in research studies,¹ was mixed with a sample of fosfosal at a concentration of 20 mM in AP buffer solution (Figure 2; this concentration was selected to ensure strong CEST signals during initial studies). A sample containing the same concentration of fosfosal without the enzyme, and a sample of 20 mM SA were prepared as controls. After adjusting the pH to reach a value of 7.4, the samples were incubated at 37°C for 30 min and then were analyzed with catalyCEST MRI. The sample containing the CIAP enzyme generated a strong 8.7% CEST signal at 8.75 ppm, which closely matched the previously reported CEST signal amplitude of SA. In contrast, the fosfosal sample without the enzyme showed very low CEST signal of 1.7%, which was considered to be negligible relative to typical contrast-to-noise generated during CEST MRI studies. This result confirmed that catalyCEST MRI can detect AP enzyme activity using fosfosal.

We performed UV-Vis spectroscopy studies to confirm that the AP enzyme caused the conversion of fosfosal to SA (Figure 3). UV-Vis absorption spectra of SA and fosfosal showed a peak at 296 and 270 nm, respectively (Figure 3a). Therefore the appearance of a peak at 296 nm can be used to indicate the conversion of fosfosal to SA. Monitoring changes in absorbance at 296 nm in a solution of fosfosal and CIAP clearly showed an increase in SA concentration over time (Figure 3b). Similar results were obtained with human liver alkaline phosphatase (HLAP; Figure S1-a). To confirm that the conversion was solely due to the presence of the enzyme, UV-Vis spectra of a fosfosal solution without AP enzyme was investigated under the same conditions (Figure S1-b). No changes in the absorbance at 296 nm over time confirmed that

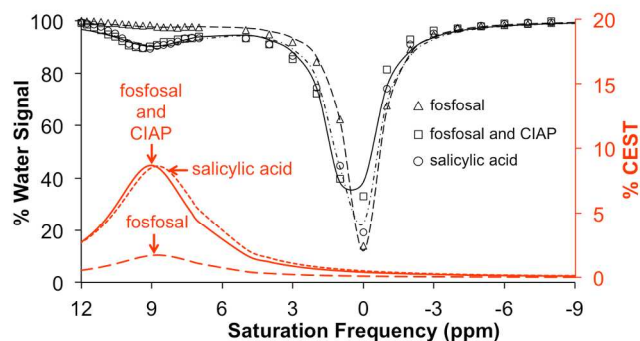


Figure 2. CEST spectra of fosfosal before and after catalysis with calf intestinal alkaline phosphatase (CIAP). Spectra are shown for samples of 20 mM salicylic acid (circle, short dashed red line), 20 mM of fosfosal incubated with CIAP (square, solid red line), and 20 mM of fosfosal with no enzyme (triangle, long dashed red line) for 30 min at 37 °C, pH = 7.4 ± 0.05, saturation power = 3.5 μ T, and saturation time = 4 s.

fosfosal was converted to SA only in the presence of the enzyme. Therefore, the UV-Vis experiment confirmed that the signal observed in the CEST MRI experiments originated from the conversion of fosfosal to SA in the presence of AP.

The saturation power and saturation time were adjusted to optimize CEST signal amplitude (Figure S2).¹¹ The CEST signal amplitude increased non-linearly with increasing saturation power or time, for concentrations of the biosensor ranging from 2.5 mM to 30 mM. However, high saturation powers may result in sample heating, and long saturation times can slow throughput in radiological clinics, each of which can limit translation of CEST MRI to clinical applications. Therefore, we performed all subsequent catalyCEST MRI studies with a 6 μ T saturation power and a 4 s saturation time that still generated strong CEST signals. We evaluated the effect of pH on the CEST signal from SA because a correlation between pH and CEST signal has been reported.¹² The CEST signal decreased significantly when the pH was lower than 6.6, and were relatively invariant between pH 6.6 and 7.96 (Figure S3). Thus the CEST signal can be detected from this biosensor within a physiologically suitable pH-range.

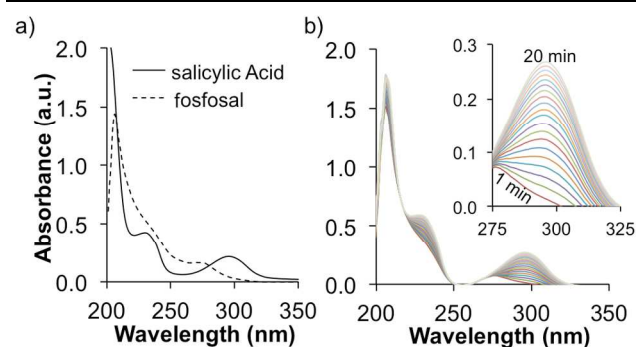


Figure 3. UV-Vis absorption studies. (a) The UV-Vis absorption spectra of 100 μ M salicylic acid or fosfosal in AP buffer at pH 7.40 ± 0.05. (b) The progress of the enzyme reaction with 0.05 Units of calf intestinal alkaline phosphatase.

We compared the kinetics of converting fosfosal to SA with CIAP and HLAP to evaluate the utility of catalyCEST MRI for detecting AP isoforms (Figure 4). These studies were performed with 0.5-1 Units of the enzymes and ≥ 2.5 mM of biosensor substrate to ensure pseudo-first-order kinetics at the

start of the reaction. The kinetics were performed for 100 minutes, considering that MRI is a slow measurement technique relative to other methods such as UV-Vis spectroscopy (Figure 4a, 4b). The CEST signal amplitude of the SA product was converted to concentration using a CEST-concentration calibration obtained under identical experimental conditions (Figure 4c). The linear regions of each concentration-time plot (Figure 4d, 4e) were used to calculate the initial velocities, which were the first 23 and 51 minutes of the reactions with CIAP and HLAP, respectively, based on analyses of R^2 values for all possible durations for each reaction. As seen in figures 4a-e, the error in determining the substrate concentration at each time point is non-negligible. This differs from typical Michaelis-Menten (MM) kinetics analyses where the substrate concentration can be determined with high accuracy and precision. Therefore, we used a Hanes-Woolf (HW) plot to determine MM kinetics parameters (Figure 4f, 4g), which cancels the error in the concentration of the substrate when determining the slope, leading to improved measurements of the maximum velocity, V_{max} , which in turn improved the determination of the catalytic rate, k_{cat} . The results showed that k_{cat} was 2.7-fold faster for CIAP than for HLAP (Table 1). The Michaelis constant, K_m , which approximates the dissociation constant, was 3.7-fold lower for CIAP relative to HLAP, demonstrating that the biosensor bound with more affinity to CIAP. The catalytic efficiency, k_{cat}/K_m , was an order of magnitude higher for CIAP than HLAP due to the superior binding and rate of catalysis. Yet both enzymes had extremely efficient catalysis for the biosensor substrate, which facilitates the detection of the activity of AP isoforms with catalyCEST MRI. This kinetic efficiency has advantages for future in vivo studies, because in vivo catalyCEST MRI depends on rapid conversion of a high concentration of biosensor concentration for adequate detection. Furthermore, this biosensor should be considered to be a non-specific substrate for detecting all alkaline phosphatases.

We performed studies with cell lysate to demonstrate that catalyCEST MRI with fosfosol can detect biologically relevant levels of AP enzymes.¹³ Confluent HEK293T cells were lysed

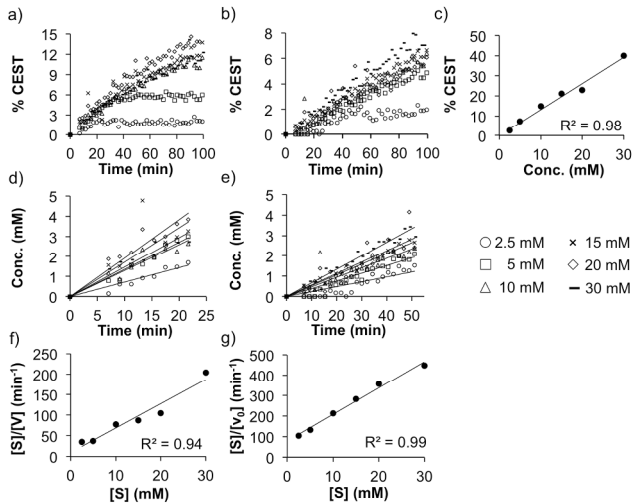


Figure 4. Michaelis-Menten kinetics of fosfosol. Fosfosol incubated with (a) calf intestine alkaline phosphatase (CIAP) and (b) human liver alkaline phosphatase (HLAP). (c) A CEST-concentration calibration for salicylic acid. Calibrated concentration-time graphs for (d) CIAP and (e) HLAP. Hanes-Woolf plots of (f) CIAP and (g) HLAP.

Table 1. Kinetics parameters for calf intestinal alkaline phosphatase and human liver alkaline phosphate enzymes at 37°C.

Enzyme	CIAP	HLAP
K_m (M)	1.63×10^{-3}	6.07×10^{-3}
V_{max} (M.s ⁻¹)	2.83×10^{-3}	1.29×10^{-3}
k_{cat} (s ⁻¹)	2.12×10^6	7.74×10^5
k_{cat}/K_m (M ⁻¹ .s ⁻¹)	1.30×10^9	1.28×10^8

by mammalian protein extraction reagent (mPER) lysis solution. This lysate was mixed with fosfosol solution and incubated for 3 h at 25°C. CEST MRI showed that the mixture produced a CEST signal at 8.75 ppm, indicating the appearance of SA (Figure 5). To confirm that the signal was produced by the reaction between AP enzyme and fosfosol, a solution containing a similar concentration of fosfosol but with no lysate and a solution containing fosfosol, lysate and a cocktail containing AP enzyme inhibitors were studied with catalyCEST MRI. Both of these additional samples showed the same negligible CEST signal amplitude, which confirmed that the observed CEST signal from fosfosol in lysate was produced by conversion of fosfosol to SA. This showed that fosfosol could be used to detect biologically relevant concentrations of AP enzymes in vitro. In particular, intracellular AP enzymes are only expected to be detected in necrotic tissues, because necrosis causes cells to release their intracellular components. Thus catalyCEST MRI with fosfosol may be useful for detecting necrotic tumor tissues after initiating cancer treatment, and before macroscopic changes in tumor size can be observed.

In conclusion, fosfosol can be used as a biosensor for the detection of AP enzymes with excellent specificity and in the presence of other biomolecules. Our results clearly showed that catalyCEST MRI can detect the activity of various isoforms of AP enzymes with fosfosol. High catalytic efficiency of the isoforms of AP enzymes for fosfosol facilitated the catalyCEST MRI detection of AP activity. Moreover, we were able to show that biologically relevant concentrations of AP enzyme can be detected by our method. In addition to biologically relevant experimental conditions, catalyCEST MRI parameters that were used in this experiment were within the range acceptable for clinical translation, which warrants future in vivo studies.

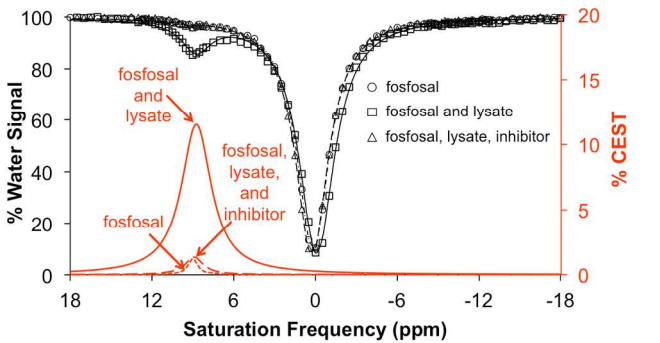


Figure 5. CEST spectra of fosfosol before and after catalysis with cell lysate. Spectra are shown for 27 mM fosfosol (triangle and short dashed red line), 27 mM fosfosol with lysate (square and solid red line), and 27 mM fosfosol with lysate pre-treated with the inhibitor cocktails (circle and long dashed red line) with pH of 7.40 ± 0.05 were incubated with lysate solution for 3 h at 25°C.

ASSOCIATED CONTENT

Supporting Information

Supporting Information is available free of charge on the ACS publications website.

Supporting Information_Alkaline Phosphatase (PDF). CEST and pH calibration graphs, UV-VIS spectra, detailed procedures of experiments and the CEST protocol.

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Author Contributions

ID and MDP designed the experiments; ID and MMG performed the studies; ID and KMJ analyzed the results; ID developed the manuscript and all co-authors approved the final version.

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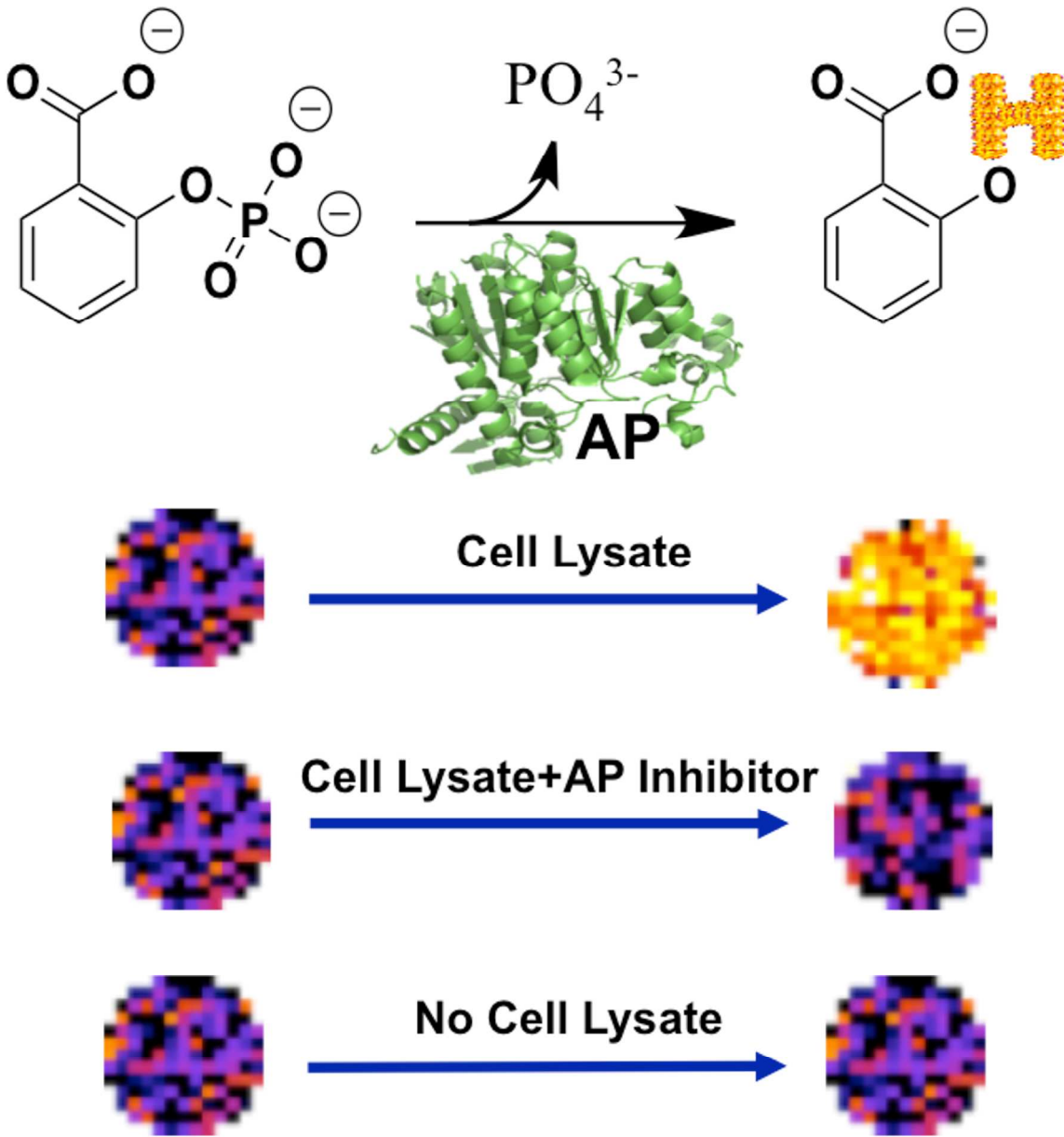
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ABBREVIATIONS

CEST, Chemical Exchange Saturation Transfer; MRI, Magnetic Resonance Imaging; AP, Alkaline Phosphatase; CIAP, Calf Intestinal Alkaline Phosphatase; HLAP, Human Liver Alkaline Phosphatase; SA, Salicylic Acid; MM, Michaelis-Menten

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Graphical Abstract