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A Hydrogen Peroxide-Responsive Hyperpolarized ^{13}C MRI Contrast Agent

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Abstract

We report a new reaction-based approach for the detection of hydrogen peroxide (H_2O_2) using hyperpolarized ^{13}C magnetic resonance imaging (^{13}C MRI) and the H_2O_2 -mediated oxidation of α -ketoacids to carboxylic acids. ^{13}C -benzoylformic acid (^{13}C -BFA) reacts selectively with H_2O_2 over other reactive oxygen species (ROS) to generate ^{13}C -benzoic acid (BA) and can be hyperpolarized using dynamic nuclear polarization (DNP), providing a method for dual-frequency detection of H_2O_2 . Phantom images collected using frequency-specific imaging sequences demonstrate the efficacy of this responsive contrast agent to monitor H_2O_2 at pre-clinical field strengths. The combination of reaction-based detection chemistry and hyperpolarized ^{13}C magnetic resonance imaging (^{13}C MRI) provides a potentially powerful new methodology for non-invasive multianalyte imaging in living systems.

Reactive oxygen species (ROS) are intimately involved in the genesis and progression of numerous ailments¹ ranging from cancer² to neurodegeneration³ to diabetes.⁴ The relative stability and diffusibility of H_2O_2 poise this ROS to act as a potential diagnostic marker for the presence and progression of a wide range of pathological states. Indeed, the measurement of H_2O_2 in exhaled breath condensates has been used as a clinical marker for illnesses associated with lung inflammation,⁵ including asthma,⁶ chronic obstructive pulmonary diseases,⁷ and cystic fibrosis.⁸ The clinical use of this direct marker for oxidative stress and inflammation in deep tissues is hampered in large part by a lack of methods to non-invasively detect H_2O_2 in thicker, non-transparent specimens.⁹ As part of a larger program in our laboratory to develop novel ways to study the chemistry and biology of H_2O_2 in living cells and animals by molecular imaging,¹⁰ we now introduce a new type of reaction-based hyperpolarized ^{13}C MRI contrast agent for the detection of H_2O_2 .

MRI offers a promising approach for precise non-invasive molecular imaging of deep tissues in real time. However, its full potential as an imaging modality has yet to be realized because of a low sensitivity¹¹ that restricts most MRI experiments to imaging highly abundant endogenous protons in water and lipids. To overcome this limitation, hyperpolarization methods¹² such as optical pumping,¹³ para- H_2 induced polarization,¹⁴ and

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Supporting Information Available. Synthetic and experimental details (PDF), including procedures for the synthesis of compounds, selectivity assays, polarization procedures, spectroscopy, and imaging. This material is available free of charge at [UUhttp://pubs.acs.org](http://pubs.acs.org).

dynamic nuclear polarization (DNP)¹⁵ can drastically enhance an MRI signal by manipulating spin states to produce samples with large non-equilibrium spin populations, leading to highly magnetized samples and signal enhancements approaching 10⁵-fold. The development of rapid dissolution procedures¹⁶ following DNP has allowed the use of hyperpolarized [1-¹³C]-pyruvate,¹⁷ [2-¹³C]-fructose,¹⁸ [5-¹³C]-glutamine,¹⁹ ¹³C-HCO₃²⁰ [1,4-¹³C₂]-fumarate,²¹ ¹⁵N-choline,²² alone and in combination²³ as metabolic probes.²⁴ These hyperpolarized probes ultimately report on the flux of specific enzymes by monitoring the conversion of isotopically labeled natural substrates to their metabolic products. On the other hand, our interest in the detection of molecular species such as H₂O₂, which can simultaneously originate from multiple cellular sources, requires the use of probes that are not prone to rapid metabolism so as to avoid false positives. In addition to being metabolically inert and non-toxic, an ideal ¹³C MRI probe of this type should also possess favorable physical properties for DNP, produce an observable chemical shift upon rapid and selective reaction with the analyte of interest, and provide a system where both reactants and products have ¹³C-labeled nuclei with long spin-lattice relaxation (T₁) times.

To this end, observations of the rapid oxidative decarboxylations of α -ketoacids²⁵ led us to investigate these species as hyperpolarizable ¹³C MRI contrast agents using a reaction-based scheme for H₂O₂ detection. Initial studies focused on benzoylformic acid (BFA) because this α -ketoacid is non-toxic and undergoes minimal metabolism in both cells²⁶ and animals. Kinetics measurements reveal that the reaction between BFA and H₂O₂ proceeds rapidly to produce benzoic acid (BA) within minutes with a second order rate constant of $10.31 \pm 0.26 \text{ M}^{-1} \text{ min}^{-1}$ (Figure S1). Moreover, the ¹³C ketone nucleus of BFA and the ¹³C carbonyl nucleus of BA are expected to have long T₁ values (on the order of tens of seconds) and the chemical shift difference between this carbon atom upon conversion of BFA to BA, the product of oxidative decarboxylation, is ca. 20 ppm. Encouraged by these initial findings, we synthesized BFA with a ¹³C-label on the ketone carbon (¹³C-BFA) using sulfur ylide acylation and oxidation chemistry (Scheme 1). Reaction of neat bromoacetonitrile with tetrahydrothiophene provided the cyanosulfonium bromide **1**. The cyanosulfur ylide formed *in situ* was then coupled with ¹³C-benzoic acid (¹³C-BA) using HATU to yield the ¹³C-cyanosulfur ylide **2**. Finally, Oxone oxidation afforded ¹³C-BFA after HPLC purification.

With this compound in hand, we proceeded to test its ability to undergo DNP under clinically relevant conditions. In these standard protocols, DNP results from the transfer of polarization from a stable organic radical species to a spin S = 1/2 nucleus by microwave irradiation at 1–2 K in a solid-state glass.¹⁵ Efficient transfer of polarization from the free radical to the nuclei of interest relies heavily on the formation of a uniform glass which is typically achieved with highly concentrated solutions; for biological use, water is an appropriate solvent so that no residual toxic organic solvents are present after rapid-dissolution of the hyperpolarized sample. We found that ¹³C-BFA is extremely soluble in water at concentrations >5 M, with solutions reproducibly forming a glassy solid upon flash-freezing in liquid N₂. Representative preparations utilize 6.0 M solutions of ¹³C-BFA and 15 mM OX063 radical in water and were hyperpolarized at 1.33 K and irradiating at 94.094 GHz using a Hypersense™ (Oxford Instruments). Under these unoptimized conditions, ¹³C-BFA polarizes with a build-up time constant of 899 s, providing 5.2% polarization and a ~5500-fold signal enhancement. The T₁ values of the labeled ¹³C₂ ketone were measured to be $24.4 \pm 0.4 \text{ s}$ at 11.7T and $18.6 \pm 0.3 \text{ s}$ at 14.1T (Figure S3).

We then evaluated hyperpolarized samples of ¹³C-BFA for spectroscopic detection of H₂O₂ (Figure 1). After hyperpolarization, the sample was rapidly dissolved in 100 mM phosphate buffered to pH 7.8 with 0.3 mM EDTA to a final concentration of 5 mM and reacted with various concentrations of H₂O₂. Spectra were acquired with a single scan in the presence of

H₂O₂ concentrations ranging from 10 to 1000 μ M. Figure 1a shows a set of spectra 21 s after rapid dissolution and reaction with H₂O₂. The labeled carboxylate ¹³C1 resonance of the ¹³C-BA product exhibits a chemical shift of 176 ppm and the *unlabeled* carboxylate C1 carbon of the starting ¹³C-BFA appears as a doublet at 173.5 ppm; the ratio of the integrated ¹³C-BA peak to the unlabeled ¹³C-BFA peak displayed a maximum at 21 s and a good linear correlation with increasing concentrations of H₂O₂ (Figure 1b), indicating that hyperpolarized ¹³C-BFA can readily detect concentrations of H₂O₂ at high micromolar levels *in vitro* that are within the range implicated in states of oxidative stress that lead to cellular senescence,²⁸ despite trace amounts of ¹³C-benzoic acid (<0.1%) produced during synthetic manipulation and rapid dissolution procedures that can reduce the accuracy of such measurements. Although we were able to detect these levels of H₂O₂ using this first-generation probe, ongoing efforts are geared toward improving sensitivity to lower physiological levels that might be encountered *in vivo* through newer probes and optimized hyperpolarization protocols.

The oxidative decarboxylation of BFA is relatively selective for H₂O₂ over other biologically relevant ROS as monitored using analytical HPLC. After reacting BFA for 20 min at room temperature, only samples treated with H₂O₂ show significant conversion to benzoic acid (Figure 2). Only upon addition of exceedingly high, non-physiological concentrations does OCl⁻ or ONOO⁻ (500 mM) show reactivity with BFA (Figure S2), producing 20% and 70% NMR conversions, respectively. We speculate that the selectivity for H₂O₂ over OCl⁻ arises from an increased nucleophilicity of the former, whereas the selectivity over ONOO⁻ is most likely due to the short lifetime of the latter under physiological conditions.²⁹

Finally, we established the ability of ¹³C-BFA to image H₂O₂ at pre-clinical field strengths. Specifically, we attained phantom images using a 14.1T micro-imager equipped with 100 G/cm gradients and millipede ¹H and quadrature birdcage ¹³C RF coils. Figure 3 displays phantoms of samples containing 20 mM hyperpolarized ¹³C-BFA with 0, 25, 50, 100, and 200 mM added H₂O₂, thermally polarized 5 M ¹³C-BA in dimethyl acetamide (DMA), and thermally polarized ¹³C-BFA in H₂O. First, we acquired ¹H spin echo images of all the tubes to indicate tube placement (Figure 3a). Next, we acquired ¹³C MRI images using frequency-specific excitation pulses to obtain images of thermally polarized ¹³C-BA in DMA (Figure 3b), thermally polarized ¹³C-BFA in H₂O (Figure 3c), hyperpolarized ¹³C-BA produced from the H₂O₂-mediated conversion of hyperpolarized ¹³C-BFA in 100 mM phosphate buffer, 0.3 mM EDTA at pH 7.8 (Figure 3d), and hyperpolarized ¹³C-BFA in 100 mM phosphate buffer, 0.3 mM EDTA at pH 7.8 (Figure 3e). DMA was necessary to dissolve the thermally polarized 5 M ¹³C-BA due to its low aqueous solubility, and an unbuffered system was used for the thermally polarized 5 M ¹³C-BFA, causing a 2–3 ppm change in the chemical shifts for these species and requiring adjustment of the corresponding frequency specific pulses for these images. ¹³C images were acquired using a frequency specific 90° pulse and GRASE type readout for each resonance.³⁰ The overall acquisition time for the ¹³C images was ~150 ms, revealing the dramatic reduction in acquisition time gained by using hyperpolarized probes. Moreover, the thermally polarized 5 M phantoms in Figures 3b and 3c are scaled by 10-fold compared to the hyperpolarized 20 mM phantoms in Figures 3d and 3e, indicating the marked signal enhancement for the hyperpolarized samples of several orders of magnitude. A clear increase in the intensity of the ¹³C-BA images with a concomitant decrease in ¹³C-BFA signals can be observed with increasing H₂O₂ concentrations, demonstrating the power of using rapid and selective reaction-based hyperpolarizable probes to image H₂O₂ levels via ¹³C MRI.

In summary, we have described a hyperpolarized reaction-based ¹³C MRI probe strategy for the selective detection and imaging of H₂O₂. This work expands the applications of

hyperpolarized ^{13}C MRI for imaging non-enzymatic species by utilizing non-endogenous and biocompatible hyperpolarized probes. This first-generation ^{13}C -BFA probe is a viable candidate for detecting H_2O_2 at oxidative stress levels, and we are currently developing H_2O_2 probes with increased sensitivity, as well as expanding the scope of other ^{13}C -labeled compounds as *de novo* MRI contrast agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(a) ^{13}C NMR spectra of hyperpolarized ^{13}C -BFA after 21 s of reaction with 10, 100, 250, 500, 750, and 1000 μM H_2O_2 at 11.7T. Spectra were acquired with a single scan every 3 s with a 5° pulse, except for 10 and 100 μM which were acquired after 21 s with a 90° pulse. (b) Linear correlation of the ratio of integrated peak intensities of the C1 (carboxylate carbon) of ^{13}C -BA to the C1 (carboxylate carbon) of ^{13}C -BFA versus the concentration of H_2O_2 ; $R^2 = 0.988$.

Figure 2.

Response of 50 μM BFA to various ROS. All ROS were added at 5 mM, except for $\text{O}_2^{\bullet-}$ which was generated enzymatically at a rate of 24 $\mu\text{mol}/\text{min}$ for 120 min (2.9 mM total). Concentrations of benzoic acid were measured by HPLC after 0, 5, 10, 15, and 20 min of reaction except for $\text{O}_2^{\bullet-}$ which was measured after 0, 30, 60, 90, and 120 min of reaction.

Figure 3. Phantom images of 5 M thermally polarized ^{13}C -BFA in H_2O , 5 M thermally polarized ^{13}C -BA in DMA, and 20 mM hyperpolarized ^{13}C -BFA in 100 mM phosphate, 0.3 mM EDTA buffered at pH 7.8 with 0, 25, 50, 100, and 200 mM H_2O_2 . (a) ^1H spin echo image. (b) Frequency specific image with selective excitation of the resonance of 5 M ^{13}C -BA in DMA. (c) Frequency specific image with selective excitation of the resonance of 5 M ^{13}C -BFA in H_2O . (d) Frequency specific image with selective excitation of the resonance of ^{13}C -BA buffered at pH 7.8 (e) Frequency specific image with selective excitation of the resonance of ^{13}C -BFA buffered at pH 7.8. Images in (b)–(e) were acquired after ~ 37 s of reaction with H_2O_2 with a $T_R = 150$ ms, FOV $40 \times 40 \times 40$ mm, $16 \times 12 \times 12$ matrix and zero-filled to a final resolution of 1.25 mm isotropic.

Scheme 1.

Design of a hyperpolarized ^{13}C MRI contrast agent for detection of H_2O_2 through H_2O_2 -mediated α -ketoacid oxidative decarboxylation and the synthesis of ^{13}C -BFA and its H_2O_2 -mediated conversion to ^{13}C -BA.