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COMMUNICATION

Hyperpolarization of ^{15}N-Pyridinium by Using Parahydrogen Enables Access to Reactive Oxygen Sensors and Pilot *In Vivo* Studies

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Abstract: Magnetic resonance with hyperpolarized contrast agents is one of the most powerful and noninvasive imaging platforms capable for investigating *in vivo* metabolism. While most of the utilized hyperpolarized agents are based on ^{13}C nuclei, a milestone advance in this area is the emergence of ^{15}N hyperpolarized contrast agents. Currently, the reported ^{15}N hyperpolarized agents mainly utilize the dissolution dynamic nuclear polarization (d-DNP) protocol. The parahydrogen enhanced ^{15}N probes have proven to be elusive and have been tested almost exclusively in organic solvents. Herein, we designed a reaction based reactive oxygen sensor ^{15}N-boronobenzyl-2-styrylpyridinium (^{15}N-BBSP) which can be hyperpolarized with para-hydrogen. Reactive oxygen species plays a vital role as one of the essential intracellular signalling molecules. Disturbance of the H_2O_2 level usually represents a hallmark of pathophysiological conditions. This H_2O_2 probe exhibited rapid responsiveness toward H_2O_2 and offered spectrally resolvable chemical shifts. We also provide strategies to bring the newly developed probe from the organic reaction solution into a biocompatible injection buffer and demonstrate the feasibility of in vivo ^{15}N signal detection. The present work manifests its great potential not only for reaction based reactive sensing probes but also promises to serve as a platform to develop other contrast agents.

Introduction

Nuclear magnetic resonance spectroscopic imaging (MRSI) with hyperpolarized contrast agents represents one of the most powerful and noninvasive techniques capable of clinically investigating *in vivo* metabolism.[1] Unlike positronemission tomography (PET), this non-radiative technology provides chemical specificity through spectra information. It elegantly addressed the inherent insensitivity issue of nuclear magnetic resonance (NMR) *via* the formation of nonequilibrium spin population. Thus, signal enhancement over 10000-fold was achieved compared to thermal equilibrium spin polarization. Hyperpolarization (HP) techniques extensively expand the scope of magnetic resonance (MR) from the most clinical used ^{1}H-MRSI, allowing the observation of metabolites in low concentrations with improved spatial and temporal resolutions. These methods are highly desirable for a multitude of preclinical and clinical investigations of metabolic processes.[2]

Among several available hyperpolarization techniques, dissolution dynamic nuclear polarization (d-DNP)[3] and para-hydrogen (pH$_2$) induced polarization (PHIP) are the two leading technologies which have been mainly applied for producing non-gaseous medically relevant contrast agents.[1b, 4] Various ^{13}C or ^{15}N-labeled metabolites can be hyperpolarized with large signal enhancements *via* d-DNP. Although d-DNP is commercially available and a widely applicable method nowadays, superconducting magnets (> 1 T) and cryogenic temperatures (< 2 K) are usually necessary for HP bolus preparation. In addition, tens of minutes are typically consumed to produce one dose of injectable metabolites.

The second hyperpolarization method, pH$_2$ induced polarization (PHIP), utilizes the inexpensive, storable and portable pH$_2$ as the polarization source. Two pathways have been explored in the context of contrast agent development using pH$_2$: 1) SABRE (Signal Amplification by Reversible Exchange), [5] during which molecules are hyperpolarized upon interaction with a catalyst and pH$_2$ and the molecule is not altered and 2) hydrogenative PHIP during which an unsaturated bond is hydrogenated and the obtained proton spin orders transferred via the *J*-coupling network to a heteronuclear (^{13}C, ^{15}N etc.) spin of interest. [6] In the early days angiography and perfusion studies were mostly pursued with pH$_2$ enhanced contrast agents[7] until recently hyperpolarized metabolites became available that have now been demonstrated in several applications including cancer

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imaging.[8] However, majorities of studies to probe metabolic events or chemical species have been conducted almost exclusively with pyruvate. A few other examples with ^{13}C and ^{15}N labelled molecules exist and designing novel hyperpolarized contrast agents and especially for PHIP are still in its infancy.[1b, 4b, 9]

In this work, we sought to develop a responsive contrast agent that can be hyperpolarized with parahydrogen and undergoes chemical alteration in the presence of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2). ROS has shown to be toxic in high concentrations but function as essential intracellular signaling molecules.[10] Compared to other ROS species, H_2O_2 is stable under physiological conditions and exists in relatively high concentrations *in vivo*.[11] Generally, the ROS signaling pathways control ROS intracellular homeostasis. Disturbance of the H_2O_2 level represents a hallmark for various diseases, such as inflammation, tissue injury, cancer, and neurodegenerative disorders.[12] It would be highly desirable to establish H_2O_2 imaging *in vivo*, which may play an important role for diagnostic and therapeutic utility. Therefore, development of H_2O_2-sensing probes viable for preclinical models is of great significance.

Currently, the hyperpolarized ROS sensing probes were mainly focused on ^{13}C nuclei[13] and ^{15}N based hyperpolarized ROS sensors are less explored.[14] Indeed, ^{15}N nuclei possess many beneficial properties for metabolic MRI, including: a) relatively long relaxation time T_1 due to its small gyromagnetic ratio γ,[15] b) ubiquitous existence in many central metabolic pathways[16] and c) a broad chemical shift window up to 900 ppm.[5b, 9a, 15, 17] Recently, some elegant ^{15}N-nuclei probes using d-DNP have been developed, enabling real-time

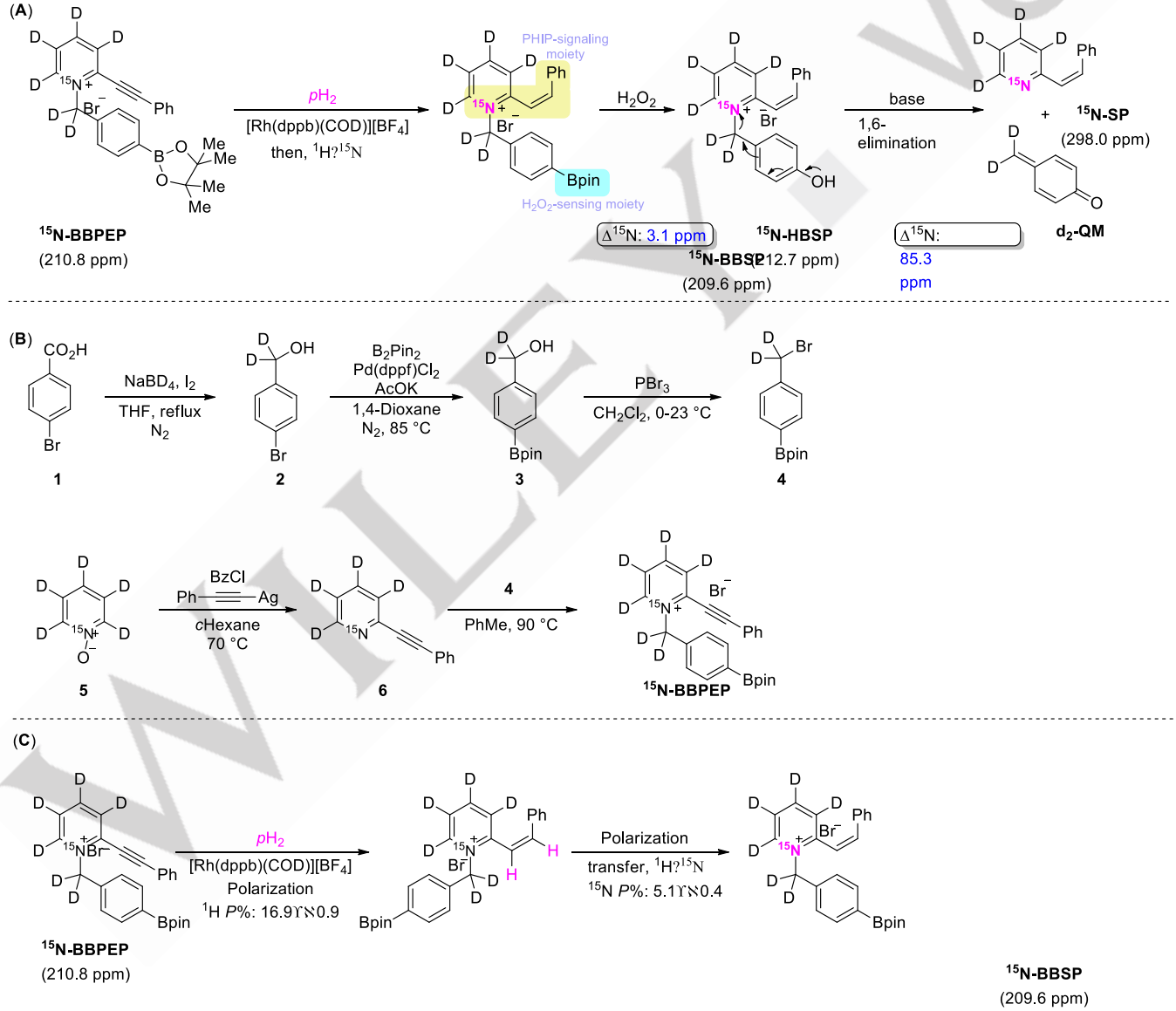

Figure 1. Design and synthesis of the PHIP ^{15}N-labeled H_2O_2-sensoring probe. (A) Proposed H_2O_2-sensoring mechanism *via* boron oxidation followed by base promoted 1,6-elimination. (B) Synthesis of PHIP ^{15}N-labeled H_2O_2-sensoring probe. (C) Proton hyperpolarization followed by polarization transfer to ^{15}N nuclei in MeOD-d_4 at 310 K. (Bpin: pinacolato boron)

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metabolic imaging *in vivo*.[14, 18] Notably, Park *et al.* disclosed a d-DNP ^{15}N-boronobenzyl-4-cyanopyridinium as a reaction-based H_2O_2-sensing probe, demonstrating the great potential of hyperpolarized ^{15}N-probes for oxidative stress in MR imaging wherein a ^{15}N-labelled nitrogen serves as a reporter group.[19] All existing hyperpolarized ROS sensors use d-DNP strategies. There are currently no known PHIP ROS sensors. Some of these studies suffer from low polarization efficiency, short T_1,[13b] tiny chemical shift changes.[14] Only a minority of these sensors have produced *in vivo* data, and to date, no one has successfully demonstrated the conversion product *in vivo* in a disease model.[13a,14] In our study, we particularly aim to investigate ^{15}N-enriched pyridinium moieties,[20] which have only been conceptually introduced and may ensure long tracing time and opportunity to provide hyperpolarization for biomedical MRI. In continuation of our hyperpolarization pyridinium project, [21] herein, we described the concise synthesis of ^{15}N-boronobenzyl-2-phenylethynylpyridinium (^{15}N-BBPEP) PHIP precursor and utilized the hyperpolarized ^{15}N-boronobenzyl-2-styrylpyridinium (^{15}N-BBSP) as an H_2O_2-responsive probe. Hyperpolarized ^{15}N-BBSP enables the detection of H_2O_2 with high sensitivity and selectivity with a significant chemical shift difference up to 88.4 ppm. Furthermore, we achieved the detection of the hyperpolarized ^{15}N-BBSP signal in aqueous media with ^{15}N-magnetic resonance spectroscopy (MRS) *in vivo*.

Results and discussion

Rational design and synthesis of PHIP precursor ^{15}N-BBPEP as a H_2O_2 sensor

The structure of hyperpolarized ^{15}N-BBSP consists of ^{15}N-2-styrylpyridin as a PHIP signaling unit and aryl boronate as a H_2O_2-sensing unit (Figure 1A). The synthesis of the labeled compound ^{15}N-BBPEP PHIP precursor was conducted as shown in Figure 1B. Starting from the reduction of 4-bromobenzoic acid **1** with $NaBD_4$, boronic ester **4** was readily prepared *via* palladium catalyzed borylation and bromination of benzyl alcohol **3**. Following a protocol developed by our group[21], we synthesized pyridine-^{15}N-oxide-d_5 **5** which could be ethynylated at the *ortho*-position *via* a N-acyloxypyridinium salt intermediate. Finally, the assembly of ^{15}N-BBPEP was achieved by conjugation of ^{15}N-2-(phenylethynyl) pyridine-d_4 **6** and boronic ester **4**. These structures have been confirmed with mass spectrometry and NMR spectra. Further experiment details could be found in the ESI.

In 2019, our group achieved efficient hyperpolarization of ^{15}N-pyridinium derivatives, a structure that is present in various bio-relevant molecules but has so far not been explored for the design of functional tracers. Furthermore, pyridinium species in the form of a salt would benefit from excellent solubility in aqueous media. Thus, we employed a pyridinium structure here, using ^{15}N-BBPEP as the H_2O_2 responsive sensor precursor (Figure 1A). After hyperpolarization with pH_2 and subsequent polarization transfer from ^{1}H to ^{15}N, hyperpolarized H_2O_2 sensor ^{15}N-BBSP would be formed. Based on previous literature reports, [19, 22] we envisioned that a phenol intermediate ^{15}N-hydroxybenzyl-2-styrylpyridinium (^{15}N-HBSP) can be released via oxidation with H_2O_2. Then a base-assisted 1,6 elimination/rearrangement of ^{15}N-HBSP was expected to take place and delivered ^{15}N-2-styrylpyridin (^{15}N-SP) as well as quinone methide-d_2 (QM-d_2). As the signaling unit, ^{15}N-2-phenylethynylpyridinium (^{15}N-PEP) possesses an unsaturated side arm which is crucial for subsequent ^{1}H/^{15}N-hyperpolarization. Due to substantial electronic density and chemical structure change among ^{15}N-BBSP, ^{15}N-HBSP and ^{15}N-SP, significant chemical shift differences would be expected. These chemical shifts are highly desirable for ^{15}N MR spectroscopy. Indeed, noticeable chemical shift differences, up to 3.1 ppm and 85.3 ppm, were observed among these hyperpolarized ^{15}N species, which could be utilized as signal readouts in the following H_2O_2 sensing studies.

Hyperpolarization of ^{15}N-BBSP and ^{15}N-T_1 investigation of ^{15}N-BBSP

With ^{15}N-BBPEP in hand, we performed hydrogenation reactions with pH_2 and investigated the spin order transfer to hetero-nucleus ^{15}N afterwards. Prior to conducting hyperpolarization experiments, we checked the stability of ^{15}N-BBPEP by pretreatment of the sample with deuterium oxide (D_2O) before the hydrogenation reactions. Although negligible hydrolysis of the boronic ester was observed (see S5), the D_2O-pretreated sample ^{15}N-BBPEP exhibited good stability and comparable hyperpolarization efficiency as normal samples. Both the boronic ester and the boronic acid substituted ^{15}N-BBPEP could be hyperpolarized and transferred into ^{15}N-HBSP equivalently upon treatment with H_2O_2 (Figure S1).

To obtain the hyperpolarized probe ^{15}N-BBSP, pyridinium ^{15}N-BBPEP was hydrogenated with pH_2 at 310 K in MeOD-d_4 using the homogeneous rhodium catalyst [Rh(dppb)(COD)][BF_4] (dppb: diphenylphosphino butane, COD: cyclooctadiene). In the ^{1}H polarization step, 16.9 ± 0.9 % polarization was achieved with three parallel experiments by comparison with the thermal spectrum (see S2). Subsequently, ^{15}N polarization was realized by utilizing the MINERVA (Maximizing Insensitive Nuclei Enhancement Reached Via parahydrogen Amplification) sequence,[23] with a final polarization of 5.1 ± 0.4 % (Figure 1C and S4). The decrease in polarization here may be due to low transfer efficiency caused by the small coupling between the ^{15}N and ^{1}H ($^3J_{N\text{-}H}$ = 1.0 Hz).**Table 1**. ^{15}N-T_1 values of ^{15}N-BBSP at 310 K in MeOD-d_4 and D_2O [a].

Entry	Mag. field in T	Average T_1 (s)[b]	Solvent
1	7	40.4 ± 3.7	MeOD-d_4
2	1	128.3 ± 26.5	MeOD-d_4
3	0.1	193.5 ± 0.7	MeOD-d_4
4	0.01	133.0 ± 10.4	MeOD-d_4
5	0.1	133.0 ± 12.8	D_2O
6	7.0	32.0 ± 2.0	H_2O
7	0.1	77.0 ± 6.0	H_2O

[a] all of the data were obtained from 3 parallel experiments in MeOD-d_4 or D_2O at 310 K unless otherwise noted.

Since we succeeded in transferring ^{1}H polarization to the ^{15}N-spin with good efficiency, we proceed with relaxation time

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measurements using low flip angle pulses of 10° at various magnetic fields (see ESI for details). As shown in Table 1, the ^{15}N-T_1 gradually increased with decreasing magnetic fields at the initial stage. An outstanding T_1 (193.5 ± 0.7 s) was achieved at a magnetic field of 100 mT (entries 1-3). The ^{15}N-T_1 decreased slightly when the field was further reduced to 10 mT (entry 4). Remarkably, a comparable ^{15}N-T_1 was maintained when we performed the T_1 measurement in D_2O buffer (entry 5). The T_1 values in H_2O were shorter than those in deuterated MeOD or in D_2O (entries 6 and 7). We also tried to test the T_1 in the presence of bovine serum albumin. However, with a flip angle of 10°, the hyperpolarized ^{15}N signal disappeared rapidly during 2D-acquisition, making T_1 determination impossible (see S7).[24] This also implies that the design of ^{15}N-probes will need to be carefully chosen to minimize albumin binding in the future. The relatively long T_1 of ^{15}N-BBSP in biocompatible aqueous solution provides the opportunity for *in vivo* application of our H_2O_2 sensor. Furthermore, given the ubiquitous presence of pyridinium moieties in various drugs or bioactive molecules, our ^{15}N-BBSP may find important utility in clinical scanners.

H_2O_2 detection of hyperpolarized ^{15}N-BBSP and its *in vivo* application

To verify the responsiveness of hyperpolarized ^{15}N-BBSP to H_2O_2, it was further tested with H_2O_2 *in vitro*. As illustrated in Figure 2A, the ^{15}N signal immediately shifted from 209.6 ppm to 212.7 ppm (Δ^{15}N = 3.1 ppm) upon addition of H_2O_2 solution. The new signal at 212.7 ppm could be assigned to ^{15}N-HBSP derived from oxidation of ^{15}N-BBSP. The H_2O_2 concentration could be reduced to the single-digit mM range while still observing a detectable conversion (Figure 2C).The cleavage product ^{15}N-SP (298.0 ppm, Δ ^{15}N = 85.3 ppm) was only detected after sequential injection of an alkaline Na_2CO_3 solution and H_2O_2 (Figure 2B).

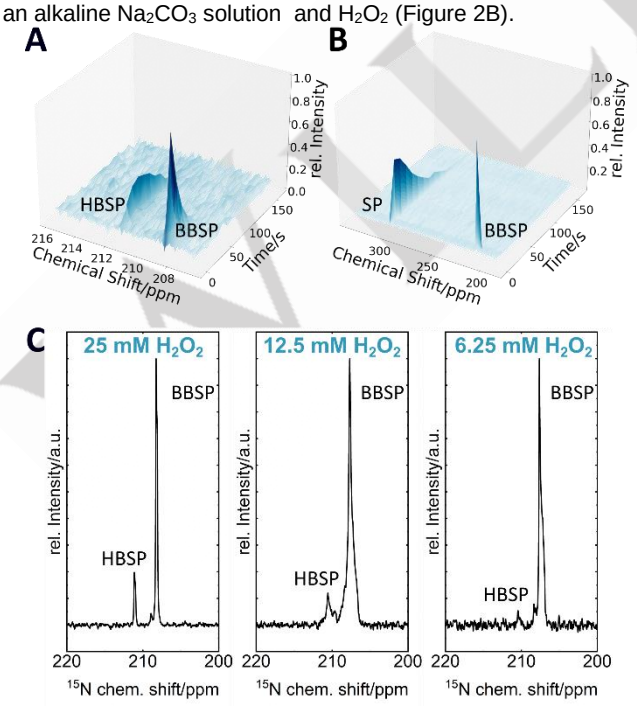

Figure 2. H_2O_2 detection of hyperpolarized ^{15}N-BBSP. Time Series spectra were recorded every 10 seconds with a flip angle of 10 degree. All spectra were recorded at 7.0 T. (A) Time series of hyperpolarized ^{15}N-NMR spectrum after addition of H_2O_2 (final concentration 100 mM). (B) Time series of hyperpolarized ^{15}N-NMR spectrum after sequential addition of H_2O_2 (1%) and Na_2CO_3 (50 mM). (C) 1D ^{15}N spectra recorded with a 90 degree pulse 30 s after H_2O_2 addition with respective final concentrations.

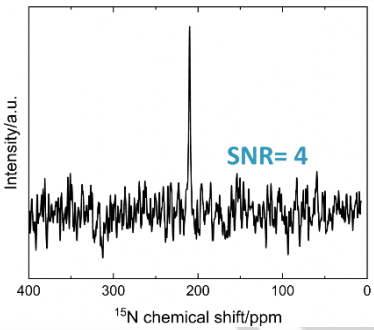

Figure 3. *In vivo* detection of hyperpolarized ^{15}N-BBSP in a wildtype mouse. ^{15}N- spectrum of ^{15}N-BBSP recorded 15 seconds after injection using a 90 degree pulse at 7.0 T. The peak has an SNR of 4 and a linewidth of 50 Hz. Additional experiments (n=3) can be found in S10.

These significant changes of chemical shifts (3.1 ppm & 85.3 ppm) as well as the relatively long T_1 of the ^{15}N-BBSP H_2O_2 sensor encouraged us to further test its performance in in vivo experiments (n=3). First, a ^{1}H-NMR image of a wildtype mousewas acquired as shown in Figure S10. Following a protocol developed by our group,[5a, 23] we evaporated MeOD-d_4 at 371 K under vacuum. The rhodium catalyst was removed *via* filtration within 40 s to obtain an aqueous solution containing ^{15}N-BBSP for injection. After tail-vein administration of the freshly prepared ^{15}N-BBSP hyperpolarized sample, the signal was detected using a nonlocalized ^{15}N spectrum recorded with a 90° pulse. The spectrum was recorded 15 s after injection of the hyperpolarized sample and showed a single peak at 210 ppm with an SNR of 4 and a linewidth of 50 Hz (Figure 3, for more information see ESI), proofing the feasibility to obtain ^{15}N tracers with pH_2 in biocompatible conditions. No oxidative or cleavage products were detected due to low H_2O_2 concentrations[25] and pH environment under psychological conditions. Future work will improve polarization and sensitivity[26] of the ^{15}N tracers and further develop the concept that *in vivo* changes become detectable. We would like to emphasize the finding that upon the cleavage of the boronate ester which is six bonds away, a chemical shift difference of the nitrogen of 3.1 ppm is observable. This is larger than the *in vivo* linewidth of 50 Hz (about 2 ppm) and therefore provides an important guideline for the development of future tracers: reactive moieties can be introduced far away from the hyperpolarized ^{15}N site putting less constraints on the molecular design.

Conclusion

In conclusion, we designed and synthesized a reaction based H_2O_2 sensor precursor ^{15}N-BBPEP. The rational designed pyridinium salt ^{15}N-BBPEP is amenable to hydrogenative PHIP polarization with high efficiency and delivered the hyperpolarized H_2O_2 sensing probe ^{15}N-BBSP. ^{15}N-BBSP exhibited rapid

responsiveness toward H_2O_2 *in vitro* and offered significant chemical shifts (3.1 ppm & 85.3 ppm) after treatment with H_2O_2 and base, which can be resolved in future MRI studies. For the first time, we successfully detected ^{15}N signal *in* vivo with a para-hydrogen enhanced tracer in biocompatible buffer. In the future we will work towards overcoming the polarization loss during the spin transfer and work-up process. This advancement will contribute to the development of a more sensitive H_2O_2 probe, potentially achieving detection levels within the pathological range. Even though improvements are needed, the present work manifests its great potential as a reaction based H_2O_2 sensing probe, which may be used for oxidative stress imaging in diagnostic and therapeutic medical applications in nearby future. Additionally, we expect that the presented *in vivo* approach can also be immediately implemented for SABRE enhanced ^{15}N molecules, thus enabling the production of more hyperpolarizeable contrast agents available in the future.

Supporting Information

The authors have cited additional references within the Supporting Information.

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Keywords: Hyperpolarization • Parahydrogen • ^{15}N-pyridinium • NMR • PHIP

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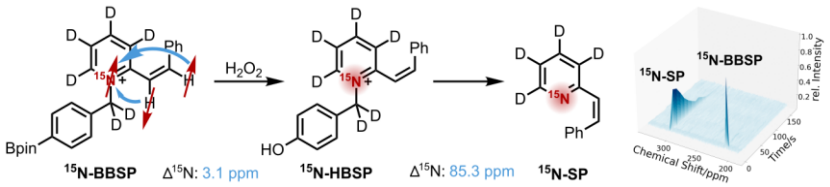

A ^{15}N-labelled tracer for magnetic resonance is introduced that is signal enhanced using parahydrogen and the feasibility for first pilot ^{15}N in vivo studies is demonstrated.