Magnetic Resonance Probes

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Design of a ^{13}C Magnetic Resonance Probe Using a Deuterated Methoxy Group as a Long-Lived Hyperpolarization Unit**

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There has been long-term effort toward molecular analysis in living systems. For such a purpose, magnetic resonance (MR) is one of the most promising techniques. Because of the good penetration of radio waves, a MR-based chemical probe (MR probe) can be detected even in a deep site of opaque bodies. Therefore, various MR probes have been designed.[1] Although they achieved good results in some cases, MR probes have an intrinsic drawback, namely, low sensitivity.

Hyperpolarization methods,[2] including dynamic nuclear polarization (DNP),[3] have been studied to overcome this drawback. The hyperpolarization methods allow polarization of nuclear spin populations, resulting in a large enhancement of MR sensitivity compared to the thermally equilibrated nuclei. It has been applied for in-cell or in vivo metabolic analyses using stable isotope-enriched natural compounds.[2]

Recently, it was demonstrated that the concept of hyperpolarization can be extended to MR probes for surveying the chemical status of living systems.[4–12] Because carbon atoms are present in almost all chemical probes, and sophisticated ^{13}C-isotope labeling procedures are available, ^{13}C-labeled probes could be a choice for designing a hyperpolarized MR probe. In practice, ^{13}C-carbon dioxide,[10] ^{13}C-benzoylformic acid,[11] and ^{13}C-ascorbic acid[12] have been successfully designed as hyperpolarized MR probes to sense pH, H_2O_2, and redox status, respectively.

Almost all of the hyperpolarized ^{13}C MR probes, however, utilized a carbonyl carbon as a hyperpolarized ^{13}C unit, because of its long hyperpolarization lifetime. In other words, the ^{13}C unit, having sufficient hyperpolarization lifetime and thus availability for designing a hyperpolarized ^{13}C MR probe, is almost limited to a carbonyl carbon. Such a strict structural limitation makes it difficult to design a variety of hyperpolarized ^{13}C MR probes. Therefore, a new hyperpolarization ^{13}C unit is highly desirable.

Deuterated ^{13}C is one of the promising candidate for hyperpolarization ^{13}C units.[13] Herein, we report a fully deuterated ^{13}C methoxy group ($^{13}CD_3O$) as a new long-lived hyperpolarization ^{13}C unit for designing a hyperpolarized ^{13}C MR probe. We successfully demonstrated the utility of this unit by designing a hyperpolarized ^{13}C chemical probe to target hypochlorous acid (HOCl).[14]

For the new hyperpolarized ^{13}C unit, we focused on alkoxy or aryloxy groups. This group is one of the key structures for designing target or environment-responsive chemical probes, where it could work as a leaving group (Figure 1 a). For example, when R^2 in Figure 1 a is a 3-methylindole quinone or

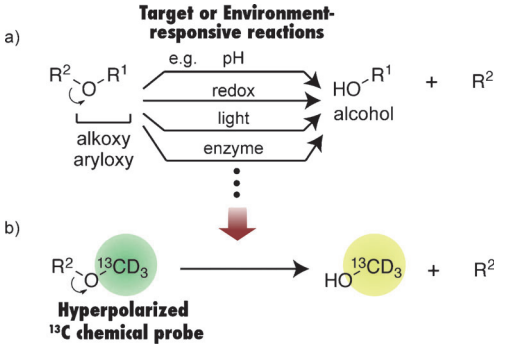

Figure 1. How a) alkoxy or aryloxy and b) deuterated ^{13}C methoxy groups can produce alcohol when R^2 is a reactive moiety that releases these groups by target or environment-responsive reactions.

4-nitrobenzyl moiety, a hypoxic condition induces electron reduction by reductase, resulting in an alkoxy/aryloxy-to-alcohol conversion.[15] When R^2 is a photoresponsive group such as the 2-nitrobenzyl moiety, photoirradiation induces alcohol release.[16] When R^2O has the galactopyranoside structure, it works as a substrate of β-galactosidase reporter protein to afford an alcohol R^1OH.[17] A similar alkoxy/aryloxy-to-alcohol conversion is utilized in many environment-responsive prodrug designs.[15,18] Therefore, if the alkoxy/aryloxy group joins in the hyperpolarization unit, it can expand the design strategy of target or environment-responsive hyperpolarized chemical probes.

We selected a fully deuterated ^{13}C methoxy group ($^{13}CD_3O$) as a candidate for the hyperpolarized alkoxy unit (Figure 1 b). A long hyperpolarized ^{13}C lifetime is achieved by nonprotonated ^{13}C because of the lack of dipole–dipole interaction with neighboring protons. In this sense, deuteration is one of the most straightforward ways to increase the hyperpolarization lifetime, as clearly demonstrated by deuterated choline chloride.[13] Furthermore, the hyperpolarization lifetime is highly dependent on molecular size. A smaller size typically produces a longer hyperpolarization lifetime. Therefore, we anticipated that a nonprotonated and small

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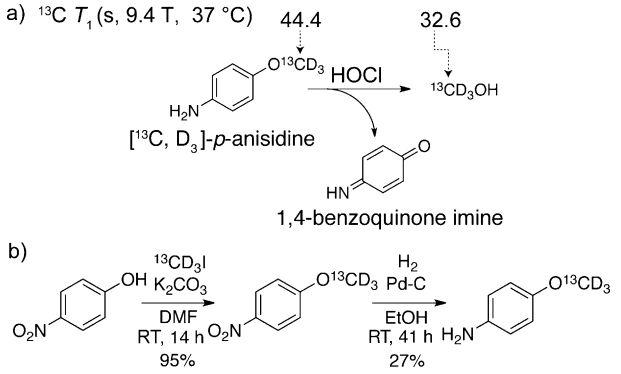
$^{13}CD_3O$ unit could retain its hyperpolarized state for a relatively long time.

The hyperpolarization lifetime of the $^{13}CD_3O$ unit was evaluated by measuring the spin–lattice relaxation time (T_1), which is directly related thereto. The T_1 values were determined by inversion recovery method using thermally equilibrated compounds. The T_1 value of the methoxy carbon in CD_3OH (3 M in $H_2O/D_2O = 9:1$, ^{13}C natural abundance) was observed as 32.9 s (9.4 T, 37 °C), which was almost the same as that of the carbonyl carbon in CH_3CO_2H (33.3 s, 9.4 T, 37 °C, ^{13}C natural abundance). These results indicate that a fully deuterated methoxy group $^{13}CD_3O$ has high potential to be used as a long-lived hyperpolarization unit.

With the promising hyperpolarization unit $^{13}CD_3O$ in hand, we then evaluated its practical utility by designing a new chemical probe. A hyperpolarized ^{13}C MR probe must satisfy the following prerequisites: The probe should 1) have a ^{13}C-labeled nucleus with long T_1 for a long hyperpolarization; 2) react with the target species rapidly within the hyperpolarization lifetime; and 3) induce a sufficiently large chemical shift change upon reaction.

We designed [^{13}C,D_3]-*p*-anisidine as a hyperpolarized ^{13}C MR probe that targets HOCl, an important ROS biomarker for inflammatory diseases (Scheme 1 a). The [^{13}C,D_3]-*p*-anisidine has the $^{13}CD_3O$ structure, which could be used as a long-lived hyperpolarization unit, on a HOCl-responsive *p*-aminophenyl scaffold.[19] It was anticipated that the [^{13}C,D_3]-*p*-anisidine would react effectively with HOCl to release $^{13}CD_3OH$ by *O*-dealkylation by an *ipso*-substitution mechanism (Scheme 1 a). Furthermore, the HOCl-responsive reaction is expected to induce a ^{13}C chemical shift change large enough to be monitored by ^{13}C NMR spectroscopy.



Scheme 1. a) Chemical structure of [^{13}C,D_3]-*p*-anisidine and its expected reaction with HOCl. b) Synthesis of [^{13}C,D_3]-*p*-anisidine.

First, we measured ^{13}C T_1 values for the probe and product. The [^{13}C,D_3]-*p*-anisidine was synthesized from 4-nitrophenol using $^{13}CD_3I$ as a [^{13}C,D_3]-labeling reagent according to the procedure in Scheme 1 b and the Supporting Information. The T_1 values of [^{13}C,D_3]-*p*-anisidine and $^{13}CD_3OH$ (50 mM in $H_2O/D_2O = 9:1$) were determined as 44.4 s and 32.6 s (9.4 T, 37 °C), respectively (Scheme 1 a). Non-deuterated *p*-anisidine showed much shorter T_1 value of 6.3 s (9.4 T, 37 °C, ^{13}C natural abundance), suggesting the favorable effect of deuteration in the methoxy group. The [^{13}C,D_3]-*p*-anisidine seems to exist stably in blood at least in DNP analysis time range (typically < 10 min) (Supporting Information, Figure S1), but the ^{13}C T_1 value of [^{13}C,D_3]-*p*-anisidine was shortened to be 23 s (9.4 T, 37 °C) in serum. Importantly however, the observed T_1 values of probe and product were comparable or longer than those of hyperpolarized ^{13}C MR probes utilizing carbonyl carbon as a hyperpolarization unit ($T_1 = 24.4$ s at 11.7 T for ^{13}C-benzoylformic acid[11] and 15.9 s at 9.4 T for [1-^{13}C]ascorbic acid[12a]), supporting our idea that $^{13}CD_3O$ can be used in MR probes as a hyperpolarization unit.

We then investigated the reaction of the probe with HOCl. The ^{13}C chemical shift of [^{13}C,D_3]-*p*-anisidine probe was observed at 56.6 ppm (Figure 2 a, top). After reaction

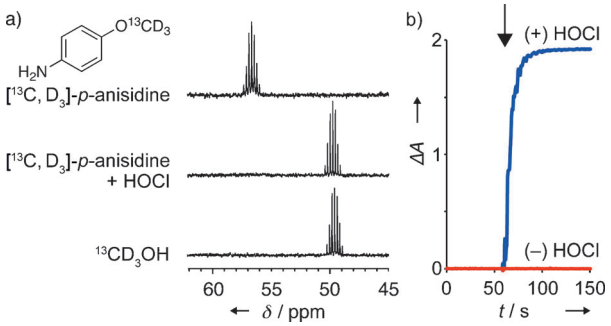


Figure 2. a) ^{13}C NMR spectra of thermally equilibrated [^{13}C,D_3]-*p*-anisidine without (top) or with (middle) 2 equiv of HOCl, and (bottom) $^{13}CD_3OH$. b) Time course of absorption changes (287 nm) of [^{13}C,D_3]-*p*-anisidine in the presence (blue) or absence (red) of 2 equiv of HOCl. The arrow indicates the addition point of HOCl.

with 2 equiv of HOCl, the peak at 56.6 ppm completely disappeared and a new peak was observed at 49.6 ppm (middle), which was assigned as $^{13}CD_3OH$ by comparison with an authentic sample (bottom). HPLC analyses showed the concomitant production of 1,4-benzoquinone imine (Supporting Information, Figure S2). These results indicate an efficient reaction of [^{13}C,D_3]-*p*-anisidine with HOCl and production of $^{13}CD_3OH$ via the *ipso*-substitution mechanism shown in Scheme 1 a. The observed ^{13}C chemical shift change of 7.0 ppm was sufficient to be monitored by ^{13}C NMR spectroscopy.

Furthermore, [^{13}C,D_3]-*p*-anisidine reacted with HOCl very quickly. When HOCl (200 μM) was added to the [^{13}C,D_3]-*p*-anisidine (100 μM) solution, a rapid enhancement of the absorption was observed and reached a maximum within about 30 s (Figure 2 b). On the other hand, no absorption change was observed without HOCl. The observed fast reaction kinetics was appropriate for DNP analysis.

Along with sufficient reactivity, [^{13}C,D_3]-*p*-anisidine also exhibited high selectivity for HOCl. [^{13}C,D_3]-*p*-Anisidine was intact after incubation with most of the biologically important ROS (H_2O_2, ROO$^{\bullet}$, $O_2^{\bullet-}$, and $^{\bullet}$OH) and reactive nitrogen species (RNS, $ONOO^-$) except for HOCl and $^{\bullet}$NO (Supporting Information, Figure S3). [^{13}C,D_3]-*p*-Anisidine reacted

with ·NO, but the main product was not 1,4-benzoquinone imine. Thus, $^{13}CD_3OH$, the target product of the present probe, was generated only when [^{13}C,D_3]-*p*-anisidine reacted with HOCl.

All of the data indicate that the designed [^{13}C,D_3]-*p*-anisidine satisfies the prerequisites: long ^{13}C T_1, rapid reaction with HOCl, and large enough chemical shift change. We then proceeded with evaluation of hyperpolarized [^{13}C,D_3]-*p*-anisidine.

Hyperpolarization was achieved by the DNP method (details on DNP procedures, experimental conditions, and instrument settings are given in the Supporting Information). Briefly, [^{13}C,D_3]-*p*-anisidine was mixed with OX63 radical in [D_6]DMSO/D_2O (1:1) and subjected to hyperpolarization at 1.4 K. After 1.5 h, the sample was dissolved rapidly using heated aqueous buffer and recovered for subsequent ^{13}C NMR experiments (Figure 3a). Although the deuterium-decoupling procedure eliminates ^{13}C–D coupling to give a ^{13}C singlet as demonstrated previously,[13] in this experiment, deuterium-decoupling conditions were not used so as to identify the deuterated ^{13}C nucleus.

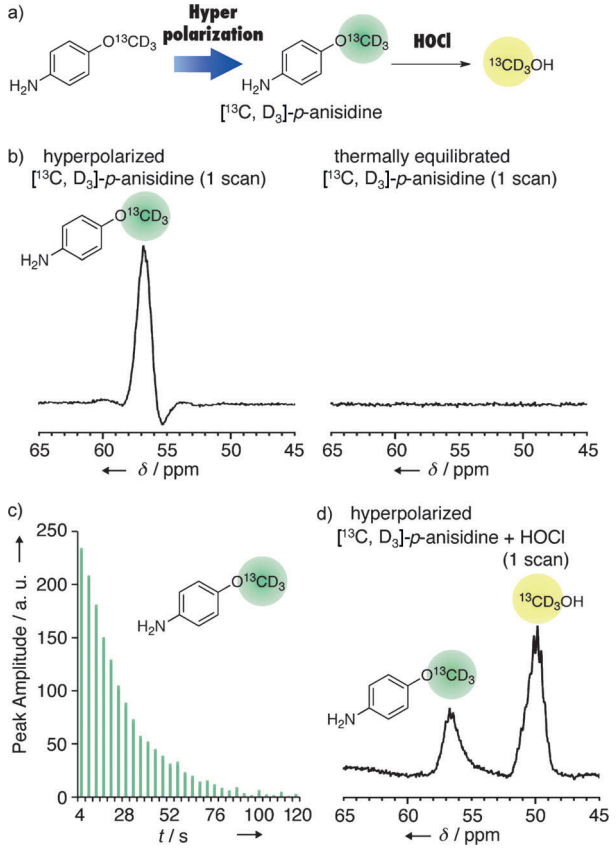


Figure 3. a) Illustration of experimental procedures. b) ^{13}C NMR spectra of hyperpolarized (4 s after rapid dissolution, left) and thermally equilibrated (right) [^{13}C,D_3]-*p*-anisidine (10 mM). c) Time-resolved ^{13}C NMR data of hyperpolarized [^{13}C,D_3]-*p*-anisidine (1.7 mM). The peak amplitude of [^{13}C,D_3]-*p*-anisidine was plotted from 4 to 120 s after initiation of the NMR acquisition. d) ^{13}C NMR spectrum of hyperpolarized [^{13}C,D_3]-*p*-anisidine (10 mM) observed 4 s after addition of HOCl (20 mM). NMR spectra were measured with a 7.05 T NMR instrument with 90° (b, d) and 18° pulse angles (c).

Polarization of 3.8% and about 6100-fold signal enhancement were achieved for [^{13}C,D_3]-*p*-anisidine, which was slightly lower but still in the comparable range with that of pyruvic acid (22000-fold signal enhancement) under the same experimental and instrument settings. A large ^{13}C NMR signal derived from hyperpolarized [^{13}C,D_3]-*p*-anisidine was observed with one scan (Figure 3b, left, 90° pulse angle). In marked contrast, no signal was obtained for thermally equilibrated [^{13}C,D_3]-*p*-anisidine under the same NMR measurement conditions (Figure 3b, right, 90° pulse angle).

The hyperpolarized [^{13}C,D_3]-*p*-anisidine showed a sufficiently long hyperpolarization lifetime. ^{13}C NMR spectra of the hyperpolarized [^{13}C,D_3]-*p*-anisidine (Ernst pulse angle, 18°) were measured every 4 s and are shown in Figure 3c. The time-resolved data revealed that a signal of hyperpolarized [^{13}C,D_3]-*p*-anisidine could be observed for about 100 s after starting the ^{13}C NMR measurement.

Finally, we applied the hyperpolarized [^{13}C,D_3]-*p*-anisidine probe to the detection of HOCl. The hyperpolarized [^{13}C,D_3]-*p*-anisidine (1.5 h polarization) was mixed with 2 equiv of HOCl and subjected to ^{13}C NMR measurements. Figure 3d shows a spectrum (90° pulse angle) observed 4 s after reaction with HOCl. Along with the signal of the hyperpolarized probe (56.7 ppm), a new signal was observed at 49.8 ppm. This new signal corresponds to $^{13}CD_3OH$, which was produced by reaction of the probe with HOCl. Because of the fast reaction, a HOCl-responsive probe-to-product conversion was successfully monitored within the hyperpolarization lifetime; in this case, the peak of the product $^{13}CD_3OH$ was observed as the main peak even 4 s after addition of HOCl. These results demonstrate that the designed [^{13}C,D_3]-*p*-anisidine, which has a fully deuterated ^{13}C methoxy group, functioned as a HOCl-responsive hyperpolarized ^{13}C MR probe.

In conclusion, we have presented a fully deuterated ^{13}C methoxy group ($^{13}CD_3O$) as a new ^{13}C hyperpolarization unit for designing a hyperpolarized ^{13}C MR probe. The significance of the designed $^{13}CD_3O$ group may be summarized as follows. First is its high performance: The non-protonated and small $^{13}CD_3O$ group was achieved a high hyperpolarization efficiency and sufficient lifetime to be used as a long-lived hyperpolarization unit. Second is its applicability: The practical utility of the unit was demonstrated successfully by developing a hyperpolarized chemical probe using the $^{13}CD_3O$ unit. The designed compound worked as a selective and quick-response chemical probe for sensing HOCl, a biomarker of inflammation. Practical in vivo applications must await further studies on detection limit, biostability, toxicity, bio-orthogonality, and distribution, although these are beyond the scope of this initial study. Third is its versatility: The alkoxy group is found in a variety of target or environment-responsive chemical probes, wherein the group could work as a leaving moiety. Therefore, the $^{13}CD_3O$ unit might be utilized as a hyperpolarization unit in diverse chemical probes apart from the present HOCl-responsive probe. Fourth is its simplicity: The $^{13}CD_3O$ unit can be easily incorporated into chemical probes using commercially available $^{13}CD_3I$ as an isotope source. All of these advantages show the high potential of the designed

$^{13}CD_3O$ group as a new hyperpolarization unit for designing various hyperpolarized MR probes.

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