

# A hyperpolarized choline molecular probe for monitoring acetylcholine synthesis

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Choline as a reporter molecule has been investigated by *in vivo* magnetic resonance for almost three decades. Accumulation of choline metabolites (mainly the phosphorylated forms) had been observed in malignancy in preclinical models, *ex-vivo*, *in vivo* and in patients. The combined choline metabolite signal appears in <sup>1</sup>H-MRS of the brain and its relative intensity had been used as a diagnostic factor in various conditions. The advent of spin hyperpolarization methods for *in vivo* use has raised interest in the ability to follow the physiological metabolism of choline into acetylcholine in the brain. Here we present a stable-isotope labeled choline analog, [1,1,2,2-D<sub>4</sub>,2-<sup>13</sup>C]choline chloride, that is suitable for this purpose. In this analog, the <sup>13</sup>C position showed 24% polarization in the liquid state, following DNP hyperpolarization. This nucleus also showed a long T<sub>1</sub> (35 s) at 11.8 T and 25 °C, which is a prerequisite for hyperpolarized studies. The chemical shift of this <sup>13</sup>C position differentiates choline and acetylcholine from each other and from the other water-soluble choline metabolites, namely phosphocholine and betaine. Enzymatic studies using an acetyltransferase enzyme showed the synthesis of the deuterated-acetylcholine form at thermal equilibrium conditions and in a hyperpolarized state. Analysis using a comprehensive model showed that the T<sub>1</sub> of the formed hyperpolarized [1,1,2,2-D<sub>4</sub>,2-<sup>13</sup>C]acetylcholine was 34 s at 14.1 T and 37 °C. We conclude that [1,1,2,2-D<sub>4</sub>,2-<sup>13</sup>C]choline chloride is a promising new molecular probe for hyperpolarized metabolic studies and discuss the factors related to its possible use *in vivo*. Copyright © 2010 John Wiley & Sons, Ltd.

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**Keywords:** hyperpolarization; magnetic resonance; choline; acetylcholine; deuterium; kinetic model; spin-lattice relaxation

## 1. Introduction

Choline is an essential nutrient that is involved in multiple cellular processes in several organs (1). The metabolism of choline is involved in neurodegenerative processes such as Alzheimer's disease, where cholinergic neurons degenerate locally (particularly in the basal forebrain nuclei) and the ability to synthesize the neurotransmitter acetylcholine is diminished (2). Choline metabolism is also known to be altered in malignancies, specifically in breast, brain and prostate cancer (3–5). Both the transport of choline into the cell and choline kinase activity have been shown to be augmented in breast cancer as well as other cancers (6–9). The formation of betaine from choline is an important step in one carbon metabolism via the betaine–homocysteine methyltransferase enzyme, which catalyzes the transfer of methyl groups from betaine to homocysteine and onwards to the methylation of DNA, proteins, lipids, and other intracellular metabolites. Abnormalities in this metabolism have been implicated in disorders ranging from vascular diseases to neural tube birth defects (10). Therefore, the ability to monitor the metabolism of choline to acetylcholine, phosphocholine or betaine holds promise as a direct diagnostic tool capable of providing important biomarkers for several conditions that affect large populations.

Today, the only technology that enables monitoring of metabolism in a non-invasive manner is magnetic resonance spectroscopy (MRS). Proton MRS can identify the combined content of choline metabolites in a tissue and this has been

shown to aid in the characterization of breast tumors and in predicting the response to treatment in breast cancer (4,6,7). However, this technology does not allow monitoring of choline's chemical evolution into its metabolites, due to both an overlap in their signals and a large background signal of endogenous water-soluble choline metabolites in tissues (mainly glycerophosphocholine and phosphocholine). In contrast, carbon-13 MRS of isotopically (<sup>13</sup>C) enriched choline has the potential to enable monitoring of the chemical evolutions of choline due to its wide spectral window (200 ppm) and low background signal (the natural abundance of <sup>13</sup>C is 1.1%). Indeed, *ex-vivo* carbon-13

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MRS of [1,2- $^{13}\text{C}_2$ ]choline had been used to monitor the conversion of choline to acetylcholine in live brain slices of rats (11), and to trace, *in vivo*, choline metabolism in breast cancer tumors implanted in nude mice (12). However, these measurements were carried out at very low temporal resolutions on the order of hours. The low sensitivity of  $^{13}\text{C}$  MRS combined with the low concentration of choline metabolites makes the *in vivo* detection of this metabolism impractical in most cases.

The choline molecular probe described herein capitalizes on the dynamic nuclear polarization (DNP) *ex-vivo* hyperpolarization of carbon-13 technology, which has been shown to provide more than 10,000-fold enhancement in carbon-13 signal (13). To date, all of the molecular probes that were utilized in this technology have contained a non-protonated carbon-13 enriched positions [e.g. either carbonyl or quaternary positions, as in pyruvate (14), fumarate (15), *t*-butanol (16), acetate (17), fructose (18) and  $\alpha$ -ketoisocaproic acid (19)]. The reason for this non-protonated positions preference is that the visibility of the hyperpolarized signals is limited in time by the decay of the hyperpolarization in the liquid state according to the spin-lattice relaxation time ( $T_1$ ) of the specific carbon position, and that non-protonated carbon-13 nuclei generally show longer  $T_1$  than protonated positions.

However, in the choline molecule, all of the carbon positions are protonated. This called for a different approach when designing this molecular probe for use as a  $^{13}\text{C}$ -hyperpolarized molecular probe. We describe a comprehensive analysis of chemical shifts and  $T_1$  properties of the various nuclei in the choline molecule. Then, we describe the replacement of proton positions with deuterium nuclei. This is a known approach to prolong the  $T_1$  of an adjacent spin  $\frac{1}{2}$  nucleus (e.g.  $^{13}\text{C}$ ), in a manner that is critically dependent on the conformation taken by the molecule in solution (20). Theoretically, substitution of one proton by one deuteron may lead to a 16-fold increase in the  $T_1$  relaxation time of adjacent carbons (21). However, the extent of this effect ranged experimentally between none and 7-fold increase in  $T_1$  (21). Moreover, the extent of this effect in specific molecules is not readily predictable. Consequently, the aim of this study was to explore the effects of deuteration on carbon positions in the choline molecule, in order to aid the design of a new choline molecular probe towards monitoring of hyperpolarized choline metabolism. The feasibility of such metabolic monitoring was demonstrated *in vitro* by an enzymatic conversion of this molecular probe in a hyperpolarized state to hyperpolarized acetylcholine.

## 2. Results

We describe the design of a choline analog which contains a nucleus with long  $T_1$  that will report on hyperpolarized metabolic chemical transformations by chemical shift changes. The  $^{13}\text{C}$  chemical shifts of the methylene positions in choline and its metabolites (acetylcholine, phosphocholine, and betaine) were previously shown to be suitable for the monitoring of the relevant metabolic pathways ( $\Delta\delta \geq 1$  ppm, Table 1) (11,12). In particular, choline's position 2 is advantageous for monitoring the metabolism to acetylcholine due to its large chemical shift difference *vis-à-vis* choline ( $\Delta\delta = 2.9$  ppm, Table 1) and the other choline metabolites – phosphocholine ( $\Delta\delta = 2$  ppm, Table 1) and betaine ( $\Delta\delta = 1.6$ , Table 1) (11).

The  $T_1$ s of the protonated methylenes in choline were, however, found to be in the range of 3–5 s (Table 2), which is too

**Table 1.** Chemical shifts of nuclei in choline and stable-isotope labeled choline analogs in water-based solutions.

Position	$\delta$ (ppm)
$^{13}\text{C}$	
Choline- $\text{CH}_2\text{-O}$	57.9 <sup>b</sup> , 56.5 <sup>a</sup>
Acetylcholine- $\text{CH}_2\text{-O}$	60.4 <sup>b</sup> , 59.2 <sup>a</sup>
Choline-N- $\text{CH}_2\text{-}$	69.6 <sup>b</sup> , 68.3 <sup>a</sup>
Acetylcholine-N- $\text{CH}_2\text{-}$	66.7 <sup>b</sup> , 65.4 <sup>a</sup>
Choline-N- $\text{CH}_3$	55.1 <sup>b</sup>
Acetylcholine-N- $\text{CH}_3$	55.1 <sup>b</sup>
Acetylcholine-CO	174.8 <sup>b</sup>
Acetylcholine- $\text{CH}_3$	22.8 <sup>b</sup>
Phosphocholine- $\text{CH}_2\text{-O}$	58.9 <sup>a</sup>
Phosphocholine-N- $\text{CH}_2\text{-}$	67.4 <sup>a</sup>
Betaine- $\text{CH}_2\text{-O}$	170.2 <sup>a</sup>
Betaine-N- $\text{CH}_2\text{-}$	67.0 <sup>a</sup>
$^{15}\text{N}$ and $^{14}\text{N}^c$	
Choline- $^{15}\text{N}$	$\sim 43.37^d$
Phosphocholine- $^{15}\text{N}$	$\sim 43.54^d$
Choline- $^{15}\text{N}$	26.740 ( $^{+}\text{NH}_4$ at 0 ppm)
Acetylcholine- $^{15}\text{N}$	26.746 ( $^{+}\text{NH}_4$ at 0 ppm)
Choline- $^{14}\text{N}$	27.3 ( $^{+}\text{NH}_4$ at 0 ppm) <sup>e</sup>
Betaine- $^{14}\text{N}$	26.6 ( $^{+}\text{NH}_4$ at 0 ppm) <sup>e</sup>
$^1\text{H}$ (TSP- $d_4$ at 0 ppm)	
Choline- $\text{CH}_2\text{-O}$	4.07
Acetylcholine- $\text{CH}_2\text{-O}$	4.57
Choline-N- $\text{CH}_2\text{-}$	3.52
Acetylcholine-N- $\text{CH}_2\text{-}$	3.74
Choline- $\text{CH}_3$	3.21
Acetylcholine- $\text{CH}_3$	3.22

<sup>a</sup>Reference (11);

<sup>b</sup>TSP- $d_4$  at  $-0.12$  ppm;

<sup>c</sup> $^{15}\text{N}$  and  $^{14}\text{N}$  are exchanged as they are generally in agreement, as previously described (40);

<sup>d</sup>reference (24);

<sup>e</sup>reference (41).

short to allow the visualization of the potentially hyperpolarized carbon-13 nuclei of this compound. Instead, full or partial deuteration of choline resulted in a 7- and 8-fold increase in the  $T_1$  of the methylene and the methyl carbons at 11.8 T and 25 °C, respectively (Table 2). Specifically, the substitution with deuterium of directly bonded protons was found to be sufficient for this  $T_1$  elongation, as evidenced by the similarity in the  $T_1$  of the methylene positions in the partly and fully deuterated analogs. The inversion recovery experiments using the fully and partially deuterated choline molecules as well as the native choline molecule were performed at  $^{13}\text{C}$  natural abundance. The combination of a long relaxation delay (in the deuterated compounds) and the fact that no deuterium decoupling was activated made the signal-to-noise ratio per acquisition time very low, therefore requiring long acquisition times of a few days (an example is provided as Supporting Information). In light of these results, a choline analog labeled with both carbon-13 and deuterium, [1,1,2,2- $\text{D}_4$ - $^{13}\text{C}$ ]choline chloride, was chosen as a candidate to demonstrate the metabolism of choline to acetylcholine.

A DNP preparation was made for [1,1,2,2- $\text{D}_4$ - $^{13}\text{C}$ ]choline chloride which takes advantage of the high water solubility of

**Table 2.**  $^{13}\text{C}$   $T_1$  relaxation times of native, fully, and partially deuterated choline<sup>a</sup>

		$T_1$ (s)		
		$C_1$	$C_2$	$C_3$
A	Choline $\text{Cl}^-$ (20 mM)	5.2 (4.5, 5.9)	4.9 (4.4, 5.4)	3.5 (2.9, 4.0)
B	$[\text{D}_{13}]$ -choline $\text{Br}^-$ (507 mM), RD = 80 s	32 (28, 35)	33 (30, 35)	27 (25, 30)
C	$[1,1,2,2\text{-D}_4]$ -choline $\text{Cl}^-$ (515 mM), RD = 130 s	29 (25, 33)	35 (26, 43)	2.4 (−7, 12)
	Elongation factor B/A	6.2	6.7	7.8
	Elongation factor C/A	5.6	7.1	0.7

<sup>a</sup>At 11.8 T, in purified water containing 10%  $\text{D}_2\text{O}$ . Curve fitting results are given with 95% confidence intervals. RD, relaxation delay in the inversion recovery study.

choline chloride. The water-based DNP preparation had the advantage that it makes a glass at low temperatures and thereby ensures a homogenous distribution of the free radical in the preparation – a prerequisite for obtaining high polarization on a reasonable time-scale. The build-up time constant for the polarization of  $[1,1,2,2\text{-D}_4,2\text{-}^{13}\text{C}]$ choline chloride in this preparation averaged at  $30 \pm 2$  min ( $n = 4$ ), meaning that 95% polarization can be achieved within about 1.5 h. The liquid state polarization of  $[1,1,2,2\text{-D}_4,2\text{-}^{13}\text{C}]$ choline was found to be  $24 \pm 8\%$  ( $n = 4$ ) at the time of measurement (*ca* 20 s following dissolution).

In order to verify that the hyperpolarized state is maintained during an enzymatic reaction and in order to monitor the hyperpolarized behavior of the resulting  $[1,1,2,2\text{-D}_4,2\text{-}^{13}\text{C}]$ acetylcholine, the reaction of carnitine acetyltransferase was employed. The choline dedicated enzyme, choline acetyltransferase, was unavailable for purchase from commercial sources, although the assay for this enzyme from rodent brain is well established (22). However, in order to avoid non-essential animal sacrifice, we searched for an alternative enzymatic acetylcholine synthesis assay. A previous study suggested that in human placental cells the carnitine acetyltransferase enzyme is capable of converting choline to acetylcholine with a similar  $K_m$  to that of the carnitine reaction but with a 10-fold lower  $V_{\text{max}}$  (23). Therefore, we investigated whether the enzyme carnitine acetyltransferase could be used to monitor acetylcholine synthesis. We note that the purpose of such investigation was not to accurately determine the rate of the carnitine acetyltransferase reaction on the labeled choline but rather to provide a tool to observe the synthesis of hyperpolarized acetylcholine in real time and determine its  $T_1$  in a hyperpolarized state.

The carnitine acetyltransferase was found to convert choline to acetylcholine albeit with a slow rate. Nevertheless, it provided an efficient way to study the properties of this enzymatic conversion in a hyperpolarized state. The conversion of native choline to acetylcholine was monitored using thermal equilibrium (conventional)  $^1\text{H}$ -NMR, as shown in Fig. 1(A, B). Four minutes after the start of the reaction (Fig. 1B, lower trace), the resonances at 3.2 ppm of the trimethylamine moieties show mainly the choline signal (3.20 ppm) with a small signal from acetylcholine at 3.21 ppm. By 45 min of the reaction, the trimethylamine signal of acetylcholine is clearly visible (Fig. 1B, upper trace). The calculated reaction rate constant averaged at  $1.2 \pm 0.4/\text{min}$  per unit  $\times 10^{-3}$  (0.9 and  $1.5/\text{min}$  per unit  $\times 10^{-3}$ ,  $n = 2$ ).

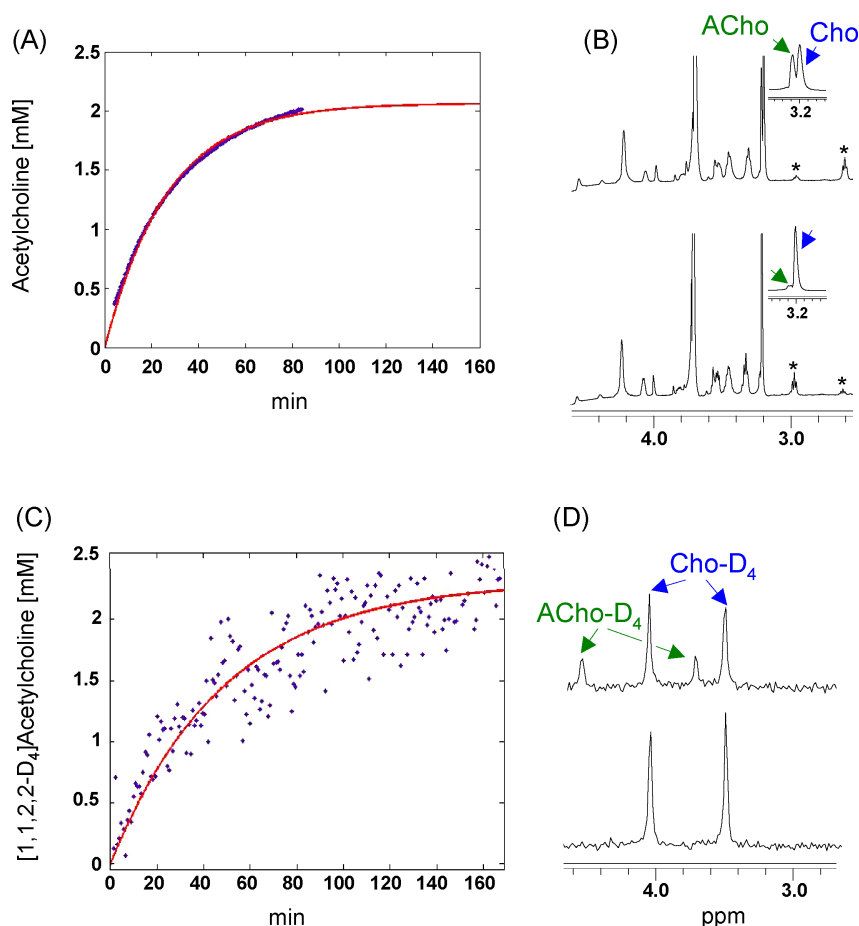
To verify that the deuteration of the methylene positions does not prohibit this conversion, the conversion of  $[1,1,2,2\text{-D}_4]$ choline to  $[1,1,2,2\text{-D}_4]$ acetylcholine was monitored using  $^2\text{H}$ -NMR. A

typical study is shown in Fig. 1(C, D). At 1.4 min from the beginning of the reaction, only the signals of  $[1,1,2,2\text{-D}_4]$ choline were observed (Fig. 1D, lower trace). By 119 min, the signals of  $[1,1,2,2\text{-D}_4]$ acetylcholine were clearly visible as well (Fig. 1D, upper trace). The time course of this reaction is shown in Fig. 1(C). The lower signal-to-noise ratio of the deuterated acetylcholine signal on the  $^2\text{H}$  spectra compared with that of the proton signals is visible when comparing the time course plots. The calculated reaction rate constant was  $0.6 \pm 0.2/\text{min}$  per unit  $\times 10^{-3}$  ( $n = 2$ ). This rate was of the same order of magnitude as that of the reaction using native choline. We note that the reaction rate constant of the carnitine acetyltransferase enzyme for its natural substrate carnitine, was much higher than the reaction rate constant for choline ( $75.3/\text{min}$  per unit  $\times 10^{-3}$ ,  $n = 1$ ).

This enzymatic reaction was utilized then on  $[1,1,2,2\text{-D}_4,2\text{-}^{13}\text{C}]$ choline in a hyperpolarized state at 14.1 T. Indeed, the conversion of  $[1,1,2,2\text{-D}_4,2\text{-}^{13}\text{C}]$ choline to  $[1,1,2,2\text{-D}_4,2\text{-}^{13}\text{C}]$ acetylcholine was visible in the hyperpolarized state as shown in Fig. 2. By fitting the data to eqn (4) (see Experimental), it was found that  $[1,1,2,2\text{-D}_4,2\text{-}^{13}\text{C}]$ acetylcholine was formed with a rate constant of  $0.7 \pm 0.2/\text{min}$  per unit  $\times 10^{-4}$  ( $n = 2$ ), which is lower than the rate of the reaction at thermal equilibrium. We attribute this difference in reaction rates to the use of enzyme from different sources (see Experimental section). We note, however, that despite the higher reaction rate in the studies at thermal equilibrium, the product was discernible after a few minutes (Fig. 1), while at the hyperpolarized state (with lower reaction rate) the product was visible already within 15 s. The curve fitting of the results yielded also the  $T_1$  value for the  $^{13}\text{C}$  nucleus of  $[1,1,2,2\text{-D}_4,2\text{-}^{13}\text{C}]$ acetylcholine, which was found to be  $34 \pm 1$  s ( $n = 2$ ). Under the same conditions, (14.1 T and  $37^\circ\text{C}$ ), the  $T_1$  of  $[1,1,2,2\text{-D}_4,2\text{-}^{13}\text{C}]$ choline was found to be  $47 \pm 2$  s ( $n = 4$ ).

### 3. Discussion

The design of a new molecular probe for metabolic studies using hyperpolarized spectroscopy is based on considerations of both the chemical shift difference between substrate and product and the  $T_1$  relaxation time of the observed nucleus in both. An attractive option with regard to  $T_1$  would be to use the  $^{15}\text{N}$  nucleus of choline as the hyperpolarized nucleus in the molecular probe since this quaternary  $^{15}\text{N}$  position in choline was previously shown to have an exceptionally long  $T_1$  *in vitro* (24) and *in vivo* (25). Unfortunately, however, the chemical shift difference for this  $^{15}\text{N}$  position between choline and its main metabolites are of the



**Figure 1.** Acetylcholine production by carnitine acetyltransferase. (A) Time course of acetylcholine production. (B) <sup>1</sup>H spectra of carnitine acetyltransferase reaction using non-labeled choline as a substrate. Cho and AChO – the trimethyl amine moiety in choline and acetylcholine, respectively. \*Signals of Acetyl Coenzyme A methylenes. Changes in intensity reflect changes in the concentrations of the acetylated (decrease) and non-acetylated (increase) in the coenzyme forms which are corresponding to the reaction progress. (C) Time course of [1,1,2,2-D<sub>4</sub>]acetylcholine production. (D) <sup>2</sup>H spectra of carnitine acetyltransferase reaction using [1,1,2,2-D<sub>4</sub>]choline as a substrate. Cho-D<sub>4</sub> – [1,1,2,2-D<sub>4</sub>]choline, AChO-D<sub>4</sub> – [1,1,2,2-D<sub>4</sub>]acetylcholine.

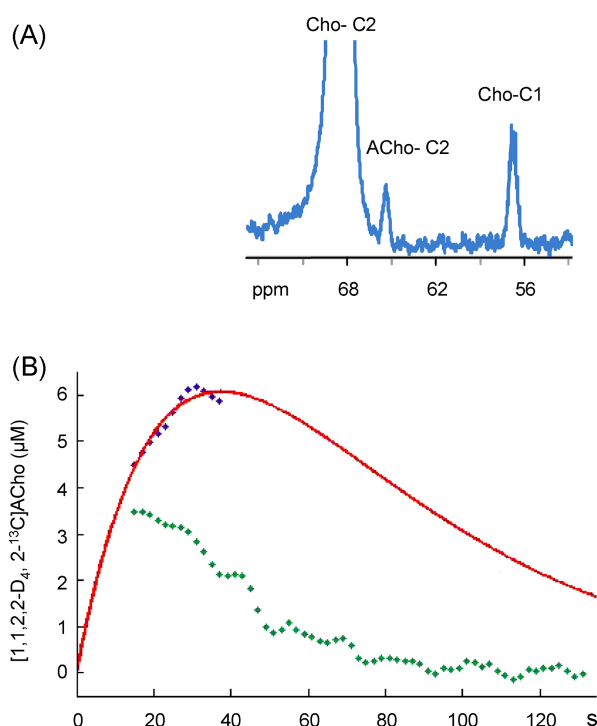
order of fractions of a ppm (Table 1). The very long  $T_1$  of the <sup>15</sup>N position in choline has prompted the suggestion to hyperpolarize <sup>15</sup>N-choline, inject, wait and transfer the polarization to protons for observations (26). The applicability of such a spectroscopic approach has, however, not been tested *in vivo* and may be challenging to combine with water-suppression.

Either protons or deuterons were excluded from serving as reporting hyperpolarized nuclei due to their short  $T_1$  (Supplementary Information). However, we note that the chemical shift differences of the methylenic positions in choline and its metabolites on proton and deuterium spectra are in principle sufficient for resolution between choline and its metabolites (Table 1 and Fig. 1). In order to identify positions for <sup>13</sup>C stable isotope labeling of the choline molecule in a manner that will allow hyperpolarized monitoring of metabolism, the chemical shifts of the various <sup>13</sup>C positions of choline, acetylcholine (see the enzymatic reaction and the nuclei numbering in Fig. 3), and other choline metabolites were characterized (Table 1).

The design of a <sup>13</sup>C molecular probe for hyperpolarized MR involves a different set of requirements in comparison to metabolic monitoring using <sup>13</sup>C at thermal equilibrium. These requirements consist of a longer rather than shorter  $T_1$ , and consequently a minimal rather than maximal number of protons

at the proximity of the labeled <sup>13</sup>C nucleus. In studies at thermal polarization, the Nuclear Overhauser Effect (NOE) of protons is used for signal enhancement of <sup>13</sup>C, while in hyperpolarized studies nearby protons serve as a cause of  $T_1$  shortening resulting from strong intramolecular dipolar interactions. For this reason, the previous molecular probes for DNP hyperpolarization utilized non-protonated carbon positions (14–19). However, the vast majority of biochemically informative molecules either do not contain such carbon positions or contain such positions but do not allow metabolic follow-up due to too small chemical shift change between substrate and product. In the case of choline, such long  $T_1$  carbon positions are not available.

In order to capitalize on the chemical shift dispersion of <sup>13</sup>C labeled choline (and metabolites) we investigated the ability to prolong the  $T_1$  of <sup>13</sup>C positions that have attached protons. To this end, the deuterium substitution effect on relaxation times (20) was investigated. The  $T_1$  relaxation times of the carbon positions in choline were found to increase substantially upon deuteration of the molecule, to the extent where the  $T_1$  of the deuterated carbon positions in choline were comparable to those of the reported hyperpolarized molecular probes. Therefore, it is demonstrated that deuteration of specific carbon positions may enable the utilization of such positions in hyperpolarization



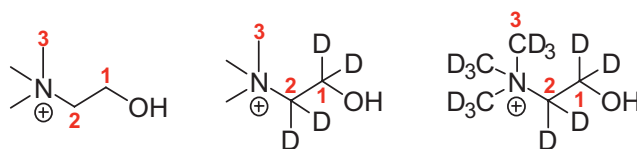
**Figure 2.** Enzymatic formation of hyperpolarized  $[1,1,2,2\text{-D}_4, 2\text{-}^{13}\text{C}]$ acetylcholine. (A)  $^{13}\text{C}$  spectrum of hyperpolarized  $[1,1,2,2\text{-D}_4, 2\text{-}^{13}\text{C}]$ choline and  $[1,1,2,2\text{-D}_4, 2\text{-}^{13}\text{C}]$ acetylcholine. This spectrum demonstrates the sum of 50 transients, each acquired with a  $10^\circ$  pulse and a repetition time of 2 s (total scan time of 100 sec). The signal of the labeled carbon in choline and acetylcholine is shown at 68.3 ppm and 65.4 ppm, respectively. The non- $^{13}\text{C}$  enriched methylene of  $[1,1,2,2\text{-D}_4, 2\text{-}^{13}\text{C}]$ choline (position 1) is seen at 56.6 ppm. Although this position is not  $^{13}\text{C}$  labeled, it is visible in the hyperpolarized state. (B) Time course of hyperpolarized  $[1,1,2,2\text{-D}_4, 2\text{-}^{13}\text{C}]$ acetylcholine formation. Each data point represents the sum of 8 spectra, in a moving averaging window. The signal was normalized to  $\mu\text{M}$  units as described in the Experimental section. The plot shows the original normalized signal intensities of  $[1,1,2,2\text{-D}_4, 2\text{-}^{13}\text{C}]$ acetylcholine (green) and the corresponding intensities after a point by point correction for the effect of  $20^\circ$  RF pulses (blue) as described in the experimental section. The corrected data points with original significant signal ( $\text{SNR} > 5$ ) were used for kinetic analysis and curve fitting was performed according to eqn (4). In this experiment, the  $T_1$  of  $[1,1,2,2\text{-D}_4, 2\text{-}^{13}\text{C}]$ acetylcholine was 35 s and the reaction rate constant ( $k$ ) was  $0.8/\text{min}$  per unit  $\times 10^{-4}$ .

studies by substantially increasing their  $T_1$  relaxation times to values that allow visualization of the hyperpolarized compound and potentially its chemical evolution. We note that this effect was quantified here at 11.8 T. Further investigation of the possible field dependence of this effect is underway.

In addition it was found that direct substitution of protons by deuterons was the most effective. In evidence, the  $T_1$  of the methylene positions was similar in the fully and the partially deuterated compounds, suggesting that in the choline molecule



**Figure 3.** The enzymatic conversion of choline to acetylcholine by acetyltransferase.



**Figure 4.** The molecular structure of choline, choline- $\text{D}_4$ , and choline- $\text{D}_{13}$ .

the deuterium substitution effect on relaxation times is predominant in the directly bonded deuterons. Therefore, we selected the partially deuterated analog for further molecular probe development. This analog has closer similarity to the natural choline molecule because the structure of its trimethylamine moiety is preserved (Fig. 4). To the best of our knowledge, this is the first report utilizing such deuterated methylenic positions in a DNP hyperpolarization study. Specifically, the methylene position adjacent to the nitrogen nucleus in choline (C2) is advantageous for monitoring the metabolic pathway of choline to acetylcholine (Table 1) (11). The methylene position adjacent to the hydroxyl moiety (C1) also shows a large chemical shift difference between choline and acetylcholine. However, the chemical shift of this position in acetylcholine is very similar to the respective position in phosphocholine ( $\Delta\delta = 0.3$  ppm, Table 1). For this reason, choline labeled with carbon-13 at the C2 position appears useful for unequivocal monitoring the conversion of choline to acetylcholine. The  $T_1$  reached in these newly designed deuterated choline analogs warranted further enzymatic studies, and also *in vivo* studies, since successfully hyperpolarized MRSI has been reported with agents of much lower  $T_1$  (16 s) (18).

Accordingly, an enzymatic conversion of choline to acetylcholine was performed using an acetyltransferase enzyme that was not dedicated to choline (but to carnitine). Therefore, the rate of this reaction was not known. Previous reports suggested that the activity of this enzyme is about 10-fold lower for choline than for carnitine (23). This trend appeared to be even greater with the purchased enzyme (showing about 60-fold higher reaction rate for carnitine). In addition, the deuteration of the methylene position may, in principle, lead to a change in the reaction rate due to the isotopic effect of the proton–deuterium substitution. For this reason it was important to demonstrate the feasibility of this particular reaction. The reaction kinetics at thermal equilibrium spin polarization showed that the linear phase of the reaction lasts about 10 min and therefore any reaction rate determined during this interval is likely to produce similar results. For the enzymatic studies at the hyperpolarized state, this has two implications: (1) at the time scale of the hyperpolarized observation (1–2 min) the reaction is at its linear phase and far from completion and (2) the level of Acetyl CoA could be lowered due to its co-factor role and the low product level that is anticipated at the time scale of the hyperpolarized observation.

For the reactions at thermal equilibrium ( $^1\text{H}$  spectroscopy), the use of  $\text{D}_2\text{O}$ -based reaction mixture was required due to the long adjustment time for the water-suppression pulse sequence on our spectrometer. To be able to monitor the first minutes of the reaction with native choline, using proton spectroscopy, we used the  $\text{D}_2\text{O}$  based preparation where water-suppression was not needed. Previous studies showed that the rate of choline acetyltransferase reaction is about 2-fold lower in  $\text{D}_2\text{O}$  compared with  $\text{H}_2\text{O}$  (27). However, the effect of  $\text{D}_2\text{O}$  on carnitine acetyltransferase reaction rate using a choline substrate is not known.



Interestingly, the  $T_1$  of hyperpolarized [1,1,2,2- $D_4$ ,2- $^{13}C$ ]acetylcholine was found to be high (34 s). This long product relaxation time in a hyperpolarized state further supports the potential of this choline molecular probe to serve as a biomarker *in vivo*, where the elongated  $T_1$  is crucial for observation of the reaction at the target area. However, the  $T_1$  of [1,1,2,2- $D_4$ ,2- $^{13}C$ ]choline was higher when measured as the decay of the hyperpolarized state (47 s) than when measured at thermal equilibrium spin polarization (35 s). We attribute this difference to the differences in temperature and magnetic field strength. The contribution of each of these parameters and possibly the effect of the medium is currently under investigation.

We conclude that the hyperpolarized [1,1,2,2- $D_4$ ,2- $^{13}C$ ]choline chloride is a promising new molecular probe for hyperpolarized MR. In particular, further studies with this probe will focus on the *in vivo* application of this probe in the brain. Based on the current study, considerations can be made which are relevant for such future studies. These include choline brain concentrations, the rate of acetylcholine synthesis, and choline toxicity relative to the sensitivity of the described method. We note that a specialized choline transporter is expressed in the blood–brain barrier, with a  $K_m$  of 39–42  $\mu M$  and a  $V_{max}$  of up to 3.1 nmol/min/g (28). Therefore it is expected that the level of choline in the brain could reach approximately 3.1  $\mu M$  following exposure to 1 min of high choline concentration in the plasma (higher than about 80  $\mu M$ ). This level appears reasonable for monitoring by hyperpolarized MR, considering that carbon-13 substrates at millimolar levels can be monitored using conventional spectroscopy and that hyperpolarization offers about four orders of magnitude increase in signal, although signal averaging considerations are different in hyperpolarized spectroscopy. The accumulation of choline in the brain following various administration routes and doses has been investigated previously (29–35). Specifically, following an intravenous bolus injection, (which is the preferred route for hyperpolarized media administration), the amount of choline that accumulated in the brain was previously found to be between 0.2 and 14% (30–32) of the total injected dose. Encouraging results have been recently shown by Cudalbu *et al.*, who have been able to monitor the signal of hyperpolarized  $^{15}N$ -choline in the rat brain (25).

The level of acetylcholine in the brain following an intravenous administration of choline was approximately 20% of the brain choline level (32). Choline acetyltransferase activity was previously determined in post-mortem brain tissue of control subjects to be  $0.70 \pm 0.21$  and  $1.24 \pm 0.59$  nmol acetylcholine/h/mg total protein, in the frontal and temporal cortex, respectively (36). Assuming about 8% of protein in brain tissue (37), it is expected that the rate of acetylcholine synthesis in the human cortex would be about 1.6 nmol/min/g. It remains to be seen whether such a level of hyperpolarized compound will be visible by  $^{13}C$  MRS.

Considering the high doses of hyperpolarized molecular probes reported to be intravenously injected in animals, it should be noted that the  $LD_{50}$  and  $LD_0$  levels for intravenous administration of choline in mice are 53 and 21 mg/kg, respectively (38). However, pretreatment with atropine was previously utilized to mitigate the cholinergic effects of high dose of choline (12) and the chloride counter ion is considered safe. Therefore, *in vivo* studies should be performed with a dose that is lower than the  $LD_{LO}$  for the particular species or under the protective effects of atropine. We note that, in the study on hyperpolarized  $^{15}N$ -choline, it was reported that a bolus injection

(9 s duration) to rats of 2.5 ml containing 90 mM choline chloride (ca 125 mg/kg) did not show any adverse effects (25).

Unfortunately, our groups are not equipped with a dissolution DNP polarizer that can be used for *in vivo* applications. Future studies using this molecular probe *in vivo* are underway in institutions where this technology is available.

## 4. Experimental

### 4.1. Materials

Choline chloride and [1,1,2,2- $D_4$ ]choline chloride were obtained from Sigma-Aldrich, (Rehovot, Israel). Choline- $D_{13}$  bromide was obtained from Cambridge Isotope Laboratories (Andover, MA, USA). The molecular structure of these compounds is shown in Fig. 4. [1,1,2,2- $D_4$ ,2- $^{13}C$ ]choline chloride was donated by BrainWatch Ltd (BW-42, BrainWatch Ltd, Tel-Aviv, Israel). Trityl free radical [OX063, trityl radical (methyl-tris[8-carboxy-2,2,6,6-tetrakis[(2-hydroxyethyl)benzo[1,2-d:4,5-d'] bis[1,3] dithiol-4-yl]trisodium salt, molecular weight = 1427 g/mol] was obtained from GE Healthcare (London, UK). ProHance was purchased from Bracco Diagnostics Inc. (Italy). Carnitine acetyltransferase from pigeon breast muscle (E.C. 2.3.1.7) was obtained from two sources: for enzymatic studies at thermal equilibrium, an enzyme from Sigma-Aldrich (Rehovot, Israel) and for the hyperpolarized enzymatic studies, an enzyme from Roche Diagnostics, (Switzerland). Acetyl CoA was obtained from Sigma-Aldrich (Rehovot, Israel).

#### 4.1.1. NMR spectrometers

Studies at thermal equilibrium were carried out on an 11.8 T high-resolution spectrometer (Varian, Palo Alto, CA, USA) with a 5 mm direct multinuclei probe. Studies at a hyperpolarized state were carried out at 14.1 T (Bruker) with a 5 mm inverse detection probe.

#### 4.1.2. $T_1$ relaxation at thermal equilibrium

$T_1$  measurements were performed with the standard inversion recovery pulse sequence. For carbon-13 at natural abundance (low signal-to-noise ratio, SNR), the number of excitations ranged between 300 and 400 per inversion delay with a total scanning time of up to 67 h due to a relaxation delay of up to 130 s. The data were fitted to the standard inversion recovery equation using the Matlab software (The MathWorks Inc., Natick, MA, USA).

#### 4.1.3. $^{13}C$ , $^2H$ , and $^1H$ NMR spectra of [1,1,2,2- $D_4$ ,2- $^{13}C$ ]choline chloride

Aliquots of 106 mM were dissolved in purified  $H_2O$  and the spectra were acquired with the following parameters:  $^{13}C$  – 3.3 s repetition time, eight transients,  $90^\circ$  flip angle, NOE and proton decoupling were applied;  $^2H$  – 2.8 s repetition time, 64 transients,  $90^\circ$  flip angle;  $^1H$  – 1.4 s repetition time, 16 transients,  $90^\circ$  flip angle. The resonances observed in these spectra were as follows.  $^{13}C$  spectrum: 55.5 ppm, singlet, methyls at natural abundance; 68.3 ppm, multiplet 1:2:3:2:1, each split to three 1:1:1,  $J_{C-D} = 22.07$  Hz,  $J_{C-14N} = 2.7$  Hz,  $N-^{13}CD_2$ -.  $^1H$  spectrum: 3.2 ppm, singlet, methyls.  $^2H$  spectrum: 3.33 ppm, doublet,  $J_{C-D} = 22.07$  Hz,  $-N-^{13}CD_2$ -. 3.88 ppm, singlet  $-CD_2-OH$  (HDO at 4.7 ppm).

## 4.2. Hyperpolarization

A typical preparation for the hyperpolarization experiment: [1,1,2,2-D<sub>4</sub>,2-<sup>13</sup>C]choline chloride (27.3 mg, 189 μmol) was mixed with 7.1 mg of an aqueous solution of OX063 (61 mM) and ProHance (2.9 mM). The concentrations in the final DNP preparation were choline 6M, OX063 14 mM, and Prohance 0.7 mM. The DNP sample was hyperpolarized in a home-build dissolution DNP polarizer at approx. 1.3 K, 93.9 GHz and 100 mW as previously described (13).

## 4.3. Enhancement factor calculation

Dissolution was performed with 4 ml of 40 mM phosphate buffer (pH 7.3) containing 100 mg/l EDTA. The sample was collected directly into a 10 mm NMR tube with a final [1,1,2,2-D<sub>4</sub>,2-<sup>13</sup>C]choline chloride concentration ranging between 20 and 46 mM. Transfer time was approximately 20 s measured as time between dissolution and first pulse taken in the spectrometer. Calculation of the enhancement and percentage polarization in solution was performed by dividing the hyperpolarized signal (single scan, 5° pulse) by the thermal equilibrium signal of the same sample and considering that the thermal polarization at 14.1 T and 37 °C is 11.7 ppm. For the thermal equilibrium measurement, 70 μl of Omniscan (0.5 M gadolinium complex) were added to the sample to ensure the decay of the hyperpolarized state and to shorten the T<sub>1</sub> to allow for fast averaging. Because of the low SNR of the sample at thermal equilibrium, this signal was averaged 512 times using the same acquisition parameters and a repetition time of 3.5 s. Signal averaging was accounted for in the enhancement factor and polarization calculation.

## 4.4. Enzymatic reactions at thermal equilibrium

### 4.4.1. Formation of acetylcholine

Forty units of carnitine acetyltransferase were dissolved in 60 mM TRIS buffer in D<sub>2</sub>O containing 5 mM Acetyl CoA in a 5 mm NMR tube. Choline chloride (5 mM) was added to the reaction mixture, and the tube was gently mixed and quickly placed in the spectrometer for monitoring by <sup>1</sup>H-NMR.

### 4.4.2. Formation of [1,1,2,2-D<sub>4</sub>]acetylcholine

Forty units of carnitine acetyltransferase were dissolved in 60 mM TRIS buffer containing 5 mM acetyl CoA in a 5 mm NMR tube. [1,1,2,2-D<sub>4</sub>]choline chloride (5 mM) was added to the reaction mixture, and the tube was gently mixed and quickly placed in the spectrometer for monitoring by <sup>2</sup>H-NMR.

## 4.5. Enzymatic reactions in the hyperpolarized state

Unfortunately, the manufacturer (Sigma-Aldrich) discontinued the supply of the enzyme in the form that was available for the studies at thermal equilibrium. The new product line contained a much diluted concentration of the enzyme in a solution of high ammonia concentration. This product was not useful for the hyperpolarized studies; therefore, the enzyme that was used in the hyperpolarized studies was purchased from a different source (Roche).

Eighty units of carnitine acetyltransferase were dissolved in 46 mM TRIS buffer containing 1.25 mM Acetyl CoA (500 μl). Hyperpolarized [1,1,2,2-D<sub>4</sub>,2-<sup>13</sup>C]choline chloride (to a final concentration of 4 mM) dissolved in 100 mM TRIS buffer

(100 μl) was added to the reaction mixture, then the NMR tube was gently mixed and quickly placed in the spectrometer. Seven seconds after mixing, a total of 128 traces were recorded at 37 °C with either 10 or 20° pulse and 2 s between pulses.

## 4.6. Enzyme kinetics of acetylcholine formation at thermal equilibrium

The time course data were fitted to the exponential equation for a first order kinetics yielding the rate constant *k*.

## 4.7. Enzyme kinetics of acetylcholine formation at hyperpolarized state

The change in the signals of hyperpolarized [1,1,2,2-D<sub>4</sub>,2-<sup>13</sup>C]choline and [1,1,2,2-D<sub>4</sub>,2-<sup>13</sup>C]acetylcholine are determined by the reaction kinetics, but also by the decay of the hyperpolarized state of both the substrate ([1,1,2,2-D<sub>4</sub>,2-<sup>13</sup>C]choline) and the product ([1,1,2,2-D<sub>4</sub>,2-<sup>13</sup>C]acetylcholine) due to both the T<sub>1</sub> relaxation and the RF pulsation. To correct for the effect of RF pulsation, a point by point correction was applied to the data prior to curve fitting and rate determination. Each data point was corrected according to  $I_n' = I_n / (\cos\theta)^n$ , where *n* is the index number of the spectra acquired (and the number of times the RF pulse was applied at the specific time point at a constant flip angle *θ*), *I<sub>n</sub>* is the measured intensity, and *I<sub>n</sub>'* is the corrected intensity, as previously described (39).

The time course data can be described by the following set of differential equations and their solutions. The change in the corrected signal (*I<sub>n</sub>'*) of hyperpolarized choline (*M<sub>z</sub><sup>cho</sup>*) with time (*t*) is dependent on *k* and the decay due to spin lattice relaxation. This dependency is described in eqn (1), where T<sub>1</sub><sup>cho</sup> is the spin lattice relaxation time of choline in the magnet.

$$\frac{dM_z^{cho}(t)}{dt} = -k \cdot M_z^{cho}(t) - \frac{1}{T_1^{cho}} \cdot M_z^{cho}(t) \quad (1)$$

The solution to this equation is given in eqn (2):

$$M_z^{cho}(t) = [cho]_0 \cdot e^{\left[-k - \frac{1}{T_1^{cho}}\right] \cdot t} \quad (2)$$

where [cho]<sub>0</sub> is the concentration of [1,1,2,2-D<sub>4</sub>,2-<sup>13</sup>C]choline at the beginning of the reaction (4000 μM).

Similarly to the choline signal, the change in the corrected signal of acetylcholine (*M<sub>z</sub><sup>Acho</sup>*) is dependent on the instantaneous signal of choline as well as on *k*, and the decay due to acetylcholine spin lattice relaxation as described in eqn (3):

$$\frac{dM_z^{Acho}(t)}{dt} = k \cdot M_z^{cho}(t) - \frac{1}{T_1^{Acho}} \cdot M_z^{Acho}(t) \quad (3)$$

The solution to this first order nonhomogenous linear differential equation is given by eqn (4):

$$M_z^{Acho}(t) = e^{-\rho^{Acho} \cdot t} \cdot \left[ \frac{k \cdot [cho]_0}{\Delta\rho - k} \cdot (e^{(\Delta\rho - k) \cdot t} - 1) \right] \quad (4)$$

where

$$\rho^{cho} = \frac{1}{T_1^{cho}}$$

and

$$\rho^{Acho} = \frac{1}{T_1^{Acho}}$$

and

$$\rho^{Acho} - \rho^{cho} = \Delta\rho = \frac{1}{T_1^{Acho}} - \frac{1}{T_1^{cho}}$$

where  $M_2^{Acho}(t)$  is the corrected signal of [1,1,2,2-D<sub>4</sub>,2-<sup>13</sup>C]acetylcholine, normalized to micromolar units using the signal of the hyperpolarized choline on the first spectrum.

## 5. Supporting information

Supporting information can be found in the online version of this article: (1) <sup>13</sup>C inversion recovery study of fully deuterated choline (2) proton and deuterium T<sub>1</sub> relaxation times in choline and acetylcholine.

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