Title
Optimization of pH Imaging Methodology for Hyperpolarized 13C MRI

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Optimization of pH Imaging Methodology for Hyperpolarized 13C MRI

by

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THESIS

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of the

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Abstract

**Background:** The acidification of the tumor microenvironment is a result of extensive metabolic reprogramming in cancer cells and is linked with tumor metastasis. Hyperpolarized MRI is a method for imaging and quantifying this change in pH, but suffers from rapid signal loss from spin-lattice ($T_1$) relaxation. We propose using hydrogen/deuterium exchange on hyperpolarized $^{13}$C probes in order to prolong hyperpolarized signal by reducing $T_1$ relaxation.

**Methods:** H/D exchange was performed on several amino acids and amino acid derivatives with utility in HP-MRI. Isotopic enrichment was evaluated using $^1$H NMR. The $T_1$ relaxation constant was quantified by analyzing the decay of hyperpolarized signal of deuterated vs non-deuterated $^{13}$C compounds. **Results:** H/D exchange was successfully used to enrich compounds with deuterium with high isotopic enrichment and moderate to high chemical yield. The $T_1$ relaxation constant of all fully analyzed $^{13}$C compounds exhibited a significant increase after deuteration at 3T: $T_1$ of $^{13}$C Gly increased from 52.0±3.2 to 65.0±1.2s, $^{13}$C Ala from 52.9±2.2 to 66.4±1.7s, $^{13}$C Val from 38.1±1.1 to 49.2±0.4s. **Conclusion:** H/D exchange method described is a viable technique for inexpensive and direct deuterium labeling. Deuterium labeling be applied to hyperpolarized $^{13}$C MRI probes to prolong HP signal by lengthening $T_1$. 
Acknowledgements

This year I had the honor of completing a thesis project for MSBI, and for this I am indebted to many people. First and foremost, Dr. Flavell has been as great an advisor as I could have hoped for, and provided excellent mentorship throughout the project; for this I owe him a tremendous debt. I also thank the other members of my thesis committee: Dr. Michael Evans, Dr. Peder Larson, and Dr. David Wilson for taking time out of their busy schedules to ensure the quality of my thesis. I have learned much from the guidance of the postdoctoral fellows that I worked with: Dr. Céline Taglang and Dr. Dave Korechan; their expertise and their patience have made a scientist out of me despite my numerous mistakes. I must also thank Dr. Subramaniam Sukumar, who provided excellent guidance for polarizer and magnet usage.

The MSBI program has been an adventure from start to finish, and my colleagues in the program have made it so. I thank all the MSBI instructors and lecturers for their imparted knowledge as well. I also owe additional gratitude to the MSBI administrators: Rukayah Abdolcader, Dr. Alastair Martin, and Dr. David Saloner, for I would not be at UCSF in the first place if not for them.

Last but not least, I owe a huge thank you to my parents, my older brother Roger, and my girlfriend Parisa, and my friends for their unwavering love and support through everything. None of this would have been possible without my loved ones motivating me to be the best person I can be.
To the fine folks on 5CH. Breathe easy.
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1 Introduction

Hyperpolarized Magnetic Resonance Imaging (HP-MRI)

Hyperpolarized $^{13}$C MRI is a powerful technique that compensates for the low sensitivity of thermal MRI techniques by boosting the polarization (the longitudinal magnetization) of an injected compound[1]. $^{13}$C labeled substrate is mixed with a free radical and cooled to 0.8-1.4K in a hyperpolarizer. The polarizer irradiates the sample with microwave length radiation, transferring magnetization from the free radical to the $^{13}$C nucleus in a process known as dynamic nuclear polarization (DNP). DNP allows for increases in polarization of up to x100,000 times higher than the equilibrium polarization at clinically relevant field strengths of 1-3T[2].

With this high polarization, spectroscopic detection of low concentration metabolites and biomarkers for disease is possible with much greater signal-to-noise ratio (SNR) than conventional MR spectroscopy. However, high polarization with DNP requires an optimized hyperpolarized prep: it must have high substrate concentration (>3M), and must form an amorphous solid (glass) when brought to cryogenic temperatures[1]. Another consideration in hyperpolarized MRI is the cost of the radical compound, which can reach thousands of dollars per gram.

Applications of hyperpolarized $^{13}$C MRI

Due to the diversity of $^{13}$C probes, the applications of HP MRI are numerous and varied. Pyruvate was the first hyperpolarized $^{13}$C probe to be used in humans; it is commonly used to measure metabolic changes in cancer (e.g. prostate cancer)[1]. Glutamine metabolism to glutamate by mitochondrial glutaminase has been measured in vitro using hyperpolarized $^{13}$C glutamine[3]. pH quantification has been demonstrated in vitro and in vivo using hyperpolarized $^{13}$C ACES and bicarbonate[4, 5]. These pH measurements can be used to probe tumor acidification, which is associated with cancer growth and metastasis[4]. The ratio of pyruvate to lactate in cells is affected by the cytosolic redox potential. Park et al. used hyperpolarized $^{13}$C alanine, which converts to pyruvate and lactate downstream in metabolism, to probe the redox potential of tissues and the effects of EtOH[6]. A longer $T_1$ on these compounds can be beneficial for monitoring their metabolism, since the conversion of the probe to its metabolic products must be observed before the
hyperpolarized signal decays.

*T*<sub>1</sub> *prolongation strategy*

One major limitation of hyperpolarized $^{13}$C spectroscopy is rapid decay of the hyperpolarized signal. Once the sample leaves the polarizer, spin-lattice ($T_1$) decay will dissipate the magnetization of the $^{13}$C nuclei. Metabolism of the probe into different products over the course of the $^{13}$C NMR experiment must be faster than the $T_1$ of the hyperpolarized $^{13}$C nucleus for useful information to be gained from the experiment. $T_1$ decay is modeled by

$$M_z(t) = M_0 + (M_{z0} - M_0) e^{-t/T_1}$$  \(1\)

where $M_0$ is the longitudinal magnetization at equilibrium, $M_{z0}$ is the longitudinal polarization at $t = 0$, and $T_1$ is the exponential decay constant[7]. A longer $T_1$ nucleus results in longer-lived magnetization, a desirable characteristic in HP-MRI. The aim of this project is to increase the $T_1$ of $^{13}$C probes by replacing nearby hydrogens with deuterium ($^2$H, or D) using an exchange method.

A major mechanism of $T_1$ relaxation at clinically relevant field strengths (1-3T) is dipole-dipole coupling[8]. Dipole-dipole coupling occurs when two nuclei with non-zero spin transfer energy between each other. The rate of energy transfer in dipole-dipole coupling is dependent on the gyromagnetic ratio $\gamma$ of each nucleus[9]. In the case of HP-MRI, the hyperpolarized $^{13}$C nucleus can relax by coupling with nearby hydrogen nuclei. Replacing nearby hydrogens with deuterium may reduce the rate of energy transfer, because the gyromagnetic ratio of hydrogen is much higher than that of deuterium (42.6MHz T<sup>-1</sup> vs 6.5MHz T<sup>-1</sup>)

H/D exchange has previously been demonstrated to increase the $T_1$ of $^{13}$C in a hyperpolarized experiment[1]. Qu, et al. synthesized [5-$^{13}$C-4-$^2$H<sub>2</sub>]-L-glutamine, and compared the $T_1$ with the non-enriched glutamine[10]. The reported $T_1$ of the deuterated glutamine was 33 seconds, compared to the protonated glutamine $T_1$ of 15 seconds. One caveat is that the enriched glutamine was synthesized via a seven step synthetic process, with a reported overall yield of 44%. Long syntheses are potentially time consuming and expensive efforts, which is why alternative methods for deuterating hyperpolarized $^{13}$C probes are worthwhile aims.
Methods of H/D exchange

We propose enriching $^{13}$C HP probes with deuterium using an H/D exchange method with ruthenium catalyst. Sajiki, et al. reported that using 5% ruthenium on carbon catalyst under H$_2$ gas, they were able to achieve deuterium enrichment of alcohols at the $\alpha$ position[11]. This study also deuterated glycine, but did not explore deuteration on the chiral amino acids. Céline Taglang PhD, a postdoctoral fellow in the Flavell lab, previously enriched amino acids and amino acid derivatives with deuterium, using ruthenium nanoparticles under D$_2$ gas, in one step with full retention of stereochemistry[12]. This labeling method enriches only carbons with directly bonded electronegative atoms, such as O or N (see Figure 1). Dr. Taglang has adapted the Ru/C method to deuterating amino acids and similar molecules $\alpha$ to the carbonyl carbon.

Many HP probes have $^{13}$C at the carbonyl, because that carbon lacks directly bonded hydrogens. $^{13}$C-H bonds would lead to $T_1$’s that are completely impractical for hyperpolarized experiments (<2s)[1]. The C$_1$ carbon of HP probes is likely to relax through coupling to the $\alpha$ carbon’s bonded hydrogen. Deuterium labeling amino acids and derivative compounds at the $\alpha$ position can mitigate C-H coupling that shortens $T_1$, and H/D exchange offers straightforward method for deuterium labeling.

Project overview

The project is comprised of three parts: 1) development of the chemistry for H/D exchange, 2) screening for potential $T_1$ increase with deuteration on natural abundance compounds, 3) measurement of $T_1$ increase of deuterated hyperpolarized $^{13}$C compounds.
2 Materials and Methods

Deuteration reaction protocol

A serum vial was charged with substrate (~1 mmol), and 5% Ru/C catalyst (40% w/w). The solid reagents were dissolved in 5 mL of D$_2$O and a magnetic stir bar was added. The vial was sealed and a H$_2$ gas balloon was inserted into the vial. The atmosphere in the vial was purged five times and replaced with hydrogen. The vial was set on a hot plate and heated at 80°C overnight while stirring. After reacting, the vial was removed from heat and allowed to come down to room temperature. The hydrogen gas was purged five times and the solution was filtered on a 0.22 µm syringe filter. After treatment with activated carbon and purification on Dowex 50WX8 resin, the product was lyophilized to give a white solid.

Determination of isotopic enrichment

Isotopic enrichment is the degree that the abundance of a particular isotope (in this case $^2$H) is altered. We measured isotopic enrichment by NMR Spectroscopy using the 400MHz Bruker spectrometer. 15 mg of compound was dissolved in 600 µL of D$_2$O. An internal standard was added to the same concentration. 1D $^1$H spectra and $^{13}$C spectra were acquired for the protonated and deuterated samples. The proton spectra peaks were assigned and the areas compared between the protonated and deuterated samples.
Figure 2: Spectrum of 2-1H alanine (black) and 2-2H alanine (red). Spectra processed with ACD/NMR Processor.

An example spectra is shown in Figure 2. The peak at 3.8ppm arises from the hydrogen bonded to the $\alpha$ carbon. This peak is split by the three methyl hydrogens, causing a quartet splitting pattern. Deuteration occurs at this position, thus the peak area is reduced from 1 to 0.03, indicating an isotopic enrichment of about 97%.
In Figure 3, the peak from the methyl hydrogens is shown close up. This peak is no longer split after deuteration because deuterium couples much more weakly with the methyl hydrogens ($\gamma_H > \gamma_D$).

**Screening $T_1$ of natural abundance deuterated compounds**

Screening was done on $^{12}$C compounds due to the expense of hyperpolarization on $^{13}$C labeled compounds. To get a preliminary measurement of $T_1$ increase, protonated and deuterated NMR samples were dissolved (1M in 600µL H$_2$O). A saturation recovery pulse sequence was applied using the 500MHz Bruker NMR spectrometer. A series of ten 90° RF pulses excited the sample with increasing TR’s, and the measured peak height was fitted to:

$$M_z(t) = M_{z0} \left( 1 - e^{-\frac{t}{T_1}} \right)$$  \hspace{1cm} (2)

within the spectrometer software, vnmrJ.

**Preparation of hyperpolarized $^{13}$C solutions**

The hyperpolarized preps were comprised of $^{13}$C labeled substrate, solvent, and radical. For
each prep described herein, $^{13}$C labeled substrate was dissolved 1-1.3 equivalents of 18.9M NaOH solution. Sonication and/or light heating was used to speed up dissolving the substrate. The solution should form a clear glass when immersed in liquid nitrogen; glassing agent (e.g. DMSO) was not necessary for alanine, glycine and valine. GE trityl radical OX63 was added to a final concentration of 20mM. The prep with radical was protected from light and kept cold until use.

<table>
<thead>
<tr>
<th>$^{13}$C Substrate</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>5.6M</td>
</tr>
<tr>
<td>Glycine</td>
<td>5.6M</td>
</tr>
<tr>
<td>Valine</td>
<td>5.9M</td>
</tr>
</tbody>
</table>

Table 1: Concentrations of hyperpolarized preps with measured $T_1$’s

**Determination of $T_1$ relaxation constant**

We used the Oxford Instruments Hypersense hyperpolarizer (3.35T) to perform our hyperpolarized experiments. For each $T_1$ measurement, 40-50µL of prep was loaded in the polarizer. The microwave sweep was performed for each prep and the peak electron paramagnetic resonance (EPR) frequency was found to be roughly 0.012-0.014GHz lower than the peak EPR frequency for pyruvic acid on the same polarizer.

The sample was polarized at this frequency for 10-30min, and then dissolved in 3.5-4mL of 50mM Tris 0.3mM EDTA dissolution buffer to a final concentration of 65-70mM. 1.5M HCl was preloaded in the collection flask to neutralize the prep to pH 7. The sample was collected in a 5mL syringe and loaded in the Bruker 3T Biospec scanner. A series of 30 5° hard RF pulses with a TR of 3s were applied and the $^{13}$C spectra were acquired. The hyperpolarized peak areas were calculated in MestReNova; the $T_1$ was calculated from the peak areas using a MATLAB function written by Dave Korenchan, PhD, postdoctoral fellow in the Flavell lab. The function automatically applies tip angle correction (Equation 4), where $S_n$ is the $n^{th}$ signal intensity, $\theta$ is the flip angle, and $S_{n,uncorrected}$ is the uncorrected signal intensity.

$$S_n = \frac{S_{n,uncorrected}}{\cos^{n-1}\theta}$$  \hspace{1cm} (3)
Figure 4: Hyperpolarized signal with $T_1$ relaxation equation fit. This measurement of protonated valine yielded a $T_1$ of 36.7s. Calculation and figure processed in MATLAB.

$N = 3$ for each set of protonated and deuterated compounds. $T_1$ uncertainty is calculated as the standard deviation of the calculated $T_1$’s.
## 3 Results

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Structure</th>
<th>Isotopic Enrichment (%)</th>
<th>Chemical Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td><img src="image1" alt="Structure" /></td>
<td>97</td>
<td>83</td>
</tr>
<tr>
<td>Valine</td>
<td><img src="image2" alt="Structure" /></td>
<td>89</td>
<td>58</td>
</tr>
<tr>
<td>Glutamine</td>
<td><img src="image3" alt="Structure" /></td>
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<tr>
<td>Glycine</td>
<td><img src="image4" alt="Structure" /></td>
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<td>79</td>
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<tr>
<td>Lactate</td>
<td><img src="image5" alt="Structure" /></td>
<td>98</td>
<td>99</td>
</tr>
<tr>
<td>Serine</td>
<td><img src="image6" alt="Structure" /></td>
<td>α: 97, β: 68</td>
<td>70</td>
</tr>
<tr>
<td>ACES</td>
<td><img src="image7" alt="Structure" /></td>
<td>α: 98, γ: 95</td>
<td>89</td>
</tr>
</tbody>
</table>

Table 2: Summary of deuteration: yield and isotopic enrichment for each target compound

Most isotopic enrichments at the α positions were greater than 95%; valine had the lowest isotopic enrichment with 89%. Glycine was successfully doubly enriched with deuterium, due to the lack
of a side chain branching from the $\alpha$ carbon. Serine and ACES were labeled at two positions within each molecule; the electronegative O and N atoms in these molecules promote deuteration at adjacent positions.

The chemical yields of the deuteration reactions were varied. The highest chemical yield was obtained with sodium lactate, with 99% of product recovered. Valine had a lower yield (58%); this may be due to its lower solubility in water. The reaction on valine required the addition of 3mL of D$_2$O (total 8mL) to fully solubilize the substrate. The lowest yield was for glutamine, at 49%. At higher temperatures, the cyclization of glutamine to pyroglutamine occurs more rapidly. This side reaction reduces the total amount of glutamine, lowering the chemical yield.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>$^1$H $T_1$ (s)</th>
<th>$^2$H $T_1$ (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>20.48±1.31</td>
<td>14.57±0.57</td>
</tr>
<tr>
<td>Glycine</td>
<td>28.88±3.16</td>
<td>32.66±4.68</td>
</tr>
<tr>
<td>Valine</td>
<td>16.32±2.80</td>
<td>12.13±1.23</td>
</tr>
</tbody>
</table>

Table 3: Summary of $T_1$’s for protonated & deuterated $^{12}$C compounds measured with saturation recovery at 11.7T

The lower observed $T_1$ of deuterated $^{12}$C alanine and valine raised concerns about paramagnetic ruthenium ions being present in the purified deuterated compounds. Paramagnetic ions shorten $T_1$ and may adversely affect the precision of the effect of deuteration on $T_1$.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>$^1$H $T_1$ (s)</th>
<th>$^2$H $T_1$ (s)</th>
<th>% increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>52.9±2.2</td>
<td>66.4±1.7</td>
<td>25</td>
</tr>
<tr>
<td>Glycine</td>
<td>52.0±3.2</td>
<td>65.0±1.2</td>
<td>26</td>
</tr>
<tr>
<td>Valine</td>
<td>38.1±1.1</td>
<td>49.2±0.4</td>
<td>29</td>
</tr>
</tbody>
</table>

Table 4: Summary of $T_1$’s for protonated & deuterated $^{13}$C compounds measured with hyperpolarized decay

Measurement of $T_1$’s on hyperpolarized $^{13}$C samples showed $T_1$ increased for deuterated substrates compared to protonated substrates. There does not appear to be a correlation between the degree of deuteration and the percent increase in $T_1$. The hyperpolarized $T_1$ measurements were highly reproducible, with a maximum uncertainty of only 3.2s out of all the measured $T_1$. All calculated regressions for $T_1$ were very tight for each of the hyperpolarized measurements.
<table>
<thead>
<tr>
<th>Molecule</th>
<th>Non-purified $T_1$ (s)</th>
<th>Chelex-purified $T_1$ (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>66.4±1.7</td>
<td>63.5±2.5</td>
</tr>
</tbody>
</table>

Table 5: Summary of $T_1$'s for protonated & deuterated $^{13}$C compounds measured with hyperpolarized decay

To elucidate the effect of lingering paramagnetic ions in our deuterated compounds, we compared the hyperpolarized $T_1$ of Chelex 100 resin-purified deuterated alanine and non-purified deuterated alanine. Chelex is a resin that would chelate metal ions in solution and remove paramagnetic impurities. No significant differences were observed between the purified and non-purified alanine.

4 Discussion

Deuteration of target compounds

The viability of the H/D exchange may be limited by substrate stability and solubility in water. As noted previously, glutamine undergoes cyclization which reduces the chemical yield of the deuteration reaction. This impact on yield is worsened by elevated temperatures during deuteration. Consideration of substrate stability and/or cross reactions will be required in planning deuteration using this method. Also, highly hydrophobic substrates may not be viable targets for H/D exchange. However, hydrophobic compounds may also be poor candidates for use as hyperpolarized imaging agents. Hydrophobicity may adversely affect achieving high concentration preps that glass for polarization[1].

$T_1$ screening on $^{12}$C compounds

The $T_1$ screening on natural abundance compounds did not produce coherent results. For alanine and valine, the apparent $T_1$ got shorter for deuterated compounds, which do not agree with previous deuteration results[10]. For glycine, the uncertainties of the $T_1$’s were too high to be able to make conclusions. The lack of reproducibility of the natural abundance compounds prompted us to abandon our reliance on the saturation recovery method for predicting $T_1$ prolongation, and move directly to deuterating and polarizing $^{13}$C compounds for $T_1$ relaxation constants.
Purification of deuterated products

There was no significant difference between the non-chelated and Chelex chelated samples. If paramagnetic ruthenium was still present in the deuterated samples, its effect on the $T_1$ relaxation was not large enough to be detected at the concentrations we used to measure hyperpolarized $T_1$ decay. Higher concentration samples would bring any ruthenium ions closer to the $^{13}$C nuclei and could potentially increase the relaxivity of the sample. To definitely prove the presence or absence of ruthenium in our final deuteration products, elemental analysis would be needed. The original observed decrease in $T_1$ in the $^{12}$C alanine and valine may be in part explained by RF pulse miscalibration on the 500MHz spectrometer, non-optimal shimming, or other errors in the NMR acquisition.

$T_1$ prolongation of protonated vs deuterated compounds

In all three tested compounds, deuteration was associated with an increase in $T_1$ greater than 25%. This is a strong result that suggests the value of H/D exchange to HP-MRI investigators. Using equation 2, for HP glycine 120s post dissolution, the protonated sample will have 10.8% of its SNR remaining, while the deuterated sample will have 16.6% SNR remaining, a 50% increase in available hyperpolarized signal. This difference only increases with longer timescales, as well. This increase in available signal would be very welcome during in vivo experiments, where data acquisition may only begin several $T_1$’s post dissolution.

Counterintuitively, our data does not show any dependence of the $^{13}$C $T_1$ on the number of nearby hydrogen or deuterium atoms. One would expect that the glycine $T_1$ would be roughly half that of alanine for both the protonated and deuterated forms, due to there being two relaxers to dissipate $^{13}$C polarization. However, our $T_1$ data show only a 1 second difference between glycine and alanine. Measuring the $T_1$ of singly labeled glycine ($2^{-1}H_1$-$2^{-2}H_1$-Gly) may yield interesting data about dipole-dipole relaxation.
5 Conclusion

H/D exchange has proved to be a viable deuterium labeling method, and can prolong the lifetime of hyperpolarized $^{13}$C signal. Currently, work is in progress measuring the $T_1$ prolongation of other hyperpolarized $^{13}$C probes, such as ACES and glutamine. Development of deuteration methods to label specific positions (such as the side chain) may be helpful for $T_1$ prolongation of substrates with other $^{13}$C positions. Investigation of the effects of deuteration on maximum % polarization may also be beneficial. The eventual goal of the project is to deploy the deuterated $^{13}$C probes in in-vivo models for cancer and other disease processes. The increase in $T_1$ of the deuterated probes may enable probing otherwise elusive mechanisms of disease beyond the capabilities of non-deuterated probes currently available.

References


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