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TITLE: Targeting the Acidic Microenvironment of Prostate Cancer Using Chemical Shift-Based, Clinically Translatable Hyperpolarized 13C MRI Biomarkers

PRINCIPAL INVESTIGATOR:  Céline Taglang

CONTRACTING ORGANIZATION: University of California, San Francisco
San Francisco, CA 94103

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Targeting the Acidic Microenvironment of Prostate Cancer Using Chemical Shift-Based, Clinically Translatable Hyperpolarized $^{13}$C MRI Biomarkers

During the first year of research period, efforts were focused on the synthesis of new classes of hyperpolarized (HP) $^{13}$C agents for probing interstitial pH ($pH_t$). Synthesis of $[^{13}$C,$^{15}$N]ACES still needs to be optimized but we already reported that deuterium labeling of one of the building blocks, $[^{1-13}$C,$^{2-2H_2}]$glycine, led to a significant increase of its relaxation time $T_1$ (+ 25% compared to the non-deuterated $^{13}$C-labeled glycine). We are convinced that application of this strategy to ACES will also improve its $T_1$, which is a critical property in the development of efficient chemical shift-dependent imaging agents. Optimization of the synthesis of $[2-^{13}$C,$^{2H_10}]$DEMA was successful with high chemical yields and purity. $[^{13}$C,$^{2H}]$labeling is also a key aspect in the potential for DEMA as a candidate for high spatial resolution in vivo $pH_t$ mapping. Indeed, DEMA has one of the longest relaxation times measured for HP molecules. We reported the development of DEMA: it exhibits a large pH-dependent $^{13}$C chemical shift over the physiological range. We demonstrated that co-polarization with $[^{1-13}$C,$^{2H_10}]$tert-butanol accurately measured pH via $^{13}$C NMR and magnetic resonance spectroscopic imaging in phantoms. In vivo experiments are currently under investigation to evaluate DEMA as a clinically translatable HP $^{13}$C MRI biomarker.
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INTRODUCTION

Prostate cancer has a heterogeneous disease course. Distinguishing between the different phenotypes of prostate cancer is an important problem for clinical oncologists. Non-invasive biomarkers that would not only characterize prostate cancer aggressiveness but also predict response to therapy would be of enormous benefit to patients. One potential prognostic imaging biomarker is acidic interstitial pH, which has been shown to be associated with local invasion and metastases in a variety of cancers. The central hypothesis of this proposal is that low interstitial pH is strongly correlated with both tumoral lactate generation and tumor aggressiveness. We propose to investigate, using hyperpolarized (HP) $^{13}$C magnetic resonance spectroscopy (MRS), the relationship of lactate export to extracellular matrix acidification, establish pH as a critical determinant of cancer aggressiveness and use new HP platforms to target tumor acidity with the long-term aim to develop new clinically-translatable HP imaging approaches. The Specific aim 1 is to develop new classes of HP $^{13}$C agents for probing interstitial pH. The Specific Aim 2 is to validate the efficiency of the new HP $^{13}$C agents for MRI. The Specific aim 3 is to investigate the relationship between HP lactate generation and acidic interstitial pH. We will correlate HP pH maps to lactate generation and efflux and show grade-dependent changes in tumoral acidity.

KEYWORDS

Prostate cancer
Hyperpolarization
Magnetic resonance imaging
Isotopic labeling
Interstitial pH
Acidic microenvironment
Carbon-13
Deuterium
N-(2-Aacetamido)-2-aminoethanesulfonic acid (ACES)
Diethylmalonic acid (DEMA)
ACCOMPLISHMENTS

What were the major goals of the project?

Specific Aim 1: Develop new classes of HP $^{13}$C agents for probing interstitial pH

- Subtask 1: Optimize HP ACES as a chemical-shift dependent $^{13}$C imaging agent
  
  Target date: February 1, 2018
  
  Completion date: -
  
  Percentage of completion: 70%

- Subtask 2: Develop dicarboxylate sensors as pH-sensitive HP imaging probes
  
  Target date: February 1, 2018
  
  Completion date: -
  
  Percentage of completion: 100%

- Milestone(s) Achieved: Synthesis and $^{13}$C, $^2$H labeling of HP $^{13}$C agents
  
  Target date: February 1, 2018
  
  Completion date: -
  
  Percentage of completion: 85%

Specific Aim 2: Validate these new HP $^{13}$C agents in vivo using a gold standard ($^{31}$P-APP)

- Subtask 1: Validate chemical shift-based pH imaging methods using $^{31}$P-APP
  
  Target date: August 1, 2018
  
  Completion date: -
  
  Percentage of completion: 0%

- Subtask 2: Demonstrate HP probe response to simple modulations of tumoral pH
  
  Target date: August 1, 2019
  
  Completion date: -
  
  Percentage of completion: 20%

- Milestone(s) Achieved: Imaging and in vivo studies on $[^{13}\text{C},^{2}\text{H}]$ ACES and $[1-^{13}\text{C}, ^{2}\text{H}_10]$ DEMA
  
  Target date: August 1, 2019
  
  Completion date: -
  
  Percentage of completion: 20%

Specific Aim 3: Investigate the relationship between HP lactate generation and acidic interstitial pH

- Subtask 1: Correlate HP pH maps to lactate generation and efflux
  
  Target date: August 1, 2019
  
  Completion date: -
  
  Percentage of completion: 0%

- Subtask 2: Show grade-dependent changes in tumoral acidity
  
  Target date: August 1, 2019
  
  Completion date: -
  
  Percentage of completion: 0%

- Milestone(s) Achieved: Rigorously investigate the precise mechanism implying lactate export and low interstitial pH
What was accomplished under these goals?

Specific Aim 1: Develop new classes of HP \(^{13}\)C agents for probing interstitial pH

- Subtask 1: Optimize HP ACES as a chemical-shift dependent \(^{13}\)C imaging agent.

Our group previously demonstrated that hyperpolarized \([^{13}\)C,\(^{15}\)N] ACES could be used to determine pH using \(^{13}\)C magnetic resonance spectroscopy [1]. Indeed this probe was applied to pH measurement in an NMR spectrometer and in a chemical shift imaging experiment on a clinical 3 T MRI scanner. However, with a relaxation time \(T_1\) (or hyperpolarized lifetime in solution, which is a major limitation in hyperpolarized \(^{13}\)C magnetic resonance spectroscopy) of 18 seconds at 11.7 T and 25 seconds at 3 T, ACES represents an interesting candidate for pH imaging via HP \(^{13}\)C MRS but needs to be improved. One approach to increase \(T_1\) is the substitution of \(^1\)H with \(^3\)H (or D) atom. In April 2018, we reported a robust and selective late-stage deuteration methodology and applied it to \(^{13}\)C-enriched amino and alpha hydroxy acids to increase spin–lattice relaxation constant \(T_1\) for hyperpolarized \(^{13}\)C magnetic resonance imaging. This methodology was based on the regioselective deuteration, at the \(\alpha\)-position of aliphatic alcohols and sugars, developed by Sajiki et al., and the use of ruthenium on carbon [2]. For the five substrates with \(^{13}\)C-labeling on the \(\alpha\)-position ([\(^{1}\)\(^{13}\)C]alanine, [\(^{1}\)\(^{13}\)C]serine, [\(^{1}\)\(^{13}\)C]lactate, [\(^{1}\)\(^{13}\)C]glycine, and [\(^{1}\)\(^{13}\)C]valine), significant increase of their \(T_1\) was observed at 3 T with deuteration labeling (+26\%, +22\%, +16\%, +25\% and +29\%, respectively). Remarkably, in the case of [\(^{2}\)\(^{13}\)C]alanine, [\(^{2}\)\(^{13}\)C]serine and [\(^{2}\)\(^{13}\)C]lactate, deuteration labeling led to a greater than four fold increase in \(T_1\), [\(^{1}\)\(^{13}\)C,\(^2\)\(^{2}\)H]alanine, produced using this method, was applied to in vitro enzyme assays with alanine aminotransferase, demonstrating a kinetic isotope effect [3, attached article in Appendix 1].

As shown in the scheme 1 of Appendix 2, [\(^{1}\)\(^{13}\)C,\(^2\)\(^{2}\)H\(_2\)]glycine was a building block in the synthetic strategy of \([^{15}\)N,\(^{13}\)C,\(^2\)H\(_4\)]ACES 2. As we showed an increase of +25\% of its \(T_1\) compared to [\(^{1}\)\(^{13}\)C]glycine 3, which is a building block of the non deuteration-labelled \([^{15}\)N,\(^{13}\)C]ACES 1, we are confident that deuteration labelling of \([^{15}\)N,\(^{13}\)C]ACES will improve its \(T_1\). The three first synthetic steps of \([^{15}\)N,\(^{13}\)C]ACES 1 (BOC-protection, amide formation and BOC-deprotection) led to the key intermediate 6 with satisfying chemical yields (scheme 1b of Appendix 2). Unfortunately, the last step (nucleophilic substitution) resulted in the formation of the desired \([^{15}\)N,\(^{13}\)C]ACES 1 with a low chemical yield. We think that the pH of the reaction, which must remain constant, is a key parameter for this reaction to succeed. Moreover, The purification step by crystallization represents also a challenge: a major quantity of the target compound stay solubilized which leads to the low chemical yield. New attempts to improve the yield of this reaction are currently under investigation in our laboratory. This step represents the only challenge of our approach in the synthesis of \([^{15}\)N,\(^{13}\)C,\(^2\)H\(_4\)]ACES 2. Indeed, we already checked the feasibility of deuteration labeling on \([^{15}\)N,\(^{13}\)C]ACES 1: the target molecule was obtained with high chemical yield (89\%) and isotopic enrichment (99\% on the \(\alpha\)-position of \(^{13}\)C).

- Subtask 2: Develop dicarboxylate sensors as pH-sensitive HP imaging probes

In 2017, we reported the development of hyperpolarized \([2\)\(^{-13}\)C,\(^{10}\)D\(_{10}\)]diethylmalonic acid, which
exhibits a large pH-dependent $^{13}$C chemical shift over the physiological range. We demonstrated that co-polarization with [1-$^{13}$C,D$_5$]tert-butanol accurately measured pH via $^{13}$C NMR and magnetic resonance spectroscopic imaging in phantoms. [4, attached article in Appendix 3]. The synthesis of DEMA was based on two steps with a total chemical yield of 75%: alkylation of [2-$^{13}$C]diethylmalonate with [D$_5$]bromoethane and saponification using NaOH. In conclusion, our synthetic strategy did provide the target probe DEMA. Synthetic access to this desired proposed substrate is a significant milestone for the progress of the project.

Specific Aim 2: Validate these new HP $^{13}$C agents in vivo using a gold standard ($^{31}$P-APP)
- Subtask 1: Validate chemical shift-based pH imaging methods using $^{31}$P-APP
  Nothing to report
- Subtask 2: Demonstrate HP probe response to simple modulations of tumoral pH
As the synthesis of [15N,$^{13}$C,$^2$H$_4$]ACES 2 is still under investigation (specific aim 1, subtask 1), we focused our efforts for the development of chemical shift-based pH imaging with DEMA.

Our group previously showed that hyperpolarized (HP) $^{13}$C MRS imaging using [13C]bicarbonate could measure pH, via changes in relative intensities of bicarbonate and CO$_2$ signal, in a phantom within 0.1 pH unit and detect spatial differences in pH in vivo in a murine model of prostate cancer [5]. We also recently identified a dicarboxylic acid as a suitable probe for physiological pH imaging via HP $^{13}$C MRS [3]. Indeed, [2-$^{13}$C,D$_{10}$]diethylmalonic acid (DEMA) demonstrated large $^{13}$C chemical shifts with pH from 6.5 to 7.4 at 37 °C ex vivo and a very long exponential decay constant $T_1$, 105.6 ± 5.2 s ($n = 3$) at 11.7 T, which is a critical limitation in the development of new probes. For the beginning of the subtask 2 of the specific aim 2 of our project we decided to demonstrate that DEMA is a strong potential candidate pH imaging probe in vivo in a healthy mouse kidney imaging experiment (Figure 1, Appendix 4).

Methods
Hyperpolarization: DEMA was prepared and co-polarized with [1-$^{13}$C,D$_5$]tert-butanol as previously described by Korenchan et al. [4].
In vivo pH measurement in mouse kidneys: a tail vein injection of HP DEMA and tBuOH (50 μL each co-polarized then diluted to 45 mM) into a healthy mouse was performed, followed by imaging of a slice containing the kidneys, 50 seconds after completion of the injection. $^{13}$C-bicarbonate (co-polarize with urea) was subsequently injected to compare pH values.

Results
A tail vein injection of HP DEMA and tBuOH into a healthy mouse showed a strong signal from the kidneys with a noticeable amount in the blood pool (Figure 1b, Appendix 4). We discovered two peaks of DEMA within the same kidney voxel forming two pH clusters (pH 7.43 and 6.41, n = 1) consistent with data from literature [6]. Indeed, zymonic acid co-polarized with urea showed peaks in the same type of imaging conditions with pH of 7.40 ± 0.01 and 6.55 ± 0.03 (n = 4 rats, mean ± s.d.). The measured pH values showed good agreement with the average voxel pH measured using HP [13C]bicarbonate (pH 6.77, Figure b).

Conclusion
The fact that DEMA, instead of using a ratiometric method, uses differences in chemical shifts to determine the pH, allows for the detection of multiple pH compartments within the same voxel, contrary to $^{13}$C-bicarbonate that shows a mean pH. Similar to the prior study employing zymonic acid, we propose that these two peaks with pH of 7.43 and 6.41 arise from the cortical and
calyx/ureter compartments, respectively. Interestingly, DEMA has a higher in vitro $T_1$ at 3 T than zymonic acid: 84 and 43-51 seconds, respectively. As polarization lifetime is a major limitation of using $^{13}$C-labelled probes for in vivo applications, DEMA represents a pH imaging candidate for clinical translation.

Specific Aim 3: Investigate the relationship between HP lactate generation and acidic interstitial pH

- Subtask 1: Correlate HP pH maps to lactate generation and efflux
  Nothing to report
- Subtask 2: Show grade-dependent changes in tumoral acidity
  Nothing to report

**What opportunities for training and professional development has the project provided?**

The purpose of this grant application was to build upon my training as a chemist by taking on a project in prostate cancer biomarker development that more heavily emphasizes magnetic resonance imaging. I joined the Wilson lab at UCSF to strengthen my training in biomarker and drug development by taking on a project that focused on studying new hyperpolarized $^{13}$C agents, their biocompatibility and their potential for near-term translation. I also deliberately chose to work on prostate cancer, owing to the established role of nuclear imaging and medicine in standard of care.

To broaden my knowledge base, there are several mechanisms available for my education inside and outside the lab. I have a serious peer group of chemists and chemical biologists that have been very helpful in teaching me radiochemistry, preclinical pharmacology and animal work. Moreover, Dr. Dave Korenchan, who obtained his Ph.D. with Pr. John kurhanewicz and has more than three years of expertise in the development of hyperpolarized $^{13}$C MRS imaging agent, taught me how to optimize the formulations ACES and DEMA, the hyperpolarization process and the treatment of the data.

During the first year of the project, I mainly focused on research and further educating myself on the background and goals of my project, but I also could summarize the data, described in the specific aim 1, for manuscript submissions [3 and 4], and submit an accepted poster to ISMRM annual meeting (Paris, June 2018).

During this first halt of funding period I will was co-mentored by Drs. Wilson (primary mentor), Flavell (co-mentor) and Kurhanewicz (secondary mentor). We will continue to meet together every week to discuss my research progress and plans to convert the data into high impact publications and long term grant support from public mechanisms.

**How were the results disseminated to communities of interest?**

Nothing to report.
What do you plan to do during the next reporting period to accomplish the goals?

With DEMA in hand, the subtask 2 of specific aim 2 is feasible in an accelerated time of 6 months. A current effort is underway to obtain \[^{15}\text{N},^{13}\text{C}\text{ACES}\] and complete the subtask 1 of specific aim 1 (new expected completion date: months 13-15). Subtask 1 of specific aim 2, which was planned to be completed on month 12 of the project, is now expected to be completed on month 18. Originally planned completion date for specific aim 3 is still August 31, 2018.

**IMPACT**

**What was the impact on the development of the principal discipline(s) of the project?**

Nothing to report.

**What was the impact on other disciplines?**

Nothing to report.

**What was the impact on technology transfer?**

Nothing to report.

**What was the impact on society beyond science and technology?**

Nothing to report.

**What was the impact on society beyond science and technology?**

Nothing to report.

**CHANGES/PROBLEMS**

**Changes in approach and reasons for change**

Nothing to report.

**Actual or anticipated problems or delays and actions or plans to resolve them**

Nothing to report.

**Changes that had a significant impact on expenditures**

Nothing to report.
Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.

Nothing to report.

PRODUCTS

Publications, conference papers, and presentations

- Journal publication


- Books or other non-periodical, one-time publications

Nothing to report

- Other publications, conference papers, and presentations

Poster presentation:

- Website(s) or other Internet site(s)

Nothing to report.

- Technologies or technique

Nothing to report.

- Inventions, patent applications, and/or licenses

Nothing to report.

- Other Products

Nothing to report.
PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

<table>
<thead>
<tr>
<th>Name</th>
<th>Céline Taglang</th>
</tr>
</thead>
<tbody>
<tr>
<td>Project role</td>
<td>Principal investigator</td>
</tr>
<tr>
<td>ORCID ID</td>
<td>0000-0002-3927-6675</td>
</tr>
<tr>
<td>Nearest person month worked</td>
<td>12</td>
</tr>
<tr>
<td>Contribution to project</td>
<td>All experiments to date</td>
</tr>
<tr>
<td>Funding support</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report.

What other organizations were involved as partners?

Nothing to report.

SPECIAL REPORTING REQUIREMENTS

Nothing to report.

REFERENCES

APPENDICES

Appendix 1

Late-stage deuteration of 13C-enriched substrates for T1 prolongation in hyperpolarized 13C MRI†


A robust and selective late-stage deuteration methodology was applied to 13C-enriched amino and aliphatic hydroxy acids to increase spin-lattice relaxation constant T1 for hyperpolarized 13C magnetic resonance imaging. For the five substrates with 13C-labeling on the C1-position (13L-Clalanine, 13L-13Cereine, 13L-Clactate, 13L-13Clycine, and 13L-13Cvaline), significant increase of their T1 was observed at 3 T with deuterium labeling (+26%, +22%, +16%, +25% and +29%, respectively). Remarkably, in the case of [2-13C]alalanine, [2-13C]cereine and [2-13C]lactate, deuterium labeling led to a greater than four fold increase in T1. 13L-13C2-2H-1alanine, produced using this method, was applied to in vitro enzyme assays with alanine aminotransferase, demonstrating a kinetic isotope effect.

Magnetic resonance imaging employing hyperpolarized substrates [HP MRI] has recently emerged as a powerful tool for studying metabolism in cells, animal models and patients.1,2 Polarization of substrates can be realized through a variety of mechanisms including dissolution dynamic nuclear polarization (DNP),3,4 spin-echo induced polarization (SIP),5-8 or signal amplification by reversible exchange (SARE).9 While these are versatile methods that allow for real-time imaging of metabolism, the short lifetime of the hyperpolarized signal, which decays exponentially based upon the spin lattice relaxation time T1, remains one of the key limiting factors in the implementation of this technology. The most widely used HP 13C probe is [1-13C]pyruvate, a key metabolic intermediate, which has a T1 of 67 s at 3 T. However, other 13C nuclei, especially those with directly attached protons, are not feasible HP 13C probes due to very short T1's (less than 5 s).10 One approach to increase T1 is the substitution of 1H with 2H or D, a quadrupolar nucleus with a gyromagnetic ratio γ about 6-fold smaller than the one for 1H.11-22 This use of deuterated substrates has proved particularly fruitful in the case of SABRE13 and PHIP14-15 methods. This approach is effective when dipolar 1H-1H coupling contributes substantially to T1 relaxation. Fortunately, in the case of pyruvate, the incorporation of deuterium is straightforward owing to lack of stereocenters.23 However, the synthesis of multiply labelled molecules containing stereocenters including both 13C and 1H is generally both expensive and time consuming, and most isotopically enriched molecules require multistep syntheses. Therefore, a robust method for incorporation of deuterium in the final step of syntheses would be generally valuable in the field of HP MRI.

For the synthesis of deuterated molecules, late-stage isotopic exchange has several advantages over a synthetic pathway from enriched building blocks. Numerous methods based on homogeneous or heterogeneous catalysts for H/D exchange have already been described, but the development of a deuteration methodology with mild reaction conditions, high selectivity and deuterium incorporation is still a challenge.24 In order to develop 13C-1H labeled probes for HP MRI, we considered the regioselective deuteration at the α-position of aliphatic alcohols and sugars, developed by Seki et al.,25 as a straightforward way to the deuterium labelling of 13C-substrates with attached O or N.25-26 In this manuscript, we report the application of this methodology to a variety of 13C-enriched compounds, enabling high incorporation yields with retention of configuration, and demonstrate a significant increase in T1 of the resulting deuterated substrates. One of the probes, [1-13C2-2H]-alanine, was studied in an in vitro enzymatic assay with alanine aminotransferase (ALT), revealing a deuterium kinetic isotope effect.

Initially, we evaluated the performance of the labelling methodology with a variety of labelled substrates including α-amino and hydroxy acids. We performed the one-step deuteration labelling reaction on position C2 of several commercial 13C-labeled substrates (Scheme 1 and Table 1). Reactions were incubated in D2O in the presence of RuC3% (40 wt%), under H2, overnight, at 80 °C (Table S1, ESI†). Efficient deuterium incorporation on position C2 (05-95%) was observed for aliphatic amino acids [1-13C2-2H] and [2-13C2-3H]alanine and [1-13C2-3H]valine, with enantiomeric...
Table 1: Structures of \(^{13}\)C-enriched molecules after deuterium enrichment. The bracketed number indicates the isotope enrichment determined by \(^{13}\)C NMR and HIMS analyses described in the ESL. ee: enantiomeric excess

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Structure</th>
<th>Chemical yield (%)</th>
<th>ee (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^{13})C on position C1 and (^{2})H on position C2</td>
<td>([1,^{13})C,(^{2})H)]alanine 1</td>
<td>97</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>([1,^{13})C,(^{3})H, (\text{isopropyl}, \text{methanol})</td>
<td>99</td>
<td>99</td>
</tr>
<tr>
<td>([1,^{13})C,(^{2})H)]alanine 3</td>
<td>98</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>([1,^{13})C,(^{2})H)]serine 4</td>
<td>99</td>
<td>99</td>
<td></td>
</tr>
<tr>
<td>([1,^{13})C,(^{2})H)]lactate 5</td>
<td>95</td>
<td>95</td>
<td></td>
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<tr>
<td>([1,^{13})C,(^{2})H)]glycine 6</td>
<td>97</td>
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<td></td>
</tr>
<tr>
<td>([1,^{13})C,(^{2})H)]valine 7</td>
<td>98</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>([2,^{13})C,(^{2})H)]alanine 8</td>
<td>99</td>
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</tr>
</tbody>
</table>

Excesses greater than 99%. Isotopic enrichments on position C2 of \([1,^{13}\)C,\(^{2}\)H\]valine 5, 53%, may be due to its lower solubility in D.O. Enantiomeric excess was 98% for both \([1,^{13}\)C,\(^{2}\)H\]serine (2 and 7) whereas chemical yields were 78% and 77%, respectively. Their lower isotopic enrichments on position C2 (52 and 90%) may be due to the additional deuterium labelling on their position C3.

In a few cases, side reactions were encountered which led to decomposition of the desired product (ESI). Taken together with prior reports,\(^{25,26}\) our data indicate that this is a versatile method for deuterium incorporation in biologically relevant molecules.

In order to determine the impact of deuterium incorporation on \(T_1\), we then prepared the labelled substrates for hyperpolarization. Solutions of 4 to 6 M substrate with 1 to 1.2 equivalents N\(\text{H}_2\)O and 23 to 24 mM free radical (OXY63) were prepared for hyperpolarization using DNP.\(^{26}\) Following polarization, \(T_1\) measurements were performed on a 3 T preclinical MR scanner. Deuterium substitution at the C2 position yielded significant improvements of the \(T_1\) with \(^{13}\)C at the C1 position, ranging from 16–29% (Fig. 1). The relatively modest improvement in \(T_1\) yielded larger signal gains at later time points. For example, in the case of \([1,^{13}\)C\]alanine, deuterium yielded an increase in signal to noise ratio of 60% at 90 s after the start of the experiment (Fig. S6, ESI). Remarkably, in the case of \([2,^{13}\)C,\(^{2}\)H\]alanine 6, \([2,^{13}\)C,\(^{2}\)H\]serine 7 and \([2,^{13}\)C,\(^{2}\)H\]lactate 8, deuterium labelling led to a greater than four-fold increase in \(T_1\). Due to rapid signal decay on \([1,^{13}\)C\]alanine, \([2,^{13}\)C\]serine and \([2,^{13}\)C\]lactate, their \(T_1\) could not be measured using a hyperpolarized method\(^{26}\) and were instead assayed using an inversion recovery sequence. Part of the reason why the \(T_1\) gains due to deuteration are relatively limited is because of chemical shift anisotropy (CSA) which is likely the dominant relaxation mechanism at 3 T.\(^{28,29}\) Therefore, at 1.5 T, there could be further improvements in \(T_1\) prolongation.\(^{26}\)

We then evaluated the \(T_1\) of one of our substrates, \([1,^{13}\)C,\(^{2}\)H\]alanine 1, in an in vivo experiment in a mouse model and compared its properties with those of \([1,^{13}\)C\]alanine. MR measurements were performed on a preclinical 3 T scanner (Fig. S7, ESI). 300 \(\mu\)L of 80 mM solutions of hyperpolarized \([1,^{13}\)C\]alanine and \([1,^{13}\)C,\(^{2}\)H\]alanine 1 were injected intravenously immediately followed by dynamic acquisition of \(^{13}\)C MRS spectra. As expected, based on the in vitro studies, we found an increase in the apparent in vivo \(T_1\) at 3 T, from 32 s, for \([1,^{13}\)C\]alanine, to 42 s, for \([1,^{13}\)C,\(^{2}\)H\]alanine 1.
As a demonstration of the utility of the deuteration method, we next applied the labelled alanine probes in an in vitro enzyme assay using alanine transaminase (ALT). ALT is an abundant enzyme and a biomarker for liver disease, which converts alanine and α-ketoglutarate to pyruvate and glutamate, respectively (Fig. 2a). Previous reports have studied this enzyme both in vitro and in vivo using hyperpolarized 13C methods. Therefore, we developed an assay for the detection of 13C pyruvate production by incubation of polarized [1-13C]alanine or [1-13C]-2,3-3H]alanine 1 with α-ketoglutarate, glutamate and ALT based on prior reports. As expected, 13C pyruvate was rapidly formed during the time course of the hyperpolarized experiment (Fig. 2b-d). Furthermore, the initial rate of pyruvate signal growth, which approximates the forward conversion rate, was about 2.42-fold lower for the [1-13C]-3H]alanine 1 as compared with the [1-13C]alanine (n = 3 each, p < 0.002, neutral pH). This agrees closely with the previously reported kinetic isotope effect of 3.3. In order to confirm these findings, we fit the dynamic alanine and pyruvate MRS data to a kinetic model accounting for HP signal exchange between protonated and deuterated [1-13C]alanine and [1-13C]pyruvate pools as well as signal loss due to HP sampling and T1 loss (Supplementary Fig. S7). We thus obtained pseudo-first order rate constants of (1.67 ± 0.174) × 10^{-3} s^{-1} (n = 3) and (0.736 ± 0.015) × 10^{-3} s^{-1} (n = 3) for protonated and deuterated alanine, respectively. This difference in kinetic rates suggested a kinetic isotope effect of 2.33, in close agreement with our previous analysis and with the literature.

In summary, these data indicate that the RuC labelling method represents a versatile method for high-yield deuteration of 13C labelled substrates, ideal for application to hyperpolarized 13C MRS. When the deuteration was incorporated adjacent to a 13C-enriched carbonyl, the effect on T1 prolongation was modest, ranging from 16-29%. In contrast, when applied to 13C nuclei with directly attached protons [2-13C]-3H]alanine 6, [2-13C]-3H]alanine 7 and [2-13C]-3H]alanine 8, an approximately 4-fold increase in T1 was observed. To further study the behavior of doubly-enriched substrates, we applied [1-13C]alanine and [1-13C]-3H]alanine 1 to an in vitro enzyme assay with purified ALT enzyme, demonstrating a kinetic isotope effect, in agreement with prior reports. We anticipate that this versatile method will find application to a variety of substrates for hyperpolarized 13C MRS.

C. T. carried out the experiments and wrote the manuscript with support from D. E. K. R. F. and D. M. W. designed and directed the project. H. V. B. and D. B. V. were supervised by R. S. and J. K. helped supervise the project and helped edit the manuscript. G. V. M., J. Y., S. W., J. E. B., S. S. and R. B. helped with characterization of obtained compounds, T1 measurements and in vivo experiments. C. N. and S. M. R. provided critical feedback and helped shape the research, notably for the study of [2-13C]-3H]enriched substrates.

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Conflicts of interest

There are no conflicts to declare.

Notes and references

Appendix 2

a)  
\[
\begin{align*}
\text{H}_2^{15}\text{N}_3\text{C} & \quad \text{H}_2\text{N}\text{SO}_3\text{H} \\
\text{O} & \quad \text{O} \\
1 & \quad \rightarrow \\
\text{D}_2\text{O}, 80^\circ\text{C}, \text{o/n} & \quad \rightarrow \\
\text{H}_2^{15}\text{N}_3\text{C} & \quad \text{H}_2\text{N}\text{SO}_3\text{H} \\
\text{D} & \quad \text{D} \\
2 & \quad \text{2g} \\
163\text{mg} - 89\% & 
\end{align*}
\]

b)  
\[
\begin{align*}
\text{H}_2^{15}\text{N}_3\text{C} & \quad \text{H}_2\text{N}\text{BOC} \\
\text{O} & \quad \text{O} \\
\text{Commercial 3} & \quad \rightarrow \\
\text{dioxane:water} & \quad \rightarrow \\
\text{BOC}_2\text{O} & \quad \text{NHBOC} \\
\text{NaOH} & \quad \text{2})^{15}\text{NH}_4\text{Cl} \\
0^\circ\text{C} > \text{RT 1h} & \quad \text{5} \\
1.9\text{g} - 16\% & \\
\text{1) CDI} & \\
\text{TFA} & \\
\text{1) CDI} & \\
\text{2})^{15}\text{NH}_4\text{Cl} & \\
\text{H}_2^{15}\text{N}_3\text{C} & \quad \text{H}_2^{15}\text{N}_3\text{C} \\
\text{N} & \quad \text{NH}_2\text{TFA} \\
\text{O} & \quad \text{O} \\
\text{1} & \quad \text{6} \\
2\text{g} &
\end{align*}
\]

Scheme 1: a) Regioselective catalytic deuterium labelling via $^1\text{H}/^2\text{H}$ exchange using ruthenium on carbon (RuC) applied on $[^{15}\text{N},^{13}\text{C}]$ACES. b) Synthesis of $[^{15}\text{N},^{13}\text{C}]$ACES.
Dicarboxylic acids as pH sensors for hyperpolarized $^{13}$C magnetic resonance spectroscopic imaging\dagger

D. E. Korenchan,\textsuperscript{a,b} C. Taglang,\textsuperscript{b} C. von Morze,\textsuperscript{b} J. E. Blecha,\textsuperscript{b} J. W. Gordon,\textsuperscript{b} R. Sriram,\textsuperscript{a} P. E. Z. Larson,\textsuperscript{a,b} D. B. Vigneron,\textsuperscript{b} H. F. VanBroekhoven,\textsuperscript{b} J. Kurhanewicz,\textsuperscript{a,b} D. M. Wilson\textsuperscript{a} and R. R. Flavell\textsuperscript{a,b}

Imaging tumoral pH may help to characterize aggressiveness, metastasis, and therapeutic response. We report the development of hyperpolarized $[2,3^{13}$C]diethylenedioxy-malic acid, which exhibits a large pH-dependent $^{13}$C chemical shift over the physiological range. We demonstrate that co-polarization with [1,2,3$^{13}$C]tert-butanol accurately measures pH via $^{13}$C NMR and magnetic resonance spectroscopic imaging in phantoms.

Introduction

Interstitial acidification, one of the hallmarks of numerous human cancers,\textsuperscript{1} has a significant impact on the tumor microenvironment. Uptregulation of aerobic glycolysis leads to proton export from tumor cells and extracellular acidification,\textsuperscript{2} leading to reduced tumor uptake of chemotherapeutics,\textsuperscript{3} decreased antitumor immune cell function,\textsuperscript{4} and tumor invasion and metastasis.\textsuperscript{5,6} Interestingly, interstitial pH heterogeneity within a tumor may contain important information about tumor behavior, especially considering that tumor cells tend to grow and migrate predominantly along gradients of decreasing pH.\textsuperscript{7} These findings suggest that pH imaging approaches may provide valuable information for clinicians wishing to grade and effectively treat tumors.

Many techniques exist for the measurement of interstitial pH in vivo,\textsuperscript{8} including fluorescence methods,\textsuperscript{9} positron emission tomography,\textsuperscript{10,11} and magnetic resonance imaging (MRI) based approaches.\textsuperscript{12,13} The two pH imaging modalities best able to capture intratumoral pH heterogeneity with potential for clinical implementation are $^1$H chemical exchange saturation transfer (CEST) MRI and hyperpolarized (HP) $^{13}$C magnetic resonance spectroscopic imaging (MRSI).\textsuperscript{7} HP $^{13}$C MRSI, enabled by MR signal enhancement on the order of $10^3$ to $10^4$ via dynamic nuclear polarization (DNP),\textsuperscript{15} has enabled the study of several metabolic and transport processes relevant to cancer, and it has been applied to human prostate cancer imaging in phase I clinical trials.\textsuperscript{16} To date, the primary HP agent for measuring interstitial pH is $^{13}$C-bicarbonate, which represents a ratiometric approach to calculating pH. Because the conjugate acid $\text{H}_2\text{CO}_3$ in rapid equilibrium with $\text{CO}_2$ and base ($\text{HCO}_3^-$) exhibit distinct MR resonances, the ratio of bicarbonate and $\text{CO}_2$ MR signal intensities can be measured in each volume element (voxel) to calculate a pH map using a modified Henderson-Hasselbalch equation.\textsuperscript{17} However, the spatial resolution is limited by the low signal-to-noise ratio (SNR) of $\text{CO}_2$, which is typically at a concentration of an order of magnitude lower than bicarbonate at physiological pH values ($pK_a = 6.17$ at $37^\circ\text{C}$)\textsuperscript{17,18}. Recently, a new class of chemical shift (CS) pH probes has been reported, in which the protonated and deprotonated forms of the molecule give rise to a single MR resonance rather than two. Such HP molecules, which include $^N$-pyridine derivatives,\textsuperscript{19} imidazole-$^N$N$_2$,\textsuperscript{20} and $^N$-O-(2-acetamido)-2-aminoethanesulfonic acid (AEBS),\textsuperscript{21} may circumvent the low SNR concerns regarding the quantification of two peak intensities.

Some dicarboxylic acids are known to have second $pK_a$ values in the physiological range,\textsuperscript{22} as well as carbon nuclei with long $T_1$ relaxation time constants, making them suitable for pH imaging via HP $^{13}$C MRSI. Therefore, the goal of this work was to identify a dicarboxylic acid that could be hyperpolarized and used for accurate pH measurement with $^{13}$C MRSI.

Experimental

Full experimental details can be found in the ESI.\dagger

Dicarboxylate screening

Eleven dicarboxylates without isotopic labeling were initially screened to measure their pH-dependent $^{13}$C chemical shifts.
Fig. 1. Investigation of dicarboxylates as $^{13}$C MR pH sensors. The downfield CS migration from pH 6.5 to 7.4 is listed near each labeled $^{13}$C nucleus. Two molecules with large CS migration over this pH range are highlighted in yellow: diethylmalonic acid (top right) and cyclopropane-1,1-dicarboxylic acid (lower left). Literature pH$^+$ values for these molecules can be found in the ESI.$^+$

(Fig. 1). Aqueous solutions of these compounds were prepared, containing 250 mM dicarboxylate and 250 mM urea (CS standard), and the pH was carefully adjusted with HCl or NaOH to either 6.5 or 7.4 using a standard laboratory pH meter. The $^{13}$C NMR spectra were acquired at 11.7 T and 37 $^\circ$C and referenced to urea at 163.7 ppm, and the CS change between these two pH values was measured.

Synthesis of [2-$^{13}$C$_5$]diethylmalonic acid and [2-$^{13}$C$_5$]cyclopropane-1,1-dicarboxylic acid

Based on the pH-dependent $^{13}$C chemical shifts obtained, enriched syntheses of both [2-$^{13}$C$_5$]diethylmalonic acid (DEMA) and [2-$^{13}$C$_5$]cyclopropane-1,1-dicarboxylic acid (CPDA) were performed (Fig. 2). Brief synthetic routes are described below, based on previously described methods.$^{23}$ [2-$^{13}$C$_5$]diethylmalonic acid [2-$^{13}$C$_5$]diethylmalonate was alkylated with [D$_9$]bromomethane and saponified using NaOH. [2-$^{13}$C$_5$]cyclopropane-1,1-dicarboxylic acid, similar to the above, but [D$_9$]bromomethane was used in place of [D$_9$]bromomethane. All compounds were characterized via standard methods, as described in the ESI.$^+$

Fig. 2. Synthesis schemes and representative HP $^{13}$C T$_1$ decay curves at 11.7 T for (a) [2-$^{13}$C$_5$]diethylmalonic acid (DEMA) 3, and (b) [2-$^{13}$C$_5$]cyclopropane-1,1-dicarboxylic acid (CPDA) 5. The measured T$_1$ values at 11.7 T for DEMA and CPDA were 105.6 ± 5.2 s and 70.2 ± 4.5 s, respectively (n = 3 each).

Hyperpolarization and characterization of $^{13}$C dicarboxylate pH sensors

Enriched $^{13}$C dicarboxylate sensors were hyperpolarized via the dynamic nuclear polarization (DNP) technique and their solution-state T$_1$ time constants were determined. A 3.8 M DEMA in N,N-dimethylacetamide was prepared with 15 mM of OX063 trityl radical and 2 mM Gd-DOTA and co-polarized with tert-butanol (tBuOH), which was formulated with OX063 in glycerol as previously described.$^{24}$ ~4 M CPDA in dimethyl sulfoxide was prepared with 15 mM OX063 trityl radical. After dissolution and NaOH titration (pH 6.6–7.5, both compounds), the HP solution-state T$_1$ values were determined via dynamic $^{13}$C MRS (5$^\circ$ hard pulses, flip angle correction, TR = 3 s, n = 3) at 11.7 T and 37 $^\circ$C.

Titration curve for $^{13}$C-enriched DEMA

Based on the T$_1$ data obtained for $^{13}$C DEMA, we obtained an NMR titration curve for this compound in preparation for imaging studies. 5 mM solutions of [2-$^{13}$C$_5$]DEMA and [1-$^{13}$C$_5$]DeBuOH were prepared ranging from pH 2.5 to 8.8. The CS difference between the labeled carbons was measured at 11.7 T and 37 $^\circ$C, plotted versus pH, and fitted to a sigmoidal…
Results & discussion

We investigated several dicarboxylic acids using $^{13}$C MRS to identify nuclei that demonstrated a pH-dependent chemical shift (Fig. 1). All the tested compounds had two carboxylic acid groups separated by either one carbon (derivatives of malonic acid) or two carbons. All molecules also had a known or predicted $pK_a$ value close to the physiological range (i.e., near 7-7.4) and contained at least one carbon nucleus without directly bonded protons, making them likely to have long $T_2$ relaxation time constants amenable to use with hyperpolarized imaging.\textsuperscript{25} Strikingly, the intermediate carbons of all malonic acid derivatives in this study demonstrated larger pH-dependent chemical shifts than did the carboxylic acid carbons themselves. This finding was somewhat surprising, considering that the carbon carbons are closer in proximity to the acidic protons in each molecule. Two of the malonic acid derivatives, highlighted in yellow in Fig. 1, demonstrated large chemical shifts over the tested pH range: diethylmalonic acid (DEMA) and cyclopropane-1,1-dicarboxylic acid (CPDA). Of the compounds with two carbons separating the dicarboxylic acid moieties, the cis enantiomers demonstrated larger pH-dependent chemical shifts than the trans. However, these molecules exhibited smaller pH-dependent carboxyl chemical shifts than the quaternary carbons in the malonates.

Following the dicarboxylate investigation, two-step synthetic routes were developed for the isotopically-enriched, deuterated versions of DEMA and CPDA (Fig. 2). These syntheses were based on a previously reported method applied to valproic acid.\textsuperscript{25} In addition to $^{13}$C labeling the pH-sensitive quaternary carbon, the functional groups were deuterated for each molecule in order to lengthen the $^{13}$C $T_2$.\textsuperscript{25} The overall reaction yields were 64% for DEMA and 45% for CPDA. The reaction products were confirmed to be the target molecules by both NMR ($^1H$, $^13C$) and high-resolution mass spectroscopy (see the ES$^\text{†}$). Based upon a preliminary $T_2$ comparison between the two synthesized compounds (Fig. 2), we chose DEMA for further development as a hyperpolarized pH probe.

The pH-dependent chemical shift behavior of the DEMA quaternary carbon was characterized via NMR spectroscopy (Fig. 3a). The CS difference between DEMA and tert-butanol (tBuOH) was plotted against the pH and fitted to a sigmoidal

\[ \text{pK}_a = 7.30 - \log \left( \frac{[\text{DEMA}]}{[\text{tBuOH}]} \cdot \frac{[\text{DEMA}]}{[\text{tBuOH}]} \right) \]

\[ \text{pH} = 7.29 \pm 0.01 \]

\[ \text{pH}_{\text{error}} < 0.1 \]
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In order to demonstrate that HP DEMA could be used with spectroscopic imaging techniques, we performed an imaging phantom experiment on a clinical 3 T MRI scanner. This allowed us to measure the pH simultaneously in several solutions (Fig. 4a). As before, the pH in three of the five tubes was measured by using the CS difference between the HP DEMA and triOH peaks (Fig. 4b), and the pH values agreed with electrode measurements within 0.1 pH units (Fig. 4c). Two tubes had pH values at the high and low ends of the measurable pH range. However, the extremely high and extremely low pH tubes demonstrated CS differences of 6.9 and 10.3 ppm, respectively, which agree with the minimum and maximum ppm values determined for the titration curve shown in Fig. 3a.

The HP agents developed in this work, in addition to others reported previously, represent a departure from previous techniques in HP pH imaging using ¹³C-bicarbonate. Important similarities exist between ¹³C pH agents that are "ratiometric" (e.g. ¹³C-bicarbonate), which quantify pH using the intensities of two separate ¹³C NMR resonances, and "chemical-shift" (e.g. ACES, DEMA), which quantify pH based upon a change in the observed ¹³C NMR frequency. In both cases, the pH-sensing molecule exists in both a protonated state and a deprotonated state, and the molecule exchanges between the two states with an overall first-order rate constant, $k_r$, representing both the forward and reverse reaction rates. The ratiometric and chemical-shift sensors differ in the magnitude of the exchange rate constant, $k_r$, relative to the CS dispersion, $\Delta \delta_r$ For ratiometric pH sensors, the exchange is much slower relative to the CS dispersion ($k_r < \Delta \delta_r$), leading to the observation of two distinct resonances via NMR spectroscopy. In the case of ¹³C-bicarbonate, the resonances for bicarbonate and CO$_2$ are separated by a large CS difference of 35.5 ppm. Furthermore, the chemical exchange between the two states is rate-limited by CO$_2$ hydration to form bicarbonate. Conversely, simple protonation-deprotonation of ACES or DEMA is fast relative to the total CS dispersion over all pH values ($k_r > \Delta \delta_r$), as is generally the case for these reactions. Therefore, these molecules exhibit one MR resonance, with a chemical shift that is a weighted average of the chemical shifts of the protonated and deprotonated molecular states.

MR chemical-shift sensors of pH possess certain advantages and disadvantages relative to ratiometric sensors. The presence of a single peak is a significant benefit concerning high spatial resolution imaging, since all HP molecules contribute to the magnitude of the single peak, and because imaging resolution is not limited by the signal of the lower of two peaks. However, these sensors also possess significant challenges. The resonant frequency, which gives a readout of pH, is also sensitive to magnetic field inhomogeneity and changes in susceptibility throughout the imaging volume. These effects can be accounted for by co-injecting a pH-insensitive HP molecule, in our case triOH, that is used as a chemical shift reference. Our experimental results in phantoms demonstrate that we can use this approach for highly accurate pH imaging. The ability to resolve different pH values in vivo will depend upon image acquisition parameters, voxel size, and $B_0$ inhomogeneity within each voxel. High-resolution pH imaging, which may be achievable using DEMA, should provide relevant data about pH gradients within tissue. As is the case with other magnetic resonance-based pH imaging approaches, the buffering capacity of DEMA could potentially alter the tissue pH. However, the signal gains resulting from hyperpolarization, and from the chemical shift imaging based approaches compared with those from a ratiometric approach, have the potential to minimize these effects.

DEMA exhibits some striking properties that make it amenable to high spatial resolution imaging. Firstly, the $T_1$ relaxation time constant is one of the longest measured for HP ¹³C compounds. Interestingly, the $T_1$ increases with field strength, as opposed to the vast majority of HP compounds ¹³C-enriched at carbonyls, whose relaxation is dominated by chemical shift anisotropy. However, the $T_1$ is still exceptionally long at a clinical field strength of 3 T. Combined with the high polarization obtainable for this compound, the long $T_1$ offers significant flexibility in terms of spatial resolution and timing of HP imaging.
Conclusions

We report a novel compound for pH measurement via \(^{13}\)C MRSI, [\(2-\text{DC}_{6}\)]diethylmalonic acid (DEMA). The pH is measured via changes in the NMR chemical shift, potentially circumventing SNR limitations found with the HP bicarbonate. The HP imaging pH accuracy and long \(T_1\) values make DEMA a strong potential candidate for high spatial resolution in vivo pH mapping.

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Notes and references

Appendix 4

Figure 1. a) Left (Chem. Commun. 2016, 52, 3030): pH phantom results at 14 T of $^{13}$C-bicarbonate. $T_2$-weighted $^1$H image shows electrode-measured pH values in white. Resonances of $^{13}$CO$_3^-$ and $^{13}$CO$_2$ from HP $^{13}$C spectra and calculated pH values for each color-coded voxel were obtained with two excitation pulses. Right (Analyst, 2017, 142, 1429): HP phantom imaging with $T_2$-weighted $^1$H image of tubes containing ~5 mM co-polarized DEMA and tBuOH at varying pH values. Overlaid $^{13}$C spectra from color-coded voxels are referenced to tBuOH peak. b) Representative kidney data from a HP $^{13}$C measurement in an axial slice overlaid on an anatomical proton image (greyscale). Simultaneously hyperpolarized and injected substances, DEMA and tBuOH, show signals in kidneys and blood pool of a healthy mouse. A voxel can contain two pairs of DEMA/tBuOH peaks leading to two pH values, contrary to the subsequent injection of $^{13}$C-bicarbonate which leads to one pair of $^1$H$^{13}$CO$_3^-$/$^{13}$CO$_2$ leading to one mean pH value.