Temperature and pH Imaging using Chemical Exchange Saturation Transfer (CEST) MRI Contrast

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A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

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TEMPERATURE AND pH IMAGING USING CHEMICAL EXCHANGE SATURATION TRANSFER (CEST) MRI CONTRAST
(Thesis format: Integrated Article)

by

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Graduate Program in Medical Biophysics, CAMPEP stream

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Abstract

Chemical exchange saturation transfer (CEST) is a novel mechanism used to generate contrast in magnetic resonance imaging (MRI). Recently, CEST contrast was proposed to noninvasively measure physiological parameters including temperature and pH. Tissue temperature and pH are known markers of pathological processes in many diseases including stroke and cancer. CEST contrast can be generated using endogenous proteins and peptides (endogenous CEST) or using exogenous paramagnetic lanthanide agents (PARACEST).

The general problem of optimizing applications of endogenous CEST and PARACEST contrast to measure temperature and pH is addressed in this thesis. Highlights of the thesis include a novel application of PARACEST contrast to measure extracellular pH and temperature in vivo and a novel ratiometric approach that uses endogenous CEST contrast to measure intracellular pH in vivo.

Using a Tm$^{3+}$-based PARACEST agent (Tm$^{3+}$-DOTAM-Gly-Lys), the PARACEST amide peak chemical shift and linewidth were shown to depend on pH and temperature in a deterministic manner. Quantitative temperature and pH maps were simultaneously measured in a normal mouse leg following agent injection using empirical relations derived in vitro.

A ratio of endogenous amide and amine proton CEST effects was developed to measure absolute tissue pH that is heavily weighted to the intracellular compartment. The technique called amine and amide concentration-independent detection (AACID) was developed using in vitro phantoms and numerical simulations. Following in vivo pH-calibration using 31P-magnetic resonance spectroscopy (MRS), tissue pH measurement was demonstrated in mice following focal cerebral ischemia. Local acidosis was measured in ischemic regions and found to correlate with regions of tissue damage.

Finally, two endogenous CEST metrics including the AACID ratio were used to monitor cancer treatment using an anticancer drug called lonidamine. Lonidamine selectively acidifies cancer cells. In vivo experiments demonstrate that endogenous CEST imaging is sensitive to intracellular acidification by lonidamine in a glioblastoma brain tumor mouse model.
Overall, the results presented in this thesis demonstrate quantitative measurement of pH and temperature using CEST and/or PARACEST contrast in vivo. Some of the novel techniques developed in this thesis were demonstrated in stroke and cancer mouse models. Future work should focus on 1) development of PARACEST agents with higher sensitivity in vivo to improve accuracy of temperature and pH maps; 2) application of AACID for absolute pH measurement to differentiate high- and low-grade tumors in vivo; and 3) application of endogenous CEST measurement to monitor tumor response to different clinically approved chemotherapy treatments.

Keywords

Magnetic resonance imaging (MRI), Chemical exchange saturation transfer (CEST), paramagnetic CEST (PARACEST), tissue pH, tissue temperature, cancer, cerebral ischemia
Co-Authorship Statement


This work was co-authored by: Nevin McVicar, Alex X. Li, Mojmir Suchy, Robert H.E. Hudson, Ravi S. Menon, and Robert Bartha.

A.L., and R.B. conceived the linewidth technique. N.M., A.L., and R.B. contributed to overall experimental design. A.L. performed preliminary in vitro experiments. N.M. performed all of the MRI experiments, analyzed the data, and wrote the manuscript; A.L. contributed to the MRI experiments, data analysis, and edited the manuscript. M.S. developed and produced the Tm$^{3+}$-based PARACEST agent (Tm$^{3+}$-DOTAM-Gly-Lys). R.H., R.M., and R.B. contributed to the manuscript writing and editing.

Quantitative tissue pH measurement during cerebral ischemia using amine and amide concentration-independent detection (AACID) with MRI (Journal of Cerebral Metabolism & Blood Flow. April 2014. DOI: 10.1038/jcbfm.2014.12)

This work was co-authored by: Nevin McVicar, Alex X. Li, Daniela F. Gonçalves, Miranda Bellyou, Susan O. Meakin, Marco A.M. Prado and Robert Bartha

N.M., A.L., and R.B. conceived the AACID technique and contributed to overall experimental design. N.M. performed the MRI experiments, analyzed the data, and wrote the manuscript; A.L. contributed to the MRI experiments, data analysis, and edited the manuscript. D.G. (MCAO and TTC staining), M.B. and S.M. completed all surgical and animal procedures and contributed to manuscript writing and editing. M.A.M.P. and R.B. contributed to the manuscript writing and editing.

Imaging chemical exchange saturation transfer (CEST) effects following tumor-selective acidification using lonidamine (Submitted to NMR in biomedicine. June 23 2014. Manuscript ID#: NBM-14-0163)
This work was co-authored by: Nevin McVicar, Alex X. Li, Susan O. Meakin, and Robert Bartha

N.M., A.L., and R.B. conceived the AACID technique and contributed to overall experimental design. N.M. performed the MRI experiments, analyzed the data, and wrote the manuscript; A.L. contributed to the MRI experiments, and edited the manuscript. S.M. contributed the brain cancer cell line and to manuscript writing and editing. R.B. contributed to the manuscript writing and editing.
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The Department of Medical Physics at Western University has been a well-organized and supportive place to earn my PhD. My advisory committee members, Dr. Neil Gelman and
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When I was young, I could not wait to get out of school. Over the years, however, I have grown to love school and this is only because I was fortunate to have had two incredible parents and so many great teachers and coaches throughout my life. I am forever grateful.
Table of Contents

Abstract........................................................................................................................................... ii
Co-Authorship Statement ................................................................................................................. iv
Acknowledgments ........................................................................................................................... vi
Table of Contents ............................................................................................................................ viii
List of Abbreviations ....................................................................................................................... xii
List of Tables ........................................................................................................................................ xiv
List of Figures ...................................................................................................................................... xv
Chapter 1 ........................................................................................................................................... 1
  1.1 Motivation and overview ........................................................................................................... 1
  1.2 Regulation of pH ...................................................................................................................... 3
    1.2.1 What is pH? ....................................................................................................................... 3
    1.2.2 Normal pH regulation ........................................................................................................ 4
    1.2.3 Regulation of pH in cancer ............................................................................................... 8
    1.2.4 Regulation of pH in cerebral ischemia ............................................................................. 10
  1.3 Temperature regulation ............................................................................................................ 11
    1.3.1 Normal temperature regulation ........................................................................................ 11
    1.3.2 Regulation of temperature in cancer ................................................................................ 11
    1.3.3 Regulation of temperature in cerebral ischemia ............................................................. 12
  1.4 Introduction to magnetic resonance imaging (MRI) .............................................................. 14
    1.4.1 Magnetic moments in an external magnetic field ........................................................... 14
    1.4.2 Excitation .......................................................................................................................... 18
    1.4.3 Relaxation ........................................................................................................................ 20
    1.4.4 Spatial-encoding magnetization using magnetic gradients ............................................. 22
### Chapter 3: Methods and Materials

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2 Methods and materials</td>
<td>85</td>
</tr>
<tr>
<td>3.2.1 CEST theory</td>
<td>85</td>
</tr>
<tr>
<td>3.2.2 AACID pH measurement</td>
<td>87</td>
</tr>
<tr>
<td>3.2.3 Numerical Simulations</td>
<td>89</td>
</tr>
<tr>
<td>3.2.4 In vitro solutions</td>
<td>90</td>
</tr>
<tr>
<td>3.2.5 MR experiments</td>
<td>91</td>
</tr>
<tr>
<td>3.2.6 Middle cerebral artery occlusion</td>
<td>94</td>
</tr>
<tr>
<td>3.2.7 Histology</td>
<td>95</td>
</tr>
<tr>
<td>3.2.8 Statistical Analysis</td>
<td>95</td>
</tr>
</tbody>
</table>

### Chapter 3.3: Results

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.3.1 Numerical Simulations</td>
<td>95</td>
</tr>
<tr>
<td>3.3.2 In vivo AACID pH-calibration</td>
<td>99</td>
</tr>
<tr>
<td>3.3.3 Acute acidosis following MCAO</td>
<td>101</td>
</tr>
</tbody>
</table>

### Chapter 3.4: Discussion

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>103</td>
</tr>
</tbody>
</table>

### Chapter 3.5: Conclusion

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>108</td>
</tr>
</tbody>
</table>

### Chapter 3.6: Acknowledgements

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>108</td>
</tr>
</tbody>
</table>

### Chapter 3.7: References

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>109</td>
</tr>
</tbody>
</table>

### Chapter 4: Imaging chemical exchange saturation transfer (CEST) effects following tumor-selective acidification using lonidamine

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1 Introduction</td>
<td>116</td>
</tr>
<tr>
<td>4.2 Theory</td>
<td>117</td>
</tr>
<tr>
<td>4.3 Experimental</td>
<td>119</td>
</tr>
<tr>
<td>4.3.1 Subjects</td>
<td>119</td>
</tr>
<tr>
<td>4.3.2 Lonidamine</td>
<td>119</td>
</tr>
<tr>
<td>4.3.3 Animal tumor preparation</td>
<td>120</td>
</tr>
</tbody>
</table>
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{18}$F FDG</td>
<td>Fluorine-18 labeled flurodeoxyglucose</td>
</tr>
<tr>
<td>AACID</td>
<td>Amine and amide concentration independent detection</td>
</tr>
<tr>
<td>AFI</td>
<td>Actual flip angle imaging</td>
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<tr>
<td>APT</td>
<td>Amide proton transfer</td>
</tr>
<tr>
<td>APT*</td>
<td>Apparent amide proton transfer</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenine triphosphate</td>
</tr>
<tr>
<td>BOLD</td>
<td>Blood oxygen level dependent</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>BW</td>
<td>Bandwidth</td>
</tr>
<tr>
<td>CA</td>
<td>Carbonic anhydrase</td>
</tr>
<tr>
<td>CEST</td>
<td>Chemical exchange saturation transfer</td>
</tr>
<tr>
<td>CNR</td>
<td>Contrast to noise ratio</td>
</tr>
<tr>
<td>CW</td>
<td>Continuous wave</td>
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<tr>
<td>DOTAM</td>
<td>1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>FBP1</td>
<td>Fructose-1,6-bisphosphatase-1</td>
</tr>
<tr>
<td>FSE</td>
<td>Fast spin echo</td>
</tr>
<tr>
<td>FOV</td>
<td>Field of view</td>
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<tr>
<td>FWHM</td>
<td>Full width at half maximum</td>
</tr>
<tr>
<td>GBM</td>
<td>Glioblastoma multiforme</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
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<tr>
<td>LND</td>
<td>Lonidamine</td>
</tr>
<tr>
<td>MCT</td>
<td>Monocarboxylate-H$^+$ efflux transporter</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MRS</td>
<td>Magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>MT</td>
<td>Magnetization transfer</td>
</tr>
<tr>
<td>MTR$_{asym}$</td>
<td>Asymmetric magnetization transfer ratio</td>
</tr>
<tr>
<td>M$_Z$</td>
<td>Longitudinal magnetization</td>
</tr>
<tr>
<td>NAD$^+$</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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</tbody>
</table>
NOE – Nuclear Overhauser effect;
PARACEST – Paramagnetic CEST
PBS – Phosphate buffered solution
PPM – Parts per million
PET – Positron emission tomography
pHₑ – Extracellular pH
pHᵢ – Intracellular pH
PTR – Proton transfer ratio
RF – Radio frequency
ROI – Region of interest
SNR – Signal to noise ratio
T₁ – Longitudinal relaxation time constant
T₂ – Transverse relaxation time constant
TE – Echo time
TI – Inversion time
Tm³⁺-DOTAM-Gly-Lys – Thulium complex with a DOTAM-Glycine-Lysine
tPA – tissue plasminogen activator
TR – repetition time
TS and T_sat – Saturation time
TTC - 2,3,5-triphenyltetrazolium chloride
WASSR – Water saturation shift referencing
List of Tables

Table 2.1: Stimulation Parameters Used for Aqueous Solutions Containing Tm$^{3+}$-DOTAM-Gly-Lys at 37 °C.................................................................60

Table 2.2: Relaxation Rates of Aqueous and 10% BSA pH 7 Solutions of Different Concentrations of Tm$^{3+}$-DOTAM-Gly-Lys (pH = 7.0, B$_0$ = 9.4 T, 37°C, Conc. = 5, 10, 15, 20 mM). .................................................................69

Table 3.1: Simulation parameters used to simulate CEST spectra for normal (NT) and ischemic (IT) cerebral tissue. .................................................................90

Table 3.2: Metabolite concentrations used for in vitro measurement of potential contaminant CEST effects.................................................................91
List of Figures

**Figure 1.1**: Schematic diagram describing pH homeostasis. Normal cellular glucose metabolism in normoxic (blue) and hypoxic (red) conditions contribute to proton, CO₂ and lactate production. Membrane ion transport channels and pHᵢ/pHₑ-buffering enzymes also contribute to normal pH maintenance. .................................................................6

**Figure 1.2**: a) An ensemble of ¹H nuclei with random orientations in the absence of a magnetic field. b) In the presence of a magnetic field (B₀), a hypothetical ensemble of ¹H nuclei have a slight excess of magnetic moments aligned in parallel with B₀ leading to a net magnetization M₀. .................................................................16

**Figure 1.3**: A hypothetical ensemble of ¹H nuclei in the rotating reference frame. a) In the presence of a magnetic field (B₀) at thermal equilibrium, the protons tend to orient in parallel or anti-parallel with B₀ leading to a thermal equilibrium net magnetization vector (M₀). b) Immediately following application of an RF excitation pulse along the x’-axis, the protons are equally distributed between parallel and anti-parallel orientations and in phase along the y’-axis. This figure was motivated by Figure 1.4 in reference [65]. ..................................................19

**Figure 1.4**: A typical CEST spectrum. A CEST effect (or CEST peak) can be observed at at 10 ppm as highlighted with the arrow. The peak at 0 ppm is due to direct saturation of the bulk water protons by the RF saturation pulse. .................................................................33

**Figure 1.5**: Numerically simulated CEST spectra (a,c,e,g) and proton transfer ratios (PTRs) (b,d,f,h) using the time-dependent analytical solutions of the two-pool version of the Bloch-McConnell equations. The effects of varying solute proton concentration (a,b), bulk water T₁ relaxation time constant (c,d), solute proton exchange rate (e,f), and RF saturation pulse amplitude (g,h) were investigated by varying the parameter of interest. For all simulations, *unless otherwise stated*: T₁w=2.0 s, T₂w = 50ms, T₁s=1.0 s, T₂s = 50ms, kₛₛ = 50 Hz, [solute proton] = 100 mM, Δωᵣ = 10 ppm, saturation pulse amplitude = 1.5 µT, saturation duration = 10 s). ...........................................................................................................38
Figure 1.6: Three CEST spectra were numerically simulated using the three-pool version of the Bloch-McConnell equations with different macromolecule concentrations expressed in % of macromolecules (% MT). The impact of magnetization transfer (MT) effects from macromolecules on CEST effects is evident when comparing CEST spectra simulated with 0% MT (a), 5% MT (b) and 10% MT (c). For all simulations, $T_{1w}=2.0$ s, $T_{2w}=50$ ms, $T_{1s}=1.0$ s, $T_{2s}=50$ ms, $T_{1m}=100$ ms, $T_{2m}=15$ ms, $k_{sw}=50$ Hz, $k_{mw}=50$ Hz, [solute proton] = 100 mM, $\Delta \omega_s = 10$ ppm, $\Delta \omega_m = 10$ ppm, saturation pulse amplitude = 1.5 $\mu$T, saturation duration = 10 s.

Figure 2.1: Molecular structure of the thulium ($\text{Tm}^{3+}$) chelate with ligand DOTAM-Gly-Lys and bound water.

Figure 2.2: a) Simulated MTR$_\text{asym}$ curves derived using the modified Bloch equations. Agent parameters used are listed in Table 1. The MRI parameters included $B_0 = 9.4$ T, $B_1 = 14$ uT, $T_S = 3$ s. pH-dependent exchange rates ($k_{SH}, k_{WS}$) were calculated using Eq. 2.9 with $k_0 = 100$ Hz, $k_b = 200 \times 10^9$ Hz and $pK_W = 15.4$. b) Linewidths plotted with pH. The exponential function (Linewidth = $3.25 + 10^{0.99pH-7.44}$) is superimposed on the simulation linewidths ($R^2 = 0.9999$). The linewidths increase monotonically and logarithmically with pH as predicted by the steady state analytical solutions.

Figure 2.3: A typical CEST spectrum of a 10 mM pH 7 solution of Tm$^{3+}$-DOTAM-Gly-Lys acquired on a 9.4 T MRI scanner at 37°C using a 14 $\mu$T 3 s saturation pulse. The linewidth of the CEST effect is defined as the full width at half maximum of the asymmetry curve generated by the subtraction of the halves of the CEST spectrum (pH = 7.0, $B_0 = 9.4$ T, $B_1 = 14$ $\mu$T, $T_S = 3$ s, 37°C).

Figure 2.4: a) The dependence of MTR$_\text{asym}$ linewidth on temperature for a series of 10 mM solution Tm$^{3+}$-DOTAM-Gly-Lys (pH: 6.0 (—), pH: 6.5 (---), pH: 7.0 (--.--), pH: 7.5 (• • •), pH: 8.0 (—)). B) Close-up of dependence of the MTR$_\text{asym}$ linewidth on temperature for pH 7 solution shown in 4A. C) The dependence of the MTR$_\text{asym}$ linewidth on concentration for the pH 7 solution of Tm$^{3+}$-DOTAM-Gly-Lys at 37°C in the absence and presence of MT effect. D) The dependence of the MTR$_\text{asym}$ linewidth on saturation power for the pH 7 solution of Tm$^{3+}$-DOTAM-Gly-Lys at 37°C ($B_0=9.4$ T, $B_1 = 14$ $\mu$T, $T_S = 3$ s for a,b,c,d). Error bars are equal to one standard deviation.
Figure 2.5: a) The dependence of MTR\_{asym} line shape on pH and b,c) the dependence of the MTR\_{asym} linewidth on pH of a 10 mM solution of Tm\textsuperscript{3+}-DOTAM-Gly-Lys at 37°C (B\textsubscript{0} = 9.4 T, B\textsubscript{1} = 14 \mu T, TS = 3 s, 37°C). .................................................................70

Figure 2.6: pH maps produced in phantom containing five tubes of solution of 10 mM Tm\textsuperscript{3+}-DOTAM-Gly-Lys with different pH at 37°C (pH = 6.0, 6.5, 7.0, 7.5, 8.0, B\textsubscript{0} = 9.4 T, B\textsubscript{1} = 14 \mu T, TS = 3 s, 37°C). The pH maps calculated using the CEST peak linewidth agree well with the actual pH measurements (white labels). .................................................................71

Figure 2.7: Temperature maps produced in phantom containing five tubes of solution of 10 mM Tm\textsuperscript{3+}-DOTAM-Gly-Lys with different pH at 37°C (pH = 6.0, 6.5, 7.0, 7.5, 8.0, B\textsubscript{0} = 9.4 T, B\textsubscript{1} = 14 \mu T, TS = 3 s, 37°C). The temperature maps calculated using the CEST peak linewidth and chemical shift agree well with the actual temperature measurements (white labels). .................................................................72

Figure 2.8: a) Average preinjection and postinjection CEST spectra, MTR\_{asym} curves on the x-axis. All curves and maps were generated using only pixels that exhibited significant PARACEST contrast (probability ≥ 95%) following injection. b) \emph{In vivo} pH map superimposed onto a preinjection anatomical image. c) \emph{In vivo} temperature map superimposed onto a preinjection anatomical image. The temperature and pH maps calculated in vivo agree well with normal temperature and pH measurements. ..........73

Figure 3.1: Illustration of chemical exchange saturation transfer (CEST) principles and the effect of pH on amine and amide CEST signal: a) Schematic diagram of relevant solute proton pools that resonate at frequencies chemically-shifted by parts per million (ppm) from bulk water. b) Irradiation of amide \textit{(top)} or amine \textit{(bottom)} protons using a frequency-selective saturation pulse. Chemical exchange transfers amide \textit{(top)} or amine \textit{(bottom)} magnetization to bulk water, resulting in indirect saturation of the bulk water (M\textsubscript{ss}). c) CEST spectra (vertically offset for clarity) measured at different pH (6.0, 6.5 and 7.0). The red and green portions of the curve illustrate the pH dependent response of the amide and amine protons. ..................................................................................................................87

Figure 3.2: Numerical simulations of AACID Dependence on pH, Macromolecule Concentration, and Relaxation Time Constants in Brain: a) Simulated CEST-spectra for
varying pH in normal brain tissue. b) Figure 3.1a magnified to highlight the amine and amide pH dependent CEST effects. Note: $M_z$ and $M_0$ represent the region of interest MRI signal acquired with and without a saturation pulse, respectively. c) Amine and amide CEST values measured directly from 2a using equation [3.3]. d) AACID values measured directly from 3.2a using equation [3.6]. e) AACID-pH relationships simulated for a range of macromolecular concentrations (% by-weight) using a 4s, 1.5 μT saturation pulse. f) AACID-pH relationships simulated for normal and ischemic brain tissue using a 4s, 1.5 μT saturation pulse. Numerical simulations predict that AACID values decrease approximately linearly with pH within the physiological pH and temperature range. Independence of physiological changes in MT and relaxation times during cerebral ischemia suggests that AACID can be used to calculate absolute pH in vivo.

Figure 3.3: Amine and Amide CEST Dependence on pH, Protein Concentration and Temperature: a) CEST-spectra of 10% (by weight) BSA dissolved in PBS with varying pH at 37 °C. b) Figure 3.2a magnified. c) Amine and amide CEST values measured directly from 3.3a using equation [3.3]. d) AACID values measured directly from 3.3a using equation [3.6]. e) AACID values measured in solutions containing a range of BSA concentrations at 37 °C using a 4s, 1.5 μT saturation pulse. f) AACID values measured in a pH 7.0 10% BSA solution for a range of physiologically relevant temperatures using a 4s, 1.5 μT saturation pulse. In vitro AACID measurements decrease linearly with pH as predicted by numerical simulations. AACID independence of protein concentration and tissue temperature suggests that AACID values can be used to calculate absolute pH in vivo.

Figure 3.4: Amine and Amide CEST Dependence on Saturation Pulse Amplitude, Duration, and Metabolite Contributions: Amine and amide CEST effects from 10% (by weight) BSA dissolved in pH 7.0 PBS at 37 °C measured following a) a 4s continuous wave RF saturation pulse with amplitudes ranging from 0-3 μT, and b) a 1.5 μT continuous wave RF saturation pulse with duration ranging from 0-6s. CEST values measured at c) 2.75 ppm and d) 3.50 ppm for major brain metabolites at physiological concentrations (37 °C using a 4s, 1.5 μT continuous wave RF saturation pulse) compared to 10% BSA. Amide and amine CEST effects approach steady state after a 4s, 1.5 μT saturation pulse. Protein is shown to be the dominant contributor to AACID values compared to other brain metabolites, which
suggests that AACID measurements are independent of changes in metabolite concentrations and can be used to calculate absolute pH in vivo. ................................................................. 100

Figure 3.5: AACID pH-Calibration In vivo using $^{31}$P-MRS: pH measurements and CEST spectra acquired using the standard $^{31}$P-MRS and $^1$H-MRI respectively in vivo and postmortem. a) In vivo (bottom) and postmortem (top) whole brain $^{31}$P-MRS spectra for one mouse. b) Corresponding CEST spectra. c) AACID and pH measurements for three different mice. In vivo AACID values decrease linearly with pH as predicted by numerical solutions and in vitro experiments. AACID pH-calibration data can now be used to calculate absolute pH in vivo. ........................................................................................................ 101

Figure 3.6: Cerebral Ischemia: Representative MR images of a mouse brain two hours (a-e) and five hours (f-j) following permanent middle cerebral artery occlusion (MCAO) in radiological orientation. a,f) $T_1$-weighted image, b,g) $T_2$-weighted image, c,h: diffusion-weighted image, d,i: pH map with ischemic (left) and contralateral (right) regions of interest (ROIs) drawn, and e) CEST-spectra measured from ischemic (red) and contralateral (blue) ROIs at 2 hours with CEST effects located at arrows (red arrow: amide CEST, green arrow: amine CEST), f) histology TTC-stained for mitochondrial cellular respiration (i.e. red stained tissue has functional mitochondrial and white un-stained tissue does not). ......................... 103

Figure 4.1: Representative set of CEST data from a healthy C57BL/6 mouse brain: CEST images acquired immediately before (a, d) and 50 min after (b, e) i.p. injection of 100 mg/kg LND. AACID and APT* values were calculated from the same z-spectra. Contrast maps (c, f) were calculated using CEST maps acquired at baseline and after LND treatment using equation [4.4]. ........................................................................................................ 125

Figure 4.2: Summary of CEST and contrast values from healthy C57BL/6 mice (N=3): a) Mean AACID and b) APT* values acquired at baseline and ~50 min after 100 mg/kg LND treatment. No significant differences were observed before and after LND treatment in both the AACID and APT* values. As a result the average LND-induced contrast was equal to background noise. ........................................................................................................ 125

Figure 4.3: Standard anatomical MR imaging of U87 GBM brain tumor before injection of lonidamine: a) $T_2$-weighted image with tumor (dashed line) and contralateral
(solid line) regions of interests (ROIs) drawn. b) T1-weighted and c) diffusion-weighted images were also acquired using the identical slice of interest. ................................. 127

Figure 4.4: Chemical exchange saturation transfer (CEST) z-spectra collected at baseline and ~50 min after administration of lonidamine (LND): Average B0-corrected z-spectra calculated from a) normal contralateral tissue-containing region of interest (ROI) and b) tumor tissue-containing ROI with error bars equal to one standard deviation. A reference line (gray dashed) was empirically superimposed onto baseline z-spectra to aid visualization of differences between z-spectra in contralateral and tumor tissue. These zoomed Z-spectra highlight the significant changes in the 1.6 to 4.5 ppm frequency range. ................................. 127

Figure 4.5: CEST data from a U87 GBM brain tumor model treated with 100 mg/kg LND: CEST images acquired immediately before (a, d) and 50 min after (b, e) i.p. injection of 100 mg/kg LND. AACID and APT* values were calculated from the same z-spectra. Contrast to noise ratio maps show the change in CEST effects caused by tumor acidification following administration of LND normalized to the background noise (c, f). ...................... 128

Figure 4.6: Summary of CEST and contrast values from NU/NU mice with U87 GBM brain tumors (N=3): a,b) Mean AACID and c,d) APT* values acquired at baseline and ~50 min after 100 mg/kg LND treatment in tumor regions of interest (ROIs) (a,c) and contralateral ROIs (b,d) with error bars equal to one standard deviation. Mean LND-induced contrast values are shown with error bars equal to one standard error................................. 129

Figure 4.7: Dose dependence of mean LND-induced contrast values calculated using each pixel in tumor regions of interest (ROIs): Mean LND-induced contrast values generated using a) AACID and b) APT* values in tumor ROIs are shown with error bars equal to one standard error for different LND doses...................................................... 131

Figure 4.8: H&E stained brain section from representative U87 GBM brain tumor model: a) Brain section corresponding to the slice of interest used for CEST MR images in Figure 3 with two regions of interest that display stain heterogeneity attributed to vasculature and/or inflammatory cells labeled b and c. b-c) Magnified regions of interest b and c respectively................................................................. 132
Chapter 1

1 Introduction

This thesis investigates the use of chemical exchange saturation transfer (CEST) magnetic resonance imaging (MRI) to produce contrast for the measurement of pH and temperature. The purpose of this chapter is to provide an introduction and motivation to the presented research. Section 1.1 outlines the motivation and general organization of the thesis. A brief survey of pH and temperature homeostasis and clinical significance is presented in Sections 1.2 and 1.3. An introduction to MRI physics is presented in Section 1.4, followed by a thorough discussion of CEST theory and applications in pH and temperature measurement in Section 1.5.

1.1 Motivation and overview

Traditionally, MRI is used to produce high-resolution images with excellent anatomical contrast that allows localization and differentiation between different types of soft tissue (e.g. muscle, fat and tumor tissue). Anatomical MRI offers clinicians valuable information and sometimes enables disease diagnosis [1, 2]. Significant advances in molecular biology have improved our understanding of the pathophysiology underlying many diseases [3, 4]. Detection of abnormal physiology often enables disease diagnosis before MRI-detectable anatomical changes occur [5]. The probability of treatment success is almost always increased with early diagnosis. Therefore, imaging physicists are highly motivated to develop novel techniques to quantify physiological processes such as metabolism. Recently, a new MRI technique called CEST has emerged to image biological targets such as specific proteins and/or metabolites as well as molecular events such as apoptosis [6] and angiogenesis [7]. CEST is a mechanism that can be used to generate contrast based on several factors that are coupled to metabolism including temperature [8], pH [9] and specific metabolite concentrations [10]. Two fundamental tissue properties, pH and temperature, are correlated with energy producing metabolic activity [9, 11]. To date, most applications of CEST to measure temperature and/or pH focus on applications in cancer and stroke. Cancer and stroke represent two common
diseases that are characterized by significant changes in metabolism resulting in altered pH and temperature as described in Sections 1.2 and 1.3 respectively. Therefore, temperature and pH are potentially valuable markers for indirect investigation of metabolic changes in cancer and stroke. In some cases, disease diagnosis and prognosis can be more accurate using metabolic imaging compared to anatomical MR imaging [5]. For example, the current gold-standard metabolic imaging device for cancer is positron emission tomography (PET). Detection of the glucose analogue called $^{18}$F-labeled fluorodeoxyglucose ($^{18}$F FDG) using clinical PET scanners enables direct measurement of glucose uptake in tumors. Quantitative parameters describing tumor uptake of $^{18}$F FDG predict tumor aggressiveness and response to therapy [12]. However, metabolic imaging using the MRI would arguably be more cost-effective, more easily accessible, and easier to interpret because metabolic maps could be easily registered with high-resolution anatomical MRI [13].

In this thesis, novel techniques are presented to measure absolute temperature, extracellular pH (pH$_e$), and intracellular pH (pH$_i$) in living biological systems using CEST MRI contrast. In all cases, the strategies are developed theoretically, tested in vitro and finally demonstrated in vivo using mouse models. Chapter 2 presents a novel strategy to measure absolute pH$_e$ and tissue temperature simultaneously using a PARACEST agent called Tm$^{3+}$-DOTAM-Gly-Lys. In vivo experiments demonstrate absolute temperature and pH$_e$ mapping after injection of Tm$^{3+}$-DOTAM-Gly-Lys into a normal mouse leg. In chapter 3, a ratiometric technique is developed to measure pH$_i$ using endogenous CEST contrast. Quantitative pH$_i$ maps are used to detect local acidosis in mouse brain during cerebral ischemia (ie. stroke). In chapter 4, tumor-selective acidification is measured using CEST contrast following injection of an anticancer drug in a glioblastoma brain tumor mouse model. The sensitivity to drug-induced changes in tumor pH$_i$ was compared for two different endogenous CEST metrics. Finally, chapter 5 offers a summary of the significant findings and conclusions of the work presented in this thesis. Experimental limitations, future experiments, and clinical applications of the novel techniques are also discussed.
1.2 Regulation of pH

1.2.1 What is pH?

Water molecules (H₂O) naturally tend to ionize, or dissociate into a proton (H⁺) and a hydroxyl ion (OH⁻) according to equation [1.1].

\[ H_2O \leftrightarrow H^+ + OH^- \]  \hspace{1cm} [1.1]

Self-ionization of water is constant and reversible. At equilibrium, the product of ion concentrations is characterized with the dissociation constant of water \( K_w \), described in equation [1.2].

\[ K_w = [H^+] [OH^-] \]  \hspace{1cm} [1.2]

At equilibrium, \( K_w \) is constant for a given temperature, for example \( K_w = 10^{-14} \) M² at 25°C [14].

The logarithmic form of \( K_w \), known as p\( K_w \), is given in equation [1.3].

\[ pK_w = -\log(K_w) \]  \hspace{1cm} [1.3]

In solution, the proton is an acid since it can donate a proton (ie. itself) or accept an electron pair, whereas the hydroxyl ion is a base since it can accept a proton or donate an electron pair. A Danish chemist named Søren Sørensen developed the pH concept that is used to quantify how acidic or basic a solution is. Solution pH is defined as:

\[ \text{pH} = -\log[H^+] \]  \hspace{1cm} [1.4]

where \([H^+]\) is the molar concentration of protons [14, 15]. Addition of other acids or bases can perturb the system shown in equation [1.1] to a new equilibrium with different \([H^+]\) and \([OH^-]\) but a constant \( K_w \). For example, at 25°C an extremely acidic solution has \([OH^-] = 1.0 \times 10^{-14} \) M, \([H^+] = 1.0 \) M, and pH = 1; a neutral solution has \([OH^-] = \)
1.0x10^{-7} \text{ M}, \left[H^+\right] = 1.0x10^{-7} \text{ M}, \text{ and pH}=7; \text{ and an extremely basic solution has } \left[OH^-\right] = 1.0 \text{ M}, \left[H^+\right] = 1.0x10^{-14} \text{ M and pH} = 14.

In biological tissue, \left[H^+\right] in intracellular or extracellular fluid can be calculated using equation [1.4] and pH_i or pH_e respectively. Furthermore, if pK_w is known, then \left[OH^-\right] may also be calculated using both pK_w and pH as shown below:

\[
\left[OH^-\right] = \frac{\left[\text{H}^+\right]\left[\text{OH}^-\right]}{\left[H^+\right]} = \frac{10^{-pK_w}}{10^{-\text{pH}}} = 10^{\text{pH}-pK_w} \tag{1.5}
\]

1.2.2 Normal pH regulation

Robust maintenance of pH is a fundamental homeostatic process in biological systems. Systemic blood pH is regulated by the synchronized function of the renal system, respiratory system and blood buffering enzymes [14]. More specifically, if the blood pH is too acidic, ventilation increased to expire more weakly acidic carbon dioxide (CO_2) and the opposite is true when blood is too alkaline. Similarly, the renal system regulates the removal or retention of acids or bases through the urine based on the blood pH. Also, several enzymes act as pH buffers by catalyzing reactions that alter the acid-base balance in blood. The pH measurement techniques developed in this thesis aim to quantify local pH_i and pH_e in diseases such as stroke and cancer. In such diseases, local changes in blood circulation, energy metabolism and ion transport membrane channels result in much larger reductions in local tissue pH (~0.3-1.0 pH units) [16-18] compared to the changes observed in systemic (ie. whole body) blood pH (~0.1 pH unit) [19]. Therefore, from this point forward, pH regulation at the cellular level is discussed with a strong focus on the role of metabolism because it changes significantly in both cancer and stroke.

Different pH balances are maintained in the two main tissue compartments, the intracellular and extracellular spaces [20]. The difference between pH_i and pH_e produces a pH gradient across the cell membrane. Under normal conditions, pH_i is slightly more acidic at ~7.2 compared to pH_e at ~7.4 [20]. Normal pH in both compartments is
maintained through the complex and coordinated interaction between several different physiological systems including cellular metabolism, the cellular membrane potential, intracellular and extracellular buffering systems, cell membrane transporters, delivery of oxygen and glucose, and removal of acid by-products by the blood [21].

Cellular glucose metabolism (Figure 1.1) plays an important role in the acid-base homeostasis of both intracellular and extracellular spaces. For example, the conversion of glucose to pyruvate via glycolysis, which occurs in the cytosol and is described by equation [1.6]. Specifically, the breakdown of one glucose molecule results in reduction of two co-enzymes called nicotinamide adenine dinucleotide (NAD\(^+\)) to their reduced form NADH, phosphorylation of two inorganic phosphate (Pi) and adenosine diphosphate (ADP) molecules to create two high-energy adenosine triphosphate (ATP) molecules as well as production of two water molecules and two protons that are mostly exported to the extracellular space by one of a variety of proton transporters located on the cell membrane [20].

\[
\text{Glucose} + 2\text{NAD}^+ + 2\text{ADP} + 2\text{P}_i \rightarrow 2\text{Pyruvate} + 2\text{NADH} + 2\text{H}^+ + 2\text{ATP} + 2\text{H}_2\text{O} \quad [1.6]
\]
**Figure 1.1:** Schematic diagram describing pH homeostasis. Normal cellular glucose metabolism in normoxic (blue) and hypoxic (red) conditions contribute to proton, CO$_2$ and lactate production. Membrane ion transport channels and pH$_i$/pH$_e$-buffering enzymes also contribute to normal pH maintenance.

In normoxic (ie. aerobic) conditions, pyruvate is transported to the mitochondrion, where it is converted to acetyl coenzyme A (acetyl-CoA) and CO$_2$. Within the mitochondrion, acetyl-CoA enters the citric acid cycle, which produces more CO$_2$ and reduces NAD$^+$ to NADH. The electron transport chain then oxidizes the NADH to its oxidized form NAD$^+$ with oxygen (O$_2$) acting as the final electron acceptor. The net result of the electron transport chain is a proton gradient across the mitochondrion inner membrane, which is used to produce three high-energy ATPs per NADH through oxidative phosphorylation [22].
In the absence of oxygen (hypoxia), the citric acid cycle and electron transport chain are inhibited, resulting in elevated glycolysis to meet energy demands. Upregulated glycolysis relies on lactic acid fermentation (equation [1.7]) to maintain a sufficient supply of NAD$^+$ for further glycolysis.

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \rightarrow \text{Lactate} + \text{NAD}^+$$ \hspace{1cm} [1.7]

Lactic acid has a $pK_a \sim 3.9$ and is mostly dissociated into the lactate anion and a proton as shown in equation [1.8] at physiological pH values leading to acidification in hypoxic conditions [23].

$$\text{Lactic acid} \leftrightarrow \text{Lactate}^{-} + \text{H}^+$$ \hspace{1cm} [1.8]

Furthermore, during hypoxia, hydrolysis of ATP (equation [1.9]) that was produced in the mitochondria also contributes to decreased cellular pH, unless the cell can export protons quickly [23].

$$\text{ATP} + \text{H}_2\text{O} \leftrightarrow \text{ADP} + \text{HPO}_4^- + \text{H}^+$$ \hspace{1cm} [1.9]

Membrane transporters such as monocarboxylate-H$^+$ efflux transporters (MCTs) are relied upon to simultaneously remove protons and lactate from the cell to avoid acidosis [12, 20]. Metabolic acids such as protons, lactate and CO$_2$ that are transported to the extracellular space diffuse into local blood vessels where they are transported to lungs (CO$_2$) or the kidneys for removal via the urine. Intracellular and extracellular acid concentration may also be buffered by different biochemical buffering systems. For example, the bicarbonate (HCO$_3^-$) buffering system regulates the CO$_2$ and HCO$_3^-$ levels. Carbonic anhydrase (CA) is an important enzyme of the bicarbonate buffering system that catalyzes the hydration of CO$_2$ to form HCO$_3^-$ (equation [1.10]) [20].

$$\text{CO}_2 + \text{H}_2\text{O} \overset{\text{CA}}{\leftrightarrow} \text{H}_2\text{CO}_3 \overset{\text{CA}}{\leftrightarrow} \text{HCO}_3^- + \text{H}^+$$ \hspace{1cm} [1.10]
Carbonic anhydrase catalyzes the consumption or production of H\(^+\) depending on substrate concentrations and is expressed in both intracellular and extracellular spaces. The bicarbonate buffering system is responsible for the vast majority of the blood buffering capacity [24-26]. Similar phosphate and ammonium (NH\(_4^+\)) buffering systems are important for intracellular buffering [12, 26].

Normal human tissue is capable of maintaining a healthy acid base balance in a variety of conditions including brief (ie. on the order of seconds) periods of hypoxia. However, in many diseases where oxygen supply does not meet the metabolic demand, elevated lactate and proton concentration can lead to abnormal pH. Importantly, many cellular macromolecules, proteins and peptides have pH-dependent structures and activities [20, 27]. Therefore, in pathological tissue, pH is a fundamental marker of disease progression.

1.2.3 Regulation of pH in cancer

Abnormal pH balance is a common trait in cancer tissue [20, 21]. Cancer cells tend to have up-regulated glycolysis in aerobic conditions, known as the Warburg effect [12]. While the origin of the Warburg effect has not been completely elucidated, several mechanisms have been shown to contribute to the observed effects. The most popular hypothesis for up-regulated glycolysis in cancer cells is that Darwin selection favours cells expressing the Warburg effect. Early in tumor progression, cancer cells proliferate and form large cellular masses, or tumors, faster than proper vasculature can be produced. Therefore, cancer cells in the central core of the tumor often lack proper circulation and cells with up-regulated glycolysis tend to have an advantage [12, 20].

Cancer cells may up-regulate glycolysis using numerous control mechanisms including pro-glycolytic enzymes such as hexokinase or increased glucose uptake [12]. For example, epigenetic mechanisms have been shown to cause increased anaerobic glycolysis in gastric cancers [28]. Epigenetics is the study of chemical processes that cause chemical changes in DNA (e.g. methylation) that alter protein expression without any change in DNA sequence [29]. Liu et al. recently demonstrated hypermethylation of a promoter for known a glycolysis antagonist called fructose-1,6-bisphosphatase-1 (FBP1) in cancer cells contributed to the observed Warburg effect [28]. Importantly, epigenetic contributions to
the Warburg effect suggest that the effect is not permanent. The end result is a tumor with a relatively large population of cells favouring glycolysis. Increased glucose uptake is a prominent phenotype in many cancers that has been observed repeatedly when imaging regional uptake of the glucose analog \(^{18}\text{F}\)-labeled fluorodeoxyglucose (\(^{18}\text{F}\) FDG) using positron emission tomography (PET) \([12]\). Therefore, cancer cells have abnormally high rates of glycolysis in both anaerobic and aerobic conditions. Anaerobic glycolysis is an inefficient way to produce ATP compared to aerobic respiration as described in Section 1.2.2. However, Vander Heiden et al. recently proposed that the Warburg effect facilitates uptake of critical components for cell proliferation such as amino acids. Therefore, the Warburg effect is used to meet energy demands while simultaneously producing cellular building blocks \([30]\).

Cancer cells with up-regulated glycolysis produce higher amounts of pyruvate leading to increased proton and lactate production \([12]\). Paradoxically, cancer cells that produce high levels of lactate tend to have an alkaline \(\text{pH}_i\) (\(\text{pH}_i > 7.4\)) compared to normal cells \([20]\). Increased \(\text{pH}_i\) in cancer cells is caused by increased expression of proton pumps on cellular membranes such as \(\text{H}^+\)-ATPases and \(\text{Na}^+\)/\(\text{H}^+\) exchangers \([20, 27]\). Cancer cells also increase expression and activity of numerous MCTs \([20]\). Increased lactate and \(\text{H}^+\) efflux is advantageous to cancer cell survival since it allows increased glycolysis that serves as an important energy source for proliferation. Furthermore, increased efflux of protons and lactate result in an acidic extracellular environment with \(\text{pH}_e < 7.1\) \([20, 21]\).

Again, reduced \(\text{pH}_e\) provides a selective advantage to tumor cells since surrounding normal cells cannot survive in the acidic extracellular space. Consequent degradation of neighbouring normal cells provides a significant opportunity for evasion and tumor growth \([20]\).

In cancer, increased \(\text{pH}_i\) and reduced \(\text{pH}_e\) both signal altered metabolism in cancer cells. Measurement of \(\text{pH}_i\) and/or \(\text{pH}_e\) may improve accuracy of diagnosis of disease progression in tumors. For example, noninvasive \(\text{pH}\) mapping may enable clinicians to predict tumor metastasis. Furthermore, \(\text{pH}\) measurement will help develop treatment strategies since many anti-cancer therapeutic agents have \(\text{pH}\)-dependent activity and pharmacokinetics \([21, 31]\). Gerweck et al. recently showed that weakly acidic
chemotherapy agents preferentially cross the cell membrane when passing through the acidic extracellular environment in cancer tissue. Upon entrance to the basic intracellular space of cancer cells, the weakly acidic agents are trapped by ionization. Previous experimental results support the use of weakly acidic chemotherapy agents when the pH balance is altered in cancer [21, 31].

1.2.4 Regulation of pH in cerebral ischemia

Cerebral ischemia is a condition characterized by insufficient blood flow to the brain to meet metabolic demand. The most common cause of stroke is the disruption of blood flow to the brain by a blood clot. Loss of blood flow to the brain not only prevents oxygen delivery to brain cells, but also inhibits removal of metabolic acids from the extracellular space. Hypoxia inhibits aerobic respiration cycles (citric acid cycle and electron transport chain) leading cells to up-regulate anaerobic glycolysis to meet energy demands. During hypoxia, pyruvate molecules are preferentially converted to lactate, resulting in both intracellular and extracellular acidosis [16]. Increased glycolysis leads to intracellular acidosis in stroke, unlike cancer, because normal brain cells do not have increased expression of H$^+$ and lactate efflux transporters. It has also been shown that cell damage is highly correlated to lactic acidosis [32, 33]. Severe acidosis occurs in the ischemic core, which is the epicenter of the disease where damage is irreversible. Severe acidosis (pH < 6.5) disrupts vascular membranes during stroke leading to increased hemorrhage and edema [34]. Specifically, acidosis leads to protonation of macromolecules and proteins causing structural changes that disrupt protein function and/or synthesis. In addition, the dissociation of iron (Fe) from proteins and production of oxygen radicals are both increased at low pH [16, 34, 35]. Oxygen radicals are highly reactive species that can cause damage to proteins, DNA and lipids. A noninvasive absolute pH measurement technique is needed to identify pH thresholds at which irreversible tissue damage occurs.

In the clinic, quantitative pH mapping techniques will enable clinicians to identify the ischemic core as well as the penumbra, which is composed of salvageable ischemic tissue surrounding the core. The current standard of care for stroke patients is administration of tissue plasminogen activator (tPA) that converts the plasminogen enzyme to plasmin,
which is responsible for breaking down blood clots. Clinicians may prescribe tPa to
dissolve the blood clot and facilitate reperfusion of the ischemic region. A recent meta-
analysis of 12 randomized clinical trials discovered that tPA treatment within 6 hours of
ischemia onset improved overall patient survival and recovery [4]. However, the same
report also found that patients treated with tPA had a higher mortality rate in the acute
phase (ie. within 7 days of treatment) due to cerebral hemorrhage. Therefore, there is
significant risk of hemorrhage when treating some patients with tPa. Knowledge of the
size, location and metabolic status of the ischemic lesion with a pH distribution map
would help guide critical treatment decisions regarding stroke patients.

1.3 Temperature regulation

1.3.1 Normal temperature regulation

Similar to pH, tissue temperature is tightly regulated around 37 °C (+/- 1.0 °C) under
normal conditions [36]. Strict regulation of core tissue temperature is important because
the function and structure of many cellular components such as protein depends on
temperature [37]. Body heat is a by-product of energy consuming metabolic processes
occurring within cells [38, 39]. Heat flow throughout the body is controlled by blood
circulation. Blood flowing through external skin and lung tissue exchanges heat with the
external environment by conduction and evaporation [38-41]. The magnitude of heat
exchange with the external environment is controlled by regulation of blood flow via
vasodilatation and/or vasoconstriction. In diseases such as stroke and cancer, abnormal
local tissue temperature is a common symptom associated with changes in heat producing
energy metabolism and/or heat removal by blood circulation [42, 43]. Inflammation can
also cause local heating due to increased blood flow to the injury as well as increased
cellular activity to repair local cells and remove necrotic cells.

1.3.2 Regulation of temperature in cancer

Temperature is altered in cancer tissue [43, 44]. Altered blood circulation and
metabolism are hallmarks of cancer leading to changes in temperature [45, 46].
Abnormal blood circulation in tumors is attributed highly branched and dilated blood
vessels [47]. Angiogenesis must occur quickly in fast growing malignant tumors
characterized by rapid tumor cell replication to meet high-energy demands resulting in disorganized and inefficient vasculature [47, 48]. As mentioned in Section 1.2.3, abnormal metabolism is also a hallmark of cancer. Importantly, different types of cancer express different metabolic profiles [49]. Therefore, since tumor temperature reflects both circulation and metabolic activity, absolute temperature mapping could tumor diagnosis, staging, and treatment strategies. Recently, Jayasundar and Singh recorded tumor temperatures ranging from ~34 to 43°C and discovered that temperature could be used to differentiate some tumor types [43]. Differences between different tumor types were hypothesized to be due to differences in vasculature, metabolic rate and the presence of necrosis. To-date, the study by Jayasundar and Singh represents the only investigation of noninvasive absolute temperature measurement within multiple types of tumors in humans and therefore, more research is required.

Absolute temperature mapping would also enable accurate monitoring of a new cancer treatment technology called thermal ablation. Thermal ablation involves focused application of extreme heat to a tumor typically using high energy radiofrequency or microwave pulses [50]. Ablation techniques enable noninvasive removal of small tumors that are deemed unfit for surgery [50]. Most thermoablative techniques aim to reach tumor temperatures of 60°C to induce cell death [50]. The most common cause of poor patient outcome following tumor ablation is incomplete tumor coverage resulting in tumor survival or recurrence [50-52]. Therefore, application of thermal ablation is limited to small (< 5 cm) tumors to avoid incomplete tumor ablation. Real-time absolute tumor temperature mapping would ensure that target temperatures are reached across the entire tumor.

1.3.3 Regulation of temperature in cerebral ischemia

As mentioned in Section 1.2.4, cerebral ischemia is a condition caused by severely or completely reduced blood delivery to brain tissue. Tissue hyperthermia has been observed in affected brain regions during the acute phase of cerebral ischemia in both animal models and humans [42, 53, 54]. Hyperthermia during cerebral ischemia is attributed to several factors including: 1) the loss of blood flow inhibits removal of heat from ischemic tissue; 2) increased heat production in ischemia tissue via elevated
anaerobic metabolic activity; 3) a release of excitatory neurotransmitters occurs upon ischemic infarct causing hyperactivity of local neurons; and 4) heat producing leukocyte activity associated with inflammation within ischemic infarct. Specific contributions of the listed factors along with a vast set of metabolic processes triggered or arrested by cerebral ischemia are poorly understood, demonstrating a need for more research [55, 56].

While acute hyperthermia is common in stroke patients, tissue damage from hyperthermia alone only occurs in severe cases (i.e. > 40°C) [36]. However, even mild hyperthermia (~38°C) can significantly enhance tissue damage caused by stroke [36, 56]. Anaerobic metabolism produces lactic acidosis during ischemia, as discussed in Section 1.2.4, and metabolic activity increases in hyperthermia [56]. The combination of hyperthermia and anaerobic metabolism leads to worse patient outcomes due to more severe lactic acidosis [53, 57]. Furthermore, hyperthermia also potentiates release of excitatory neurotransmitters [36, 57] and synthesis of damaging reactive oxygen radicals [36, 58]. Therefore, high-resolution quantitative temperature maps in stroke patients will offer valuable information regarding the metabolic status of ischemic tissue that would identify tissue that could be rescued with treatment.

Several reports have demonstrated that inducing mild hypothermia after stroke by reducing the core body temperature by approximately 1-6°C [42, 59] leads to improved neurological outcomes in animals and humans [36, 42, 59, 60]. For example, a recent clinical trial showed that reducing core body temperature to 32°C approximately 3 hours after thrombolysis treatment with tPA and 6 hours after stroke onset led to improved outcomes in stroke patients [60, 61]. Controlled hypothermia offers neurological benefits to some patients however, it is difficult to induce and maintain mild hypothermia and then re-warm a critically ill patient without encountering severe side effects that may negate any potential benefits. There are many side effects during prolonged hypothermia including bradycardia, increased blood pressure, shivering that reverses body cooling, insulin-resistance that leads to hyperglycemia, as well as during re-warming including high extracellular potassium [60-63]. Quantitative brain temperature mapping would enable more accurate selection of patients that would benefit from hypothermia (ie.
patients with hyperthermic lesions), and clinical monitoring of tissue temperature in the ischemic lesion during hypothermia maintenance and re-warming. Currently, scientists and clinicians monitor hypothermia using the core body temperature. Whole-brain absolute temperature maps will offer a more detailed assessment of local ischemic tissue status and may enable the discovery of an optimal ischemic lesion temperature that leads to the best long-term outcome in patients while minimizing adverse side effects.

1.4 Introduction to magnetic resonance imaging (MRI)

Magnetic resonance imaging (MRI) is an established diagnostic imaging tool that is used in clinical medicine. Traditionally, clinical MRI produces high-resolution anatomical images based upon magnetic signals from hydrogen ($^1$H) associated with bulk water in soft tissue. This section briefly outlines the fundamental principles underlying $^1$H MRI. The purpose of this section is to offer a basic understanding of MRI, to develop the theory for chemical exchange saturation transfer (CEST) MRI techniques described in the following sections.

1.4.1 Magnetic moments in an external magnetic field

A hydrogen ($^1$H) nucleus is composed of a positively charged proton and will be referred to as a proton from this point forward. An intrinsic property called spin characterizes the angular momentum of the proton. The nuclear spin quantum number ($I$) for the proton is equal to $1/2$. The angular momentum of the proton (a charged particle) gives rise to a magnetic field, known as a magnetic moment ($\mu$). In MRI, protons are placed into a static external magnetic field ($B_0$) with a fixed axis. Conventionally, the fixed axis of $B_0$ is considered to be the $z$- or longitudinal axis. Magnetic moments $\mu$ experience a torque ($\tau$) when placed in $B_0$, which causes them to precess about $B_0$. The motion of a magnetic moment $\mu$ in the presence of $B_0$ is described by the differential equation:

$$\frac{d}{dt}\bar{\mu} = \bar{\mu} \times \bar{B}_0$$  \hspace{1cm} [1.11]

with the following solution:
\[ \vec{\mu} = \vec{\mu}_0 e^{-i\vec{B}t} \]  

[1.12]

that describes the magnetic moment \( \mu \) rotating about \( B_0 \) in the clockwise direction with an angular frequency equal to:

\[ \vec{\omega}_0 = \gamma \vec{B}_0 \]  

[1.13]

where \( \omega_0 \) represents the Larmor frequency in rads s\(^{-1}\) and \( \gamma \) is the gyromagnetic ratio in radians per second tesla \( (10^6 \text{ rad s}^{-1} \text{ T}^{-1}) \) and \( B_0 \) is the main magnetic field strength (in T). It is common to express the Larmor frequency in s\(^{-1}\), or Hz, using the following conversion:

\[ f_o = \frac{\vec{\omega}_0}{2\pi} = \frac{\gamma}{2\pi} \vec{B}_0 \]  

[1.14]

In the absence of an external magnetic field, the alignment of proton magnetic moments \( \mu \) are randomly distributed in all directions. This condition leads to no net magnetization (Figure 1.2a). In the presence of an external magnetic field \( (B_0) \), the distribution of proton magnetic moments \( \mu \) alignments slightly favours the direction of \( B_0 \). This leads to a net magnetization \( M_0 \) in the same direction as \( B_0 \) (Figure 1.2b). In a hypothetical spin system, where the protons do not interact with each other, proton magnetic moments \( \mu \) precess at a fixed angle about \( B_0 \) in one of two possible orientations [64]. Protons precess with the \( z \)-component of \( \mu \) (\( \mu_z \)) either parallel or anti-parallel with the direction of \( B_0 \) (Figure 1.2). In reality, protons are constantly colliding with surrounding nuclei resulting in protons aligned in every direction with slight excess in the direction of \( B_0 \). The interested reader is referred to a thorough description of the more realistic spin system [64]. The hypothetical spin system offered here is a simple description that allows the reader to more easily envision the production of a net magnetization in the presence of an external magnetic field, excitation (section 1.4.1) and saturation (section 1.5.1).
Figure 1.2: a) An ensemble of $^1$H nuclei with random orientations in the absence of a magnetic field. b) In the presence of a magnetic field ($B_0$), a hypothetical ensemble of $^1$H nuclei have a slight excess of magnetic moments aligned in parallel with $B_0$ leading to a net magnetization $M_0$.

The orientation of $\mu_z$ with respect to $B_0$ depends on its energy and for this reason, the orientations have different energy states. Protons precessing with $\mu_z$ parallel with $B_0$ reside in the low energy state ($E_L$), while protons with $\mu_z$ oriented anti-parallel with $B_0$ occupy the high energy state ($E_H$). The energy difference between the two states is equal to:

$$\Delta E = \gamma \frac{h}{2\pi} \bar{B}_0$$ \[1.15\]

where $h$ is Planck’s constant. When placed in a magnetic field, protons tend to orient in the low energy state $E_L$, in parallel with $B_0$. However, at room temperature, the thermal energy of the protons favours an equal distribution between energy states. In reality, a very small excess of protons on the order of parts per million (ppm) reside in the low
energy state $E_L$. The distribution of protons between the two energy states at thermal equilibrium is calculated using the Boltzmann distribution given by:

$$
\frac{N_L}{N_H} = e^{-\frac{\hbar \gamma}{2\pi \hbar kT}}
$$

[1.16]

where $N_L$ and $N_H$ represent the number of protons in the lower and higher energy states respectively, $k$ is Boltzmann’s constant and $T$ is absolute temperature. The fraction of excess protons in the lower energy state accounts for a net polarization ($P$) given by:

$$
\tilde{P} = \frac{N_L - N_H}{N_L + N_H} = e^{\frac{\hbar \gamma}{2\pi \hbar kT}} - e^{-\frac{\hbar \gamma}{2\pi \hbar kT}}
$$

[1.17]

Curie’s Law states that for laboratory magnetic field strengths at room temperature, $\frac{\hbar \gamma}{2\pi \hbar kT} \ll 1$ and consequently the polarization $P$ is well approximated by:

$$
\tilde{P} \approx \frac{\gamma \hbar \bar{B}_0}{4\pi kT}
$$

[1.18]

The excess protons in the lower energy state produces the thermal equilibrium net magnetization $M_0$ calculated using the following product:

$$
\tilde{M}_0 = N \cdot \tilde{\mu} \cdot \tilde{P}
$$

[1.19]

where $N$ is number of proton nuclei per unit volume. The ensemble of magnetic moments $\tilde{\mu}$ creates a net magnetization $M_0$ that is parallel with the fixed axis of $B_0$ ($z$-axis). Therefore, at thermal equilibrium the summation of all magnetic moments $\mu$ produces a net longitudinal magnetization denoted as $\tilde{M}_0^z$, which for protons is equal to:

$$
\tilde{M}_0^z = \frac{N \cdot \left(\frac{\gamma \hbar}{2\pi}\right) \cdot \bar{B}_0}{4kT}
$$

[20]

It is important to note that while the net magnetization vector $\tilde{M}_0^z$ is composed of magnetic moments $\mu$ precessing at identical Larmor frequencies, there is no phase
coherence between the magnetic moments $\mu$ in the $xy$- or transverse plane. Therefore, at thermal equilibrium, there is no net transverse magnetization. To summarize, in the presence of a static external magnetic field $B_0$, proton magnetic moments $\mu$ precess about $B_0$ at the Larmor frequency $\omega_0$ and tend to align with $B_0$ forming a net longitudinal magnetization vector $\vec{M}_z^0$ at thermal equilibrium.

### 1.4.2 Excitation

MRI signal detection is based upon Faraday’s Law of induction. Faraday’s law states that rotating magnetization induces a corresponding voltage in electric radiofrequency (RF) receive coils near the sample. RF receive coils are placed in the transverse plane and detect rotating magnetization in the transverse plane, or transverse magnetization $\vec{M}_{xy}$. As described in Section 1.2.1, at thermal equilibrium the proton magnetic moments $\mu$ form a static net magnetization vector $\vec{M}_z^0$ aligned with $B_0$ (Figure 1.2a). In most MRI applications, $\vec{M}_z^0$ is excited or tipped into the transverse plane using an additional external magnetic field $B_1$. The additional $B_1$ field is removed once the net magnetization vector is rotated into the transverse plane, creating a transverse magnetization $\vec{M}_{xy}$ (Figure 1.2b). By Faraday’s Law, RF receive coils detect a voltage induced by $\vec{M}_{xy}$ precessing about $B_0$ at the Larmor frequency.
Figure 1.3: A hypothetical ensemble of $^1$H nuclei in the rotating reference frame. a) In the presence of a magnetic field ($B_0$) at thermal equilibrium, the protons tend to orient in parallel or anti-parallel with $B_0$ leading to a thermal equilibrium net magnetization vector ($\vec{M}_z^0$). b) Immediately following application of an RF excitation pulse along the $x'$-axis, the protons are equally distributed between parallel and anti-parallel orientations and in phase along the $y'$-axis. This figure was motivated by Figure 1.4 in reference [65].

In MRI, RF coils not only receive magnetic signals by Faraday’s Law but may also produce magnetic fields by Faraday’s Law. The RF coils are used to create a magnetic field for short intervals, known as RF pulses, to excite spins and create transverse magnetization $\vec{M}_{xy}$. RF pulses create an oscillating magnetic field with amplitude $B_1$ and a frequency $\omega_{RF}$. To achieve rotation of the net magnetization into the tranverse plane, the applied magnetic field $B_1$ must be 1) tuned to the Larmor frequency $\omega_0$ ($\omega_{RF} = \omega_0$) and 2) applied perpendicular to $B_0$. A rotating frame of reference ($x'y'z'$) that is rotating in a clockwise direction at the Larmor frequency $\omega_0$ about the longitudinal axis is often used to demonstrate the effect of RF pulses. In such a rotating frame of reference, the macroscopic motion of a net magnetization $\vec{M}$ in a magnetic field $\vec{B}$ can be described in the same way as a single magnetic moment $\vec{\mu}$ in a magnetic field was described in equation [11], as shown by:
\[ \frac{d}{dt} \mathbf{M} = \mathbf{M} \times \gamma \mathbf{B}_1 \]  

[1.21]

when off-resonance effects are ignored. In the same way that the magnetic moment \( \mathbf{\mu} \) precessed about the main magnetic field \( \mathbf{B}_0 \), the net magnetization vector \( \mathbf{M} \) rotates around \( \mathbf{B}_1 \) (eg. \( x' \)-axis) towards the \( y' \)-axis in the \textit{transverse} plane. If a rectangular RF pulse is applied with an amplitude of \( \mathbf{B}_1 \), the angle at which the net magnetization vector \( \mathbf{M} \) rotates away from the \textit{longitudinal} axis is called the flip-angle, \( \alpha \), and can be calculated using:

\[ \alpha = \gamma \mathbf{B}_1 T \]  

[1.22]

where \( T \) represents the RF pulse duration and \( \mathbf{B}_1 \) is the amplitude of the applied magnetic field. From the classical mechanics perspective, immediately following a 90° excitation and removal of \( \mathbf{B}_1 \), the net \textit{transverse} magnetization vector \( \mathbf{M}_{xy} \) is along the \( y' \)-axis and precessing about \( \mathbf{B}_0 \) in the laboratory frame of reference. From the quantum mechanics perspective, the protons are equally distributed between both orientations (eg. energy states) and they are in phase coherence along the \( y' \)-axis. As discussed earlier in this section, the rotating \textit{transverse} magnetization \( \mathbf{M}_{xy} \) can be detected by the RF receive coil.

1.4.3 Relaxation

The previous section described how an appropriate RF magnetic pulse could be used to generate an applied magnetic field \( \mathbf{B}_1 \) perpendicular to the main magnetic field \( \mathbf{B}_0 \). Such an applied magnetic field \( \mathbf{B}_1 \) causes the magnetization vector \( \mathbf{M} \) to rotate into the \textit{transverse} plane, resulting in transverse magnetization \( \mathbf{M}_{xy} \), that is detected by the RF receive coil. However, immediately following an excitation RF pulse, the \textit{transverse} magnetization \( \mathbf{M}_{xy} \) immediately begins to decrease in amplitude as magnetic moments dephase and the \textit{longitudinal} magnetization \( \mathbf{M}_z \) begins to grow back to the thermal equilibrium magnetization \( \mathbf{M}_z^0 \). The process, though which magnetization \( \mathbf{M} \) returns to
\( \tilde{M}_z^0 \), is called relaxation. *Longitudinal* and *transverse* relaxation occurs by different physical processes.

*Longitudinal* or spin-lattice relaxation describes the process by which the *longitudinal* magnetization \( \tilde{M}_z \) returns to \( \tilde{M}_z^0 \) after any perturbation, such as excitation by an RF pulse. Specifically, spin-lattice relaxation is induced by dipole-dipole interactions between neighboring protons that result in a net energy transfer from an excited magnetic moment (spin) to its surrounding environment (lattice). The time-dependent relaxation of the *longitudinal* magnetization \( \tilde{M}_z \) can be described using an exponential re-growth function given by:

\[
\tilde{M}_z(t) = \tilde{M}_z(0) \cdot e^{-t/T_1} + \tilde{M}_z^0 (1 - e^{-t/T_1})
\]

[1.23]

where \( \tilde{M}_z(0) \) is the magnitude of the *longitudinal* magnetization \( \tilde{M}_z \) at a reference time \( t = 0 \) and \( T_1 \) is the spin-lattice relaxation time constant.

As mentioned in the previous section, protons are in phase coherence following a 90\(^{\circ}\) RF excitation pulse. *Transverse* or spin-spin relaxation describes the process by which magnetic moments dephase and the *transverse* magnetization \( \tilde{M}_{xy} \) decays to zero. In contrast to *longitudinal* relaxation, *transverse* relaxation is a result of energy transfer between neighboring protons with no loss of energy to the surrounding lattice. Specifically, *transverse* relaxation can be defined as the loss of phase coherence resulting from the exchange of energy between spins. For example, following excitation, high energy spin may transfer energy to a low energy spin to cause both spins to change states and lose their phase coherence. Spin-spin relaxation of the *transverse* magnetization \( \tilde{M}_{xy} \) is described using the exponential decay function given by:

\[
\tilde{M}_{xy}(t) = \tilde{M}_{xy}(0) \cdot e^{-t/T_2}
\]

[1.24]

where \( \tilde{M}_{xy}(0) \) is the magnitude of *transverse* magnetization \( \tilde{M}_{xy} \) at a reference time \( t = 0 \) and \( T_2 \) is the spin-spin relaxation time constant.
Following an excitation pulse, the *transverse* magnetization $\vec{M}_{xy}$ decays more quickly than would be explained by the $T_2$ time constant. This is primarily due to time-independent variations in local magnetic field within the sample itself. The net result is that all spins experience a slightly different magnetic field, resulting in a range of Larmor frequencies from equation [1.13]. Local variation in Larmor frequencies results in dephasing of the *transverse* magnetization $\vec{M}_{xy}$ leading to additional loss of the *transverse* magnetization $\vec{M}_{xy}$. Dephasing due to time-independent local magnetic field inhomogeneities is described by an additional decay constant $T_2'$. Therefore the apparent *transverse* magnetization relaxation is described by Equation [1.25]:

$$\vec{M}_{xy}(t) = \vec{M}_{xy}(0) \cdot e^{-t/T_2'}$$ \[1.25\]

where

$$1/T_2' = 1/T_2 + 1/T_2.$$

[1.26]

In summary, following excitation by an RF pulse, the magnetization vector $\mathbf{M}$ immediately begins to re-grow in the longitudinal direction and dephase in the *transverse* plane. The time-dependent motion of the magnetization vector $\mathbf{M}$ immediately after excitation is described by the Bloch equations [27], which accounts for precession about $\mathbf{B}_0$ and relaxation.

$$\frac{d}{dt} \begin{bmatrix} \vec{M}_x \\ \vec{M}_y \\ \vec{M}_z \end{bmatrix} = \begin{bmatrix} -1/T_2' & \gamma \vec{B}_0 & 0 \\ -\gamma \vec{B}_0 & -1/T_2' & 0 \\ 0 & 0 & -1/T_1 \end{bmatrix} \begin{bmatrix} \vec{M}_x \\ \vec{M}_y \\ \vec{M}_z \end{bmatrix} + \begin{bmatrix} 0 \\ 0 \\ \vec{M}_0/T_1 \end{bmatrix}$$ \[1.27\]

### 1.4.4 Spatial-encoding magnetization using magnetic gradients

In the previous section, the Bloch equations were introduced to describe both precession and relaxation of the net magnetization vector $\vec{M}$ in the presence of $\vec{B}_0$ immediately following an excitation RF pulse. This section describes how external magnetic fields are used to produce magnetic gradients that spatially-encode magnetic moments. First, the
transverse magnetization notation is defined using complex numbers to facilitate description of the effects of magnetic gradients.

The transverse magnetization \( \vec{M}_{xy} \) can be described as a complex quantity:

\[
\vec{M}_{xy} = \vec{M}_x + i\vec{M}_y
\]  

[1.28]

Combining equation [28] with the Bloch equations described in equation [27] results in:

\[
\frac{d\vec{M}_{xy}}{dt} = \frac{d\vec{M}_x}{dt} + i\frac{d\vec{M}_y}{dt} = -\left(i\gamma\vec{B}_0 + \frac{1}{T_2}\right)\vec{M}_{xy}
\]  

[29]

which can be solved to:

\[
\vec{M}_{xy}(t) = \vec{M}_{xy}(0) \cdot e^{-i\omega T} \cdot e^{-i\gamma B_0 t} = \vec{M}_{xy}(0) \cdot e^{-i\omega T} \cdot e^{-i\gamma B_0 t}
\]  

[1.30]

Equation [1.30] represents the signal observed immediately following a 90° RF excitation pulse and is called the free induction decay (FID) signal. While the FID signal can be used to estimate the Larmor frequency \( \omega_0 \) and the apparent transverse relaxation time constant \( T_2^* \), it provides no spatial information. In MRI, external magnetic field gradients (eg. a magnetic field with spatially varying strength) are used to spatially encode the transverse magnetization \( \vec{M}_{xy} \). MRI scanners are typically equipped with three orthogonal gradient RF coils that produce magnetic gradients in the \( x \)-, \( y \)- and \( z \)-directions, denoted as \( \vec{G}_x = d\vec{B}_x/dx \), \( \vec{G}_y = d\vec{B}_y/dy \), and \( \vec{G}_z = d\vec{B}_z/dz \).

For example, if an external magnetic gradient, \( \vec{G}_r \), is applied in the \( r \)-direction then the net magnetic field \( \vec{B} \) becomes a function of the space vector \( r \) and is represented as:

\[
\vec{B}(r) = \vec{B}_0[z] + \vec{G}_r(r)[z]
\]  

[1.31]

The Larmor frequency \( \omega_0 \) is dependent on the magnetic field strength \( \vec{B} \) as was shown in equation [13]. Upon the application of the external magnetic gradient, \( \vec{G}_r \), the Larmor frequency \( \omega_0 \) becomes a spatially varying function of \( r \) described by:
\[ \vec{\omega}(r) = \gamma B(r) = \gamma (\vec{B}_0 + \vec{G}_r \cdot r) = \vec{\omega}_0 + \gamma \vec{G}_r \cdot r \]  

[1.32]

Magnetic gradients modulate the Larmor frequency (equation [1.13]) and introduce an accumulated frequency difference, or phase \( \phi \) that is dependent on \( r \) as described by:

\[ \vec{\phi}(r, t) = \gamma \int_0^t \vec{G}_r \cdot r \, d\tau \]  

[1.33]

where \( t \) is the duration of the applied magnetic gradient \( G_r \).

In MRI, magnetic gradients are used to spatially encode the transverse magnetization \( \vec{M}_x \). The spatially dependent integral of the gradient represents a point in the \( k \)-space domain as shown for the \( x \)- and \( y \)-direction denoted by:

\[ \vec{k}_x(t) = \frac{\gamma}{2\pi} \int_0^t \vec{G}_x(x, \tau) \, d\tau \]  

[1.34]

\[ \vec{k}_y(t) = \frac{\gamma}{2\pi} \int_0^t \vec{G}_y(y, \tau) \, d\tau \]  

[1.35]

Therefore, the total phase \( \phi \) accrued throughout application of magnetic gradients \( G_x \) and \( G_y \) is expressed in terms of the \( k_x \) and \( k_y \) as:

\[ \vec{\phi}(x, y, z, t) = 2\pi \left( \vec{k}_x \cdot x + \vec{k}_y \cdot y \right) \]  

[1.36]

The \( k \)-space domain represents the Fourier transform of the object. In other words, the two-dimensional Fourier transform of an object in the space domain (eg. \( xyz \) space) produces the spatial frequency representation of the object in \( k \)-space. Conversely, the inverse Fourier transform of the \( k \)-space signal generates the spatial domain representation of the object.

Another application of a magnetic gradient used in MRI is for slice selection. The excitation of a specific, or ‘selected’ slab is called slice selection. Slice selection is achieved using a magnetic gradient \( G \) that spatially varies the Larmor frequency. Magnetic moments \( \mu \) precessing with Larmor frequencies that are excited by the RF
pulse produce \textit{transverse} magnetization $\mathbf{M}_{xy}$. The slice is selected by simultaneously applying the magnetic gradient $\mathbf{G}$ and an RF excitation pulse with a bandwidth equal to $\text{BW}_{\text{RF}}$. The BW of an RF excitation pulse represents the range of frequencies, $\Delta \omega$, centered on the RF pulse frequency $\omega_{\text{RF}}$ (for on-resonance excitation $\omega_{\text{RF}} = \omega_0$) that are excited by the pulse. For example, a slice in the $xy$-plane of thickness $\Delta z$ can be selected using an RF pulse with $\text{BW}_{\text{RF}}$ and a magnetic gradient $\mathbf{G}_z$, with the thickness determined by:

$$
\Delta z = \text{BW}_{\text{RF}} \left( \frac{\gamma}{2\pi} \mathbf{G}_z \right) \quad [1.37]
$$

In two-dimensional MRI, the \textit{transverse} signal is spatially encoded by \textit{phase-} and \textit{frequency}-encoding gradients using the orthogonal $\mathbf{G}_x$ and $\mathbf{G}_y$ gradients. For clarity, it helps to define the $y$-direction of $k$-space $k_y$ as the \textit{phase}-direction and the $x$-direction of $k$-space $k_x$ as the \textit{frequency}-direction. Similarly, \textit{phase-} and \textit{frequency}-encoding gradients will be described in the space domain along the $y$- and $x$-direction respectively. Application of a \textit{phase}-encoding magnetic gradient $\mathbf{G}_y$ causes the magnetic moments $\mu$ in the system to precess at different frequencies based on position $y$. During the time that the \textit{phase}-encoding magnetic gradient $\mathbf{G}_y$ is on, the accumulated phase depends on the $y$-position according to equation [36]. The \textit{phase}-encoding magnetic gradient $\mathbf{G}_y$ is typically turned off prior to signal acquisition, which causes the magnetic moments $\mu$ to precess at the initial frequency (ie. Larmor frequency $\omega_0$) but the $y$-dependent accumulated phase shift remains fixed. Hence, the \textit{phase}-encoding gradients spatially encode the $y$ position of magnetic moments $\mu$ in the sample through the $y$-dependent phase accumulation. Subsequent application of a \textit{frequency}-encoding magnetic gradient $\mathbf{G}_x$ causes the magnetic moments $\mu$ in the system to precess at different frequencies based on position $x$. The \textit{frequency}-encoding gradients are applied throughout the signal acquisition. Similar to \textit{phase}-encoding, the \textit{frequency}-encoding gradients spatially encode the $x$ position of magnetic moments $\mu$ in the sample through the $x$-dependent phase-accumulation.
Application of phase- and frequency-encoding gradients alters the transverse magnetization $\tilde{M}_{xy}$ according to [38]:

$$\tilde{M}_{xy}(t) = \tilde{M}_{xy}(0) \cdot e^{-\frac{i}{T_2^*}} \cdot e^{-i\omega_0 t} \cdot e^{-\frac{i2\pi}{k_x} x(t)} \cdot e^{-\frac{i2\pi}{k_y} y(t)}$$

[1.39]

The amplitude of the magnetization at value of $k_x$ and $k_y$ represents the inverse Fourier transform of the object. The signal collected by the RF receiver coil is used to fill this $k$-space using a sequence of magnetic gradients, as will be discussed using a pulse sequence in the next section.

### 1.4.5 Pulse sequences

Most applications in MRI employ a sequence of RF pulses and external magnetic gradients, known as a pulse sequence, to manipulate the thermal equilibrium net magnetization vector $\tilde{M}_z^0$ into the transverse plane, and generate spatially encoded signals from the sample. Pulse sequences have several parameters including the repetition time (TR), echo-time (TE), and the RF excitation pulse flip-angle ($\alpha$), which was defined in Section 1.4.2. Pulse sequences typically repeat or loop through the same pattern of RF pulses and gradients with a specified time of repetition (TR). Pulse sequences that generate signal echoes, defined below, at specific echo times (TE) are very common in MRI applications.

Spin-echo pulse sequences are based on the creation of signal echoes (or spin-echo) using $180^\circ$ refocusing RF pulses after an initial $\alpha=90^\circ$ excitation pulse along the $x$-axis. Immediately after excitation, the transverse magnetization $\tilde{M}_{xy}$ is composed of spins that are in phase and pointing along the $y'$-axis. The spins immediately begin to dephase due to $T_2$ and $T_2'$ decay, as described in Section 1.4.3, resulting in decay of the transverse magnetization $\tilde{M}_{xy}$. It is important to recall that the $T_2'$ signal decay is due to local static field inhomogeneities. After a dephase period equal to $TE/2$, a $180^\circ$ RF refocusing pulse inverts the spins about the $y'$-axis. Following the $180^\circ$ pulse, spins immediately begin to rephase, forming a spin-echo at a time defined as TE. The refocusing pulse does not affect the precession frequency of the spins, however their phase angle is inverted in the
transverse plane. Briefly, spins that exist in a slightly higher magnetic field, due to inhomogeneities, precess relatively fast. Conversely, spins in slightly lower magnetic fields precess relatively slow. Phase dispersion occurs due to magnetic fields inhomogeneities and consequent variations in the Larmor frequency. It is the refocusing 180° RF pulse that reverses the position of the ‘fast’ and ‘slow’ frequency spins relative to each other. The spin-echo is formed when the ‘fast’ spins catch up and re-phase with the ‘slow’ spins. The spin-echo signal amplitude still decays due to T₂ relaxation. Only the time-independent magnetic field inhomogeneity effects (T₂’) are eliminated using the refocusing pulses. Fast pulse sequences are typically preferred to reduce MRI acquisition times. A fast spin-echo (FSE) pulse sequence consists of multiple 180° refocusing RF pulses that result in multiple spin-echoes. Each spin-echo is recorded and is accompanied by a specific set of phase- and frequency-encoding gradients to fill one line in k-space. The filling of k-space will be described for a typical gradient-echo pulse sequence below.

Like spin-echo imaging, the gradient-echo imaging pulse sequence is commonly used. While three-dimensional pulse sequences exist, the current explanation describes the generation of a two-dimensional image only. A typical two-dimensional gradient-echo pulse sequence begins with slice selection using a z-direction magnetic gradient G_z and a 90° RF excitation pulse with a bandwidth equal to BW_{RF} as described in Section 1.4.2. The transverse magnetization \( \vec{M}_{xy} \) created in the slice volume is immediately phase-encoded using phase-encode magnetic gradients G_y for a duration \( \tau_y \) as discussed in Section 1.4.4. Following removal of the phase-encode gradient G_y, a negative frequency-encode magnetic gradient (- G_x) is applied for a duration \( \tau_x/2 \). During application of the frequency-encode magnetic gradient G_x, spins dephase rapidly due to T₂ and T₂’ decay along with dephasing due to the magnetic gradient G_x. After applying the negative frequency-encode G_x gradient for duration \( \tau_x/2 \), an equal magnitude but positive magnetic gradient (eg. G_x = + G_x \cdot x) is applied for \( \tau_x \). The positive gradient reverses the dephasing caused by the negative gradient, resulting in the formations of a gradient-echo exactly \( \tau_x/2 \) after the start of the positive gradient. In terms of k-space, the maximum durations \( \tau_{x/y} \) and maximum magnitude G_{x/y} of the frequency-/phase-encode G_{x/y} gradients are determined based on the desired field of view (FOV_{x/y}) as shown by:
\[
\text{FOV}_x = \frac{1}{\frac{\gamma}{2\pi} \cdot \vec{G}_x \cdot \tau_x}
\]  \[1.40\]

and

\[
\text{FOV}_y = \frac{1}{\frac{\gamma}{2\pi} \cdot \vec{G}_y \cdot \tau_y}
\]  \[1.41\]

### 1.5 Chemical exchange saturation transfer (CEST)

Chemical exchange saturation transfer (CEST) represents a relatively new class of MRI contrast. CEST enables detection of small (~ mM) solute proton pools that exchange with the bulk water proton pool (~110 M). Typical solute protons pools include protons associated with exogenous paramagnetic (PARACEST) agents as well as protons associated with endogenous molecules (endogenous CEST). A unique and powerful advantage of CEST contrast is the ability to switch CEST contrast on and off, which enables the use of multiple CEST agents (e.g. multiple PARACEST agents and/or endogenous molecules) simultaneously for labeling.

Investigations into the effects of chemical exchange on pools of nuclei have been ongoing for more than 50 years. McConnell and Thompson introduced a modified version of the Bloch equations known as the Bloch-McConnell equations that include chemical exchange effects in 1957. Balaban and Ward coined the term CEST more recently in 2000 when they described a new class of MRI contrast agents that are sensitive to their surrounding environment. Balaban and Ward immediately recognized and investigated applications of CEST agents to measure tissue temperature and pH. Fast-forward roughly 15 years and CEST remains a rapidly growing field in the MR community. The collection of CEST applications is diverse including cell labeling, monitoring glucose uptake, detection of the ischemic penumbra following stroke, detection of zinc ions and many more.
In this section, fundamental principles of CEST are presented. Specifically, the mechanisms leading to CEST are discussed qualitatively referencing quantum theory followed by a mathematical description using the Bloch-McConnell equations. A description of some marked differences between PARACEST and endogenous CEST is followed by a review of the background theory regarding CEST dependence on pH and temperature.

1.5.1 CEST mechanism

CEST contrast is generated by saturation of a solute (S) proton pool that is 1) chemically-shifted from bulk water protons and 2) in chemical exchange with bulk water (W) protons. The process of chemical exchange transfers high energy protons to the bulk water proton pool, saturating this pool, resulting in a reduced net magnetization that leads to a decrease (darkening) in MR image intensity. A proper description of the CEST mechanism requires definition of chemical shift, saturation and chemical exchange.

Chemical shift is defined as the difference in Larmor frequency of two nucleus pools. The Larmor frequency was defined in equation [1.13] as the product of the nucleus specific gyromagnetic ratio $\gamma$ and the main magnetic field $B_0$. While the gyromagnetic ratio $\gamma$ is identical for all protons, the local magnetic field depends on a proton’s chemical environment such as the solute molecule with which it is associated. The electron cloud surrounding the nucleus causes changes in local magnetic field. Electron clouds shield the nucleus from the main magnetic field $B_0$ resulting in an effective main magnetic field ($B'_0$) expressed as:

$$B'_0 = B_0(1 - \sigma_s) \quad [1.42]$$

where $\sigma_s$ is the solute-dependent shielding coefficient. Protons associated with a specific solute molecule tend to have unique Larmor frequencies expressed as:

$$\tilde{\omega}_s = \gamma B'_0 \quad [1.43]$$
For CEST applications, the Larmor frequency of bulk water protons is set as the reference frequency $\omega_{\text{ref}}$. The chemical shift, or frequency offset, of solute protons from bulk water protons is defined in ppm according to:

$$\delta = \frac{\omega_s - \omega_{\text{ref}}}{\omega_{\text{ref}}} \cdot 10^6$$

[1.44]  

Saturation is achieved using RF pulses. As mentioned in Section 1.4.1, the $z$-component of the proton magnetic moments $\mu_z$ tend to align parallel with the direction of $B_0$ in the presence of a static external magnetic field ($B_0$). For clinical magnetic field strengths at room temperature, there is a slight excess of protons oriented parallel with $B_0$ in the low energy state $E_L$ compared to protons oriented anti-parallel with $B_0$ in the high energy state $E_H$. Saturation is achieved by applying an RF excitation pulse with energy equal to the energy difference between the two proton energy states $\Delta E$ described in Section 1.4.1, which drives enough low energy protons to the high energy state such that the populations in each energy state are equal. More specifically, for the RF pulse to excite protons from $E_L$ to $E_H$, or vise versa, the pulse energy $h\omega_{\text{RF}}$ must equal the energy required to transition between the states, which is equal to $\Delta E$. Considering equations [1.13] and [1.15], it is shown that the applied RF pulse frequency $\omega_{\text{RF}}$ must be equal to the Larmor frequency $\omega_0$ because:

$$h\tilde{\omega}_{\text{RF}} = \Delta \tilde{E} = \gamma h \tilde{B}_0 = h\tilde{\omega}_0$$

[1.45]  

It is important to note that in CEST applications, an RF pulse is typically used to saturate a pool of solute protons resonating at a Larmor frequency $\omega_S$ that is chemically shifted from the bulk water protons. Hence, application of an RF pulse of amplitude $B_1$ and tuned to the frequency offset of the solute proton pool (eg. $\omega_{\text{RF}} = \omega_S$) leads to a net excitation of protons in the lower energy state $E_L$ into the higher energy state $E_H$. If the RF pulse has enough energy, the number of protons at each energy level will equalize (eg. $N_L = N_H$) and the proton pool is saturated. A long RF pulse designed to saturate a solute proton pool and not generate phase coherence is referred to as an RF saturation pulse from this point forward.
Chemical exchange occurs when a nucleus freely passes between two different chemical environments. For CEST applications, the most common pathways of chemical exchange include proton exchange, molecular exchange or a combination of both. An example of proton exchange is when a proton from a protein amide group (NH₂) moves to bulk water (H₂O). An example of molecular exchange is a water molecule moving between free water and a paramagnetic lanthanide metal associated with a PARACEST agent. In order to observe CEST contrast, chemical exchange must occur on the slow-to-intermediate NMR time-scale defined as:

\[ \Delta \omega_s \geq k_{sw} \]  

[1.46]

where \( k_{sw} \) represents the chemical exchange rate from the solute to the bulk water pool and \( \Delta \omega_s \) is the frequency offset between the solute protons and bulk water protons. Note that the chemical shift \( \delta \) is the frequency offset \( \Delta \omega_s \) normalized by the reference frequency \( \omega_{\text{ref}} \). Importantly, both the chemical exchange rate \( k_{sw} \) and the chemical shift \( \delta \) often depend on pH and temperature.

In summary, CEST contrast is generated in a system with a chemically shifted solute proton pool that is in chemical exchange with the bulk water proton pool. A frequency-selective RF saturation pulse is applied to saturate the solute proton pool. During saturation, chemical exchange transfers an increased number of high energy protons (e.g. aligned anti-parallel with \( B_0 \)) from the saturated solute pool to bulk water pool. Throughout a long (e.g. several seconds) RF saturation pulse, high energy protons accumulate in the bulk water pool leading to partial saturation. Bulk water proton pool saturation is limited by ongoing longitudinal relaxation. The simultaneous saturation and relaxation of the bulk water proton pool drives the system to a steady state \( \tilde{M}_{zw}^\infty \) during a long RF saturation pulse. The CEST effect is the resulting partial saturation of the bulk water proton pool due to saturation of the solute proton pool. The proton transfer ratio (PTR) is common metric used to quantify CEST effects by calculating the reduced steady state bulk water magnetization \( \tilde{M}_{zw}^\infty \) relative to the equilibrium magnetization \( \tilde{M}_{zw}^0 \) as shown by:
CEST contrast is measured as the reduction in the bulk water proton MRI signal generated by saturating a much smaller solute proton pool that is exchanging with bulk water protons. Chemical exchange acts as sensitivity amplification mechanism because bulk water saturation accumulates throughout the long RF saturation pulse resulting in significantly amplified bulk water reduction.

A typical CEST MRI experiment consists of the acquisition of a series of MR images that are each preceded by a RF saturation pulse with an incrementally different frequency. The normalized MRI signal intensity ($\frac{\tilde{M}_{zw}^*}{M_{zw}^0}$) is then plotted as function of the RF saturation pulse frequency offset relative to bulk water resonance to create a CEST spectrum. If experimental parameters are appropriate then a reduction in MRI signal intensity referred to as a ‘CEST peak’ may be observed in the CEST spectrum. A CEST spectrum is shown in Figure 1.4 with a CEST peak at 10 ppm highlighted by the arrow for illustrative purposes.
Figure 1.4: A typical CEST spectrum. A CEST effect (or CEST peak) can be observed at at 10 ppm as highlighted with the arrow. The peak at 0 ppm is due to direct saturation of the bulk water protons by the RF saturation pulse.

1.5.2 Mathematical description of CEST

CEST contrast is the result of a complex interplay of proton pool parameters as well as RF saturation pulse parameters and mathematical descriptions are often useful to understand the impact of individual parameters. The Bloch-McConnell equations are used to describe multi-pool exchange systems during the saturation pulse. In this section, Bloch-McConnell equations are introduced for two-pool and three-pool exchange systems. The two-pool version of the Bloch-McConnell equations describes a system with one solute ($s$) dissolved in water ($w$) and is written as:

$$\frac{d}{dt} \begin{bmatrix} M_{sw} \\ M_{ws} \\ M_{ws} \\ M_{sz} \\ M_{sz} \end{bmatrix} = \begin{bmatrix} \frac{1}{T_{1w}} - k_{sw} & k_{sw} & -\Delta\omega_{rf,w} & 0 & 0 \\ k_{sw} & \frac{1}{T_{1w}} - k_{sw} & 0 & -\Delta\omega_{rf,s} & 0 \\ \Delta\omega_{rf,w} & 0 & \frac{1}{T_{2w}} - k_{sw} & 0 & -\omega_i \\ 0 & \Delta\omega_{rf,s} & 0 & \frac{1}{T_{2s}} - k_{sw} & 0 & -\omega_i \\ k_{sw} & 0 & 0 & 0 & \omega_i \end{bmatrix} \begin{bmatrix} M_{sw} \\ M_{ws} \\ M_{ws} \\ M_{sz} \\ M_{sz} \end{bmatrix} \begin{bmatrix} 0 \\ 0 \\ 0 \\ 0 \\ 0 \end{bmatrix}$$

$M^0_{zi}$ is the thermal equilibrium longitudinal magnetization and the chemical exchange rate from pool $i$ to $j$ is $k_{ij}$. The Larmor frequency of pool $i$ is denoted as $\omega_{0i}$. The longitudinal and transverse relaxation time constants for pool $i$ are $T_{1i}$ and $T_{2i}$ respectively. RF saturation pulse parameters include the amplitude $\omega_1 = \gamma B_1$, the frequency offset from pool $i$ denoted as $\Delta\omega_{rf,i} = \omega_{rf} - \omega_{0i}$, and the duration ($t_{sat}$) for a continuous wave rectangular pulse that is assumed to be along the $x'$-axis in the rotating frame of reference.

Analytical steady-state solutions derived using reasonable assumptions provide insight into the effects of proton pool and RF saturation parameters. Zhou and Van Zijl
introduced the weak saturation pulse (WSP) approximation that is often used to derive analytical solutions for the steady state bulk water magnetization after a long RF saturation pulse [66]. Briefly, the WSP approximation assumes that RF saturation pulse acts on the solute proton pool (eg. \(\Delta \omega_{rf,s} = 0\)) and not the bulk water pool (eg. \(\Delta \omega_{rf,w} \to \infty\)). Under WSP conditions, the two-pool system reaches a steady state and it is possible to derive analytical solutions. At steady state, the time derivatives in equation [1.48] are zero and the exact steady state PTR can be solved as:

\[
\text{PTR}(\Delta \omega_{rf,s}) = \frac{k_{ws}}{R_{1w} + k_{ws}} \cdot \frac{\omega_{1}^{2}}{\omega_{1}^{2} + pq + \frac{p}{q} \Delta \omega_{rf,s}^{2}}
\]  

[1.49]

where,

\[
p = R_{2s} + k_{sw} - \frac{k_{sw} k_{ws}}{R_{2w} + k_{ws}}
\]  

[1.50]

and

\[
q = R_{1s} + k_{sw} - \frac{k_{sw} k_{ws}}{R_{1w} + k_{ws}}
\]  

[1.51]

and the rate of spin-lattice (\(R_{1}\)) and spin-spin (\(R_{2}\)) relaxation are defined as:

\[
R_{1,2} = \frac{1}{T_{1,2}}
\]  

[1.52]

The labeling fraction or saturation efficiency \(\alpha\) is defined as:

\[
\alpha(\Delta \omega_{rf,s}) = \frac{\omega_{1}^{2}}{\omega_{1}^{2} + pq + \frac{p}{q} \Delta \omega_{rf,s}^{2}}
\]  

[1.53]

and represents the fraction of solute protons that are saturated at steady state as shown by:
\[
\frac{M^{ss}}{M^{0}} = 1 - \alpha \quad [1.54]
\]

Hence, at steady state PTR can be described as:

\[
\text{PTR} \left( \Delta \omega_{rf,s} \right) = \frac{k_{ws}}{R_{1w} + k_{ws}} \alpha \left( \Delta \omega_{rf,s}^2 \right) \quad [1.55]
\]

Using the WSP approximation and assuming that the solute proton pool reaches steady state instantaneously Zhou et al. also derived the time-dependent PTR solutions:

\[
\text{PTR} \left( t_{sat} \right) = \frac{k_{ws} \alpha}{R_{1w} + k_{ws}} \left[ 1 - e^{-\left( R_{1w} + k_{ws} \right) t_{sat}} \right] \quad [1.56]
\]

The chemical exchange rates obey the rate equation:

\[
k_{ws} M^{0}_{zw} = k_{sw} M^{0}_{zs} \quad [1.57]
\]

Using equations [1.56-1.57] a more insightful version of the time-dependent PTR solutions is given as:

\[
\text{PTR} \left( t_{sat} \right) = \frac{k_{ws} M^{0}_{zw} \alpha}{(R_{1w} + k_{ws}) M^{0}_{zs}} \left[ 1 - e^{-\left( R_{1w} + k_{ws} \right) t_{sat}} \right] \quad [1.58]
\]

The time-dependent PTR solution in equation [1.58] is often used to highlight the effects of several CEST parameters including solute concentration, bulk water $T_1$ and the exchange rate from solute to water pool, $k_{sw}$. For example, based on equation [1.58] one may predict that CEST contrast is linearly dependent on the solute concentration since $M^{0}_{zs}/M^{0}_{zw} \propto \text{[solute protons]/[water protons]}$.

The fundamental relations identified in the analytical steady state solutions are a useful theoretical platform for designing optimal CEST experiments by modeling changes in specific sample parameters such as concentration or pH-dependent exchange rate. However, it is important to understand the limitations of the WSP assumption and the two-pool model. For example, the chemical shift $\delta$ of solute protons must be large (>10
ppm) in order avoid violation of the WSP approximation by perturbing the bulk water protons during RF saturation. Whereas, endogenous CEST contrast is typically generated using solute protons less than 5 ppm away from bulk water protons and therefore the WSP approximation is not reasonable for typical RF saturation pulse amplitudes $B_1$ ($\sim 2 \mu$T) with adequate durations to achieve steady state.

In order to avoid restrictive assumptions, Woessner et al. introduced numerical simulations using the Bloch-McConnell equations. Briefly, the Bloch-McConnell equations (equations [1.48]) are given in the form:

$$\frac{d\bar{M}}{dt} = A \cdot \bar{M} + B$$

[1.59]

and therefore have the time-dependent analytical solution:

$$\bar{M} = \left(M_0 + \frac{B}{A}\right) \exp(A t) - \frac{B}{A}$$

[1.60]

Woessner and colleagues used numerical solutions to investigate effects of the chemical exchange rate on CEST contrast [60] in order to optimize CEST contrast and improve the application of pH-dependent chemical exchange rates for pH measurement. It was shown that an optimal RF saturation amplitude $B_1$ exists for a given exchange rate $k_{sw}$, and is equal to $2\pi B_1 = k_{sw}$ [60]. The time-dependent analytical solutions numerical solution approach is more powerful and applicable since they do not require any assumptions or approximations.

Numerical solutions can be used to simulate CEST spectra for a range of parameters to identify a parameter set that generates optimal PTR. For example, after setting all chemical and RF saturation pulse parameters, the effect of varying the solute concentration on the CEST spectrum and PTR can be numerically simulated as shown in Figure 1.5a,b. Similarly, the effects of varying the bulk water longitudinal relaxation time constant $T_{1w} = 1/R_{1w}$ (Figure 1.5c,d), the solute proton exchange rate $k_{sw}$ (Figure 1.5e,f) and finally the RF saturation pulse amplitude (Figure 1.5g,h) are also shown.
Figure 1.5: Numerically simulated CEST spectra (a,c,e,g) and proton transfer ratios (PTRs) (b,d,f,h) using the time-dependent analytical solutions of the two-pool version of the Bloch-McConnell equations. The effects of varying solute proton concentration (a,b), bulk water $T_1$ relaxation time constant (c,d), solute proton exchange rate (e,f), and RF saturation pulse amplitude (g,h) were investigated by varying the parameter of interest. For all simulations, unless otherwise stated: $T_{1w}=2.0$ s, $T_{2w}=50$ms, $T_{1s}=1.0$ s, $T_{2s}=50$ms, $k_{sw}=50$ Hz, [solute proton] = 100 mM, $\Delta\omega_s=10$ ppm, saturation pulse amplitude = 1.5 $\mu$T, saturation duration = 10 s).

Two-pool steady state solutions may only be used to simulate very simple CEST experiments with aqueous solutions in vitro. In vivo CEST experiments are more accurately described by incorporating the presence of magnetization transfer (MT) processes between bulk water protons and protons associated with endogenous macromolecules (m). For example, RF saturation pulse amplitudes that generate optimal contrast in aqueous solutions in vitro are typically not optimal in vivo because significant contrast is loss in vivo due to MT effects. Therefore, a three-pool or four-pool version of the Bloch-McConnell equations is generally used to describe CEST effects and inherent macromolecule effects MT effects. The macromolecules represent the semi-solid component of tissue, which is modeled using very short transverse relaxation. To simulate the endogenous macromolecular MT effect, the macromolecule proton pool is described mathematically using a super-Lorentzian line shape across a very broad range of frequencies (~200 ppm) centered at bulk water protons. Specifically, the super-Lorentzian line shape is used since Li et al. found that the super-Lorentzian line shape simulates the endogenous macromolecule MT effects observed in biological tissue better than the Lorentzian or Gaussian line shape. The interested reader is referred to [67, 68] for a comprehensive explanation of mathematical descriptions of macromolecular MT effects. Since the macromolecule and solute proton pools are very small compared to the bulk water pool, chemical exchange between the solute and macromolecule pools is negligible and ignored. The three-pool version of the Bloch-McConnell equations is:
Using identical parameter definitions as the two-pool version and the super-Lorentzian line shape $g$ defined as:

$$
g(\Delta \omega_{J,M}) = \sqrt{\frac{2}{\pi}} \int_{0}^{\pi/2} \frac{\sin^2 \theta}{\cos^2 \theta - 1} e^{\left[\frac{-2 (\Delta \omega_{J,M}) T_{1w}}{3 \cos \theta - 4}\right]} d\theta \quad [1.62]
$$

It is not possible to derive exact equations for the three-pool version of the Bloch-McConnell equations. However, again the Bloch-McConnell equations are of the form shown in equation [1.59] leading to the time-dependent analytical solution shown in equation [1.60]. The analytical solutions derived using the three-pool model provide a more accurate description of CEST contrast in vivo. The effect of incorporating the macromolecule pool in a simulated CEST contrast experiment is demonstrated in Figure 1.6. It is important to note that a fourth pool may be added in cases where two CEST generating solutes are simultaneously modeled in vivo, as was done in chapter 3.
Figure 1.6: Three CEST spectra were numerically simulated using the three-pool version of the Bloch-McConnell equations with different macromolecule concentrations expressed in % of macromolecules (% MT). The impact of magnetization transfer (MT) effects from macromolecules on CEST effects is evident when comparing CEST spectra simulated with 0% MT (a), 5% MT (b) and 10% MT (c). For all simulations, $T_{1w}=2.0$ s, $T_{2w}=50$ ms, $T_{1s}=1.0$ s, $T_{2s}=50$ ms, $T_{1m}=100$ ms, $T_{2s}=15$ μs, $k_{sw}=50$ Hz, $k_{mw}=50$ Hz, [solute proton] = 100 mM, $\Delta \omega_s=10$ ppm, $\Delta \omega_m=10$ ppm, saturation pulse amplitude = 1.5 μT, saturation duration = 10 s).

1.5.3 Endogenous CEST and PARACEST

The work presented in this thesis focuses on two different types of CEST known as endogenous CEST and PARACEST. Endogenous CEST contrast is generated using protons associated with endogenously expressed solutes such as proteins and peptides, or metabolites such as glucose, glutamate, and creatine. Specifically, work reported in this thesis uses amide ($\Delta \omega = 3.50$ ppm) and amine protons ($\Delta \omega = 2.75$ ppm) that are associated with proteins and peptides (Chapters 2 and 3). Exchangeable endogenous solute protons typically have frequency offsets within 5 ppm of bulk water protons. Therefore, CEST contrast can only be generated using exchangeable protons with exchange rates slower than 2000 Hz at 9.4 T, determined using the slow-to-intermediate exchange condition in equation [1.46]. PARACEST contrast is generated using protons
associated with exogenous paramagnetic lanthanide complexes. In 2001, Aime et al. [69] and Zhang et al. [70] separately discovered that protons associated with Europium (Eu$^{3+}$) bound water molecules could be detected at a frequency offset $\sim 50$ ppm away from water. Europium bound water molecules exchange with bulk water at an exchange rate of 2630 Hz, which is well within the slow-exchange regime due to the large chemical shift. Other lanthanide metals such as Tm$^{3+}$ and Yb$^{3+}$ have bound water molecules with chemical shifts greater than 200 ppm, which is outside of the endogenous macromolecule MT range [71]. Hence, the large chemical shift of lanthanide PARACEST agents offers two important advantages that should theoretically improve sensitivity. First, the increased chemical shift away from bulk water enables detection of protons with much faster exchange rates while still satisfying the slow-to-intermediate exchange condition and the CEST contrast increases with exchange rate $k_{sw}$, according to equation [1.46]. Second, the large chemical shift enables CEST detection in the presence of endogenous MT effects, which was shown to significantly reduce CEST contrast in Figure 1.6. Zhang et al. used the Bloch-McConnell equations to demonstrate that PARACEST agents with large chemical shifts and optimal exchange rates could generate detectable CEST contrast at $\mu$M concentrations [71]. While theoretical models suggest that PARACEST agents could have greater detection sensitivity than typical Gd$^{3+}$-based relaxivity contrast agents, PARACEST agents have suffered from low-detection sensitivity due to a severe loss of contrast as a result of bulk water saturation from endogenous macromolecules, which reduces the dynamic range of the CEST experiment. Furthermore, as previously mentioned very fast exchange rates require high amplitude $B_1$ saturation RF pulses to achieve optimal CEST contrast, which can exceed clinical specific absorption rate (SAR) limits. Currently, several groups are exploring the addition of different ligands (eg. side arms) to lanthanide complexes to alter both the chemical shift and proton exchange rate. For example, the DOTAM-Gly ligand was first introduced by Aime et al. in 2002 [72]. Lanthanide complexes with DOTAM-Gly ligands have exchanging bound water protons and amide protons giving rise to two different chemical exchange sites. A lanthanide complex developed by Hudson and colleagues [73] called Tm-DOTAM-Gly-Lys is used in Chapter 4 to measure pH using the amide proton CEST effects in vivo.
1.5.4 Proton exchange rate and pH measurement

The proton exchange rate affects the magnitude of the CEST effect as was shown in figure 1.5e,f. Importantly, the proton exchange rate $k_{ex}$ is pH-sensitive for several types of exchangeable protons including amine and amide groups. The proton exchange rate $k_{ex}$ depends on pH according to equation [1.65]:

$$k_{ex} = k_0 + k_a [H^+] + k_b [OH^-] = k_0 + k_a 10^{-pH} + k_b 10^{pH-pK_w}.$$  \[1.65\]

where $k_0$ is the rate constant of the spontaneous proton exchange reaction between water and the amide nitrogen nucleus, $k_a$ is the rate constant of the acid-catalyzed protonation of the amide nitrogen nucleus, $k_b$ is rate constant of the base-catalyzed proton exchange reaction between hydroxyl ion ($OH^-$) and the amide nitrogen nucleus, and pK$_w$ is the ionization constant of water (pK$_w$ = 15.4 at 37 $^\circ$C) from equation [1.3] in Section 1.2.1. However, proton exchange is predominantly base-catalyzed for amide and amine protons and the expression for proton exchange rate is often simplified as:

$$k_{ex} = k_b 10^{pH-pK_w}.$$  \[1.66\]

The pH-dependence of proton exchange has been known since before the origin of CEST imaging in 2000 [74, 75]. Since CEST contrast is dependent on proton exchange rate it is inherently sensitive to pH. However, CEST contrast also depends on other factors including: solute proton concentration, temperature, $B_1$ amplitude, as well as bulk water relaxation time constants ($T_1$ and $T_2$). The difficulty in quantitative pH measurement using CEST contrast is isolating the pH contribution to the CEST contrast effects on CEST measurements. For example, in some cases, increasing pH will cause the CEST contrast to increase. However, an increase in solute proton concentration could also induce an identical increase in CEST effect. Therefore, it is tedious and difficult to properly measure pH without contamination from other concurrent effects. Isolation of pH effects on CEST contrast is further complicated in vivo by the endogenous macromolecule MT effects and because the exact solute proton concentration is unknown. Different strategies to measure pH with CEST have been developed for endogenous CEST and PARACEST.
1.5.4.1 Using endogenous CEST to measure pH

Endogenous CEST contrast generated using amide protons resonating at 3.50 ppm downfield (ie. higher frequency) from bulk water protons has been used to measure pH [33]. The amide protons visible at 3.50 ppm are associated with mobile proteins and peptides. Approximately 90% of proteins and peptides originate from the intracellular compartment and consequently, pH measured using amide proton CEST effects is considered to be heavily weighted to the intracellular space and is referred to as intracellular pH. Zhou et al. first developed a method to measure intracellular pH using amide proton transfer (APT) imaging, however this APT pH measurement required several limiting assumptions [33]. First, Zhou et al. subtracted the macromolecule MT effects from the CEST contrast using the asymmetric magnetization transfer ratio (MTR_{asym}) curve by subtracting the negative frequency offsets from the positive frequency offsets of the CEST spectrum with bulk water proton frequency referenced to 0 ppm using equation [1.67].

\[
\text{MTR}_{asym}(\Delta \omega) = \frac{M_z(\Delta \omega) - M_z(-\Delta \omega)}{M_z(-\Delta \omega)} \quad [1.67]
\]

where \(M_z(\Delta \omega)\) represents the bulk water magnetization after saturation at the frequency offset \(\Delta \omega\). Furthermore, Zhou et al. defined the MTR_{asym} curve as the combination of an inherent and pH-insensitive MTR_{asym} curve defined as MTR’_{asym} and a pH-sensitive amide proton transfer ratio (APTR) as shown by:

\[
\text{APTR}_{sat}(t_{sat}) = k_{ex} \frac{[\text{amide proton}]}{[\text{water proton}]} R_1^w \cdot [1 - e^{-R_1^w}] \quad [1.68]
\]

Equation [1.68] is clearly derived from the time-dependent steady state solutions from equation [1.58], which was derived from the two-pool exchange model using the WSP approximation. Using water exchange filter spectroscopy (WEX) experiments, Zhou et al. measured amide proton exchange rates in rat brains in different physiological states including healthy, during cerebral ischemia and post mortem, and also measured intracellular pH using the chemical shift between inorganic phosphate (Pi) and
phosphocreatine (PCr) obtained by $^{31}$P NMR. The proton exchange rates and pH values were fit to the theoretical equation [1.69] giving the in vivo relationship described by:

$$k_{ex} = 5.57 \times 10^{\text{pH}-6.4}$$  \[1.69\]

Quantitative pH measurement using APT imaging is quite limited because the pH-sensitive parameter APTR is also affected by amide proton concentration, cellular water content, and longitudinal relaxation. Also the MTR$_{\text{asym}}$ measurement may be contaminated by nuclear Overhauser effects (NOEs) that occur between -2.0 and -4.0 ppm, directly opposite to the amide protons at 3.50 ppm. Therefore, pH measurement using APT is susceptible to error.

Recently Cai et al. demonstrated that amine protons associated with glutamate have pH-sensitive exchange rates [76]. Similarly, several groups showed that hydroxyl protons associated with glucose exchange with water in a pH-dependent manner [13, 77, 78]. However, these CEST effects are also affected by changes in longitudinal relaxation and concentration limiting their utility for absolute pH measurement.

1.5.4.2 Using PARACEST to measure pH

In 2000, Ward and Balaban demonstrated absolute pH measurement using PARACEST contrast in vitro [75]. A novel ratiometric approach was developed to measure pH independent of agent concentration or longitudinal relaxation using two different base-catalyzed exchange sites. Briefly, two pH-sensitive effects can be observed from solutions containing either a single agent with two chemically shifted exchange sites or a mixture of two agents, each with one chemically shifted exchange site. When using a single agent, the ratio of the two CEST effects was shown to be independent of agent concentration and longitudinal relaxation. When using a mixture of two PARACEST agents, the ratio of both agents’ concentration is assumed to be constant, which is only appropriate if the two PARACEST agents have identical pharmacokinetic properties. Recently, Sheth et al. demonstrated extracellular pH measurement in a mouse tumor model using a ratiometric approach with a single PARACEST agent that possessed exchangeable amine protons resonating at 10 ppm and amide protons resonating at -10
ppm relative to water [79-81]. In Chapter 4, we developed a novel method to measure extracellular pH using a PARACEST agent using the line shape of the CEST effect. This approach is independent of agent concentration and T1 relaxation.

1.5.5 Chemical shift: pH and temperature effects

The chemical shift (δ) of protons associated with lanthanide PARACEST agents can be sensitive to both pH and temperature [82, 83]. As mentioned in Section 1.3.1, the chemical shift is determined by the distribution of the electron clouds. The chemical shift is often divided into the diamagnetic component and the paramagnetic component, with the latter describing the effects of the unpaired electrons. Importantly, the paramagnetic component of the chemical shift of a nucleus is often much larger than the diamagnetic component [83]. The paramagnetic chemical shift depends on the position of unpaired electrons relative to the nucleus, which in turn depends on temperature and sometimes pH [83]. Zhang et al. first introduced the idea of measuring the chemical shift using PARACEST contrast to measure temperature [82]. Our group recently developed a Eu3+-based PARACEST agent called Eu3+-DOTAM-Glycine(Gly)-Phenyalanine(Phe) with a temperature-dependent and pH-independent bound water chemical shift. Using in vitro and ex-vivo phantoms, the chemical shift was shown to be independent of agent concentration, local pH, and endogenous macromolecule MT effects within physiologically relevant ranges [84]. In Chapter 4, the bound water proton chemical shift of a new Tm3+-based PARACEST agent is shown to depend on pH and temperature. Temperature and pH we calculated simultaneously by first obtaining pH using the line shape of the CEST effect and then the temperature using both the pH and the chemical shift.
1.6 References


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Simultaneous in vivo pH and temperature mapping using a PARACEST-MRI contrast agent

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Abstract: Altered tissue temperature and/or pH is a common feature in pathological conditions where metabolic demand exceeds oxygen supply such as in tumors and following stroke. Therefore in vivo tissue temperature and pH may become valuable biomarkers for disease detection and the monitoring of disease progression or treatment response in conditions with altered metabolic demand. In the current study, pH is measured using the amide protons of a thulium (Tm³⁺) complex with a DOTAM-Glycine-Lysine (ligand: Tm³⁺-DOTAM-Gly-Lys). The pH was uniquely determined from the linewidth of the asymmetry curve of the chemical exchange saturation transfer (CEST) spectrum, independent of contrast agent concentration or temperature for a given saturation pulse. pH maps with an inter-pixel standard deviation of less than 0.1 pH units were obtained in 10 mM Tm³⁺-DOTAM-Gly-Lys solutions with pH ranging from 6.0 to 8.0 pH units at 37 °C. Temperature maps were simultaneously obtained using the chemical shift of the CEST peak. Temperature and pH maps are demonstrated in the mouse leg (N=3), where the mean and standard deviation for pH was 7.2 ± 0.2 pH unit and temperature was 37.4 ± 0.5 °C.
2.1 Introduction

Contrast agents for magnetic resonance imaging (MRI) are often used in clinical medicine. Most MRI contrast agents are gadolinium-based compounds that enhance image contrast by shortening the bulk water longitudinal relaxation time through rapid exchange of inner-sphere water molecules with bulk water [1, 2]. However, most gadolinium-based agents have limited responsiveness to their environment. In contrast, MRI contrast agents based on chemical exchange saturation transfer (CEST) [3, 4] may be used to measure pH [5-8], track genetically altered glioma cells expressing lysine-rich protein [9], and track the metabolism of glycogen in mammalian tissue [10]. Furthermore, paramagnetic CEST (PARACEST) contrast agents may be used to measure physiological parameters [11-26], including tissue pH based on the pH dependence of the exchange rate of amide protons with bulk water protons [11-15, 27], and tissue temperature using the linear dependence of the exchangeable proton chemical shift on temperature [16, 17]. PARACEST agents can also be made sensitive to enzymatic activity [18, 19], proteins [20], and metabolites [21-23].

Altered tissue pH is a common feature in pathological conditions such as stroke and tumors when metabolic demand for oxygen exceeds supply. The reduced buffering capacity of tumor interstitial fluid, reduced tumor perfusion, and the increased lactic acid secreted by tumor cells results in an acidic pH (i.e. 6.4 ~ 7.0) that is lower than normal tissue pH (i.e. 7.0 ~ 7.4) [5, 6, 28-31]. Therefore in vivo tissue pH may be a valuable biomarker of disease progression and/or a predictor of treatment response. Extracellular pH could also be used to measure physiological changes due to radiation treatment and chemotherapy. Accurate in vivo pH measurements could significantly improve cancer and stroke treatment strategies.

Several methods to measure pH using PARACEST agents [11-15] have been demonstrated based on the pH dependence of the CEST effect of amide protons. Initial attempts were based on the CEST peak amplitude [13, 14, 32]. However, the amplitude of the CEST effect depends on many factors including the saturation pulse power, and duration, which are controlled, but also agent concentration, temperature, and pH, which
are typically unknown \textit{in vivo}. Therefore, direct application of the CEST effect amplitude for \textit{in vivo} pH measurement is difficult.

To overcome the concentration dependence, Aime \textit{et. al.} [11] proposed the use of ratiometric methods developed by Ward \textit{et. al.} [3] using a PARACEST agent with a bound water and an amide proton exchange site, or utilizing the bound water exchange site from one PARACEST agent and the amide proton exchange site from another. This approach is possible because the CEST effect arising from bound water is unaffected by changes in pH over the physiological pH range (i.e. 6 - 8), but the CEST effect from the amide protons is sensitive to pH changes. Therefore the ratio of the amide proton CEST effect to the bound water CEST effect provides a concentration independent method to measure pH. However the CEST effect from these two pools is acquired in two separate experiments using different saturation powers because the exchange rate of bound water with bulk water is significantly different than that of amide protons. A limitation of this approach is the assumption that endogenous magnetization transfer (MT) effects will not affect the ratio of the measured CEST effects from bound water and amide protons. Since the MT effect is typically different at the chemical shifts of the amide proton and bound water protons, regional differences in MT could alter the measured ratio of the CEST peaks. Altered temperature is also a property of pathological tissues [17, 33]. Therefore pH measurements must be independent of temperature. Temperature has negligible effects on pH measurements calculated using ratiometric methods within physiological temperature ranges [34, 35]. The method presented in the current study is expected to be less sensitive to temperature since linewidth measurements are independent of chemical shift. Recently \textit{in vivo} extracellular pH maps were demonstrated based on the ratiometric method [32, 36].

Another method to measure pH using only the amide proton site of a PARACEST agent was proposed by Wegh \textit{et. al.} [27]. The approach involves taking the ratio of the CEST effect at the resonance frequency of the amide protons and a frequency several parts per million (ppm) closer to the water frequency [37]. This ratio was pH-sensitive and concentration independent. However, the method assumed that the temperature dependent resonance frequency of the amide protons was constant.
In the current study, a novel method was developed to measure pH using the glycine amide proton site of a thulium (Tm³⁺) complex with a DOTAM-Glysine-Lysine (ligand: Tm³⁺-DOTAM-Gly-Lys) [38]. With this compound, pH was determined from the linewidth of the asymmetric magnetization transfer ratio (MTR\textsubscript{asym}) curve, independent of agent concentration and temperature for a given saturation pulse. Temperature was measured simultaneously using the dependence of the CEST peak chemical shift on temperature. This novel method is demonstrated \textit{in vivo} in the murine leg muscle following intramuscular injection.

2.2 Theory

CEST contrast is generated by the saturation of the exchangeable chemically shifted amide proton pool associated with a PARACEST agent using a long continuous wave radio frequency pulse [39]. These saturated protons then transfer to the bulk water pool by chemical exchange. The net transfer of high-energy protons to the bulk pool leads to a reduction of the bulk water net magnetization and produces negative contrast in MRI.

The proton transfer ratio (PTR) (equation [2.1]) is used to quantify CEST contrast. The steady state bulk water magnetization following a presaturation pulse of frequency $\Delta \omega$ is given by $M^0_Z(\Delta \omega)$ and $M^0_Z$ is the equilibrium bulk water magnetization.

$$\text{PTR}(\Delta \omega) = \frac{M^0_Z - M^0_Z(\Delta \omega)}{M^0_Z}$$  \[2.1\]

To model the effect of pH on the PTR, we consider a two-site exchanging system with protons occupying the bulk water pool (W) and the solute/CEST pool (S). At equilibrium, protons within the bulk water and CEST pool have a net magnetization of $M^0_w$ and $M^0_s$, respectively. The Bloch equations, modified with exchange terms, describe such systems of exchanging magnetic spins [39, 40, 41]. Under appropriate conditions, it can be assumed that steady state is achieved upon irradiation to derive analytical solutions [26]. Using the analytical solutions, Zai et al. described the PTR with respect to presaturation pulse frequency ($\Delta \omega$) [42]. Assuming steady state, it was derived that

$$\text{PTR}(\Delta \omega) = \frac{k_{WS}}{R_{1W}k_{WS}} \cdot \frac{\omega^2}{\omega^2 + pq + \Delta \omega^2 \frac{q}{p}}$$  \[2.2\]
with:

\[ p = R_{2S} + k_{SW} - \frac{k_{SW} k_{WS}}{R_{2W} + k_{WS}} \]  \hspace{1cm} [2.3]

\[ q = R_{1S} + k_{SW} - \frac{k_{SW} k_{WS}}{R_{1W} + k_{WS}} \]  \hspace{1cm} [2.4]

where \( \omega_1 \) is the saturation pulse power, \( k_{SW} \) is the exchange rate of protons from the agent-bound proton pool to bulk water and \( k_{WS} = k_{SW} \frac{M_0^S}{M_0^W} \) is the exchange rate in the reverse direction, and \( R_{1/2,WS} / T_{1/2,WS} \) are the relaxation rates of the proton pools [42].

The Lorentzian line shape of the PTR curve implies a linewidth measured as the full-width half maximum (FWHM) given by:

\[
\text{Linewidth} = 2 \sqrt{\omega_1^2 \frac{p^2 + q^2}{p}} \]

[2.5]

Rearranging Eqs. 3 and 4, gives,

\[ p = R_{2S} + \frac{R_{2W} k_{SW}}{R_{2W} + k_{WS}} \]  \hspace{1cm} [2.6]

\[ q = R_{1S} + \frac{R_{1W} k_{SW}}{R_{1W} + k_{WS}} \]  \hspace{1cm} [2.7]

The exchange rate (\( k_{SW} \)) of amide protons bound to Tm\(^{3+} \) agents is approximately \( \sim 2500 \) Hz [13]. Thus, at a concentration of 10 mM it follows by definition that \( k_{WS} \approx 0.182 \) Hz. Relaxation rates of PARACEST aqueous solutions are on the order of \( R_{1W} / R_{2W} = 1/10 \) Hz and \( R_{1S} / R_{2S} = 10/10 \) Hz [41, 43]. Based on these estimates, we can assume that (1) \( k_{SW} \gg k_{WS} \), (2) \( \frac{k_{SW}}{R_{1S,2S}} \gg R_{1W,2W} \gg k_{WS} \). Under such conditions, \( p \) and \( q \) are approximately equal to \( k_{SW} \) and

\[
\text{Linewidth} = 2 \sqrt{\omega_1^2 + k_{SW}^2} \]

[2.8]

Recently, Van Zijl et al. and coworkers [5] introduced the magnetization transfer ratio asymmetry curve (MTR\(_{\text{asym}}\)), defined by equation [2.9], as a practical measure of PTR.

\[
\text{MTR}_{\text{asym}}(\Delta \omega) = \frac{M_{z}^{S}(\Delta \omega) - M_{z}^{S}(\Delta \omega)}{M_{z}^{S}(-\Delta \omega)} \approx \text{PTR}(\Delta \omega) \]

[2.9]
The MTR asym curve is created by subtracting the positive frequencies from negative frequencies of a CEST spectrum, with water referenced to 0 ppm to remove the effect of the direct saturation of bulk water [5]. Hence, MTR asym linewidth is linearly proportional to exchange rate as shown in equation [2.8]. Furthermore, when the CEST pool consists of protons coordinated with an amide group, the exchange rate is base-catalyzed and sensitive to pH [5]. With respect to pH, a base-catalyzed exchange rate is described as:

$$k_{SW} = k_0 + k_b \times 10^{pH-pK_w}$$

[2.10]

where $k_0$ is the spontaneous exchange rate, $k_b$ is the base-catalyzed exchange rate, and $pK_w$ is the ionization constant of water ($pK_w=15.4$ at 37 oC) [5, 44]. Hence, MTR asym linewidth has an exponential relation with pH as shown in equation [2.11] and described by

$$\text{Linewidth} \propto k_{SW} = k_0 + k_b \times 10^{pH-pK_w}$$

[2.11]

2.3 Methods

2.3.1 PARACEST Agent Synthesis

The synthesis method of Tm$^{3+}$-DOTAM-Gly-Lys has been previously described [38]. Briefly, the DOTAM-Gly-Lys was synthesized by tetraalkylation of cyclen with the corresponding iodoacetyl dipeptide sequence (C-terminal ester). After hydrolysis of the ester groups, Tm$^{3+}$-DOTAM-Gly-Lys was prepared by mixing an aqueous solution of ligand and TmCl$_3$ in a 1:1 molar ratio. The reaction mixture was treated with NaOH (2.5 M solution) to adjust the pH to 9 and was stirred for 18 hours at room temperature followed by size exclusion chromatography to purify the compound. The formation of the complex was confirmed using high-resolution mass spectrometry (found: 1311.5773, calculated: 1311.5750 for C$_{48}$H$_{86}$N$_{16}$O$_{16}$Tm) and high-resolution proton NMR spectroscopy. The structure of Tm$^{3+}$-DOTAM-Gly-Lys is given in Figure 2.1.
**Figure 2.1:** Molecular structure of the thulium (Tm$^{3+}$) chelate with ligand DOTAM-Gly-Lys and bound water.

### 2.3.2 Bloch Simulations

Using the two-pool modified Bloch equations, the effect of pH on the MTR$_{asym}$ linewidth was studied. Simulations were performed using MATLAB (MATLAB release 14; The MathWorks Inc., Natick, MA, USA). CEST spectra were simulated by modeling the bulk water signal intensity as a function of presaturation frequency using the parameters listed in Table 2.1. Parameters remained constant for all simulations with the exception of the pH dependent exchange rates ($k_{SW}$, $k_{WS}$). Exchange rates were calculated using equation [2.9] with varying pH (6.0 - 8.0 pH units). MTR$_{asym}$ curves were created using the simulated spectra and fitted to a Lorentzian function in MATLAB to determine the linewidths. Linewidth was defined as the Full Width at Half Maximum (FWHM). A base-10 exponential function was fitted to the resulting linewidth-pH curve using the MATLAB *lsqcurvefit* function.
<table>
<thead>
<tr>
<th>Simulation parameters (Units)</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_{1w}/T_{2w}$ (s)</td>
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</tr>
<tr>
<td>$T_{1s}/T_{2s}$ (s)</td>
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</tr>
<tr>
<td>Concentration (mM)</td>
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</tr>
<tr>
<td># Amide proton sites</td>
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</tr>
<tr>
<td>Chemical shift (ppm)</td>
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<td>Spontaneous exchange rate (Hz)</td>
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<tr>
<td>Base-catalyzed exchange rate (Hz)</td>
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<tr>
<td>pHs</td>
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<tr>
<td>Saturation power ($\mu$T)</td>
<td>14</td>
</tr>
<tr>
<td>Saturation duration (s)</td>
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</tr>
</tbody>
</table>

**Table 2.1**: Stimulation Parameters Used for Aqueous Solutions Containing Tm$^{3+}$-DOTAM-Gly-Lys at 37 °C.

### 2.3.3 In vitro Experiments

To study the effect of pH on the linewidth of the CEST effect, a series of 10 mM solutions (600 μL) of Tm$^{3+}$-DOTAM-Gly-Lys were produced with pH ranging from 6.0 to 8.0 pH units in steps of 0.5 pH units. Sample pH was controlled by dissolving Tm$^{3+}$-DOTAM-Gly-Lys into 600 μL of 20 mM phosphate buffer solution ($\text{NaH}_2\text{PO}_4 + \text{Na}_2\text{HPO}_4$) with different pH (6.0, 6.5, 7.0, 7.5, and 8.0). The pH value of the buffer solution was confirmed with a standard pH electrode (Cole-Parmer, QC, Canada). To study the effect of agent concentration on the linewidth of the CEST effect, a series of pH 7 solutions (600 μL) of Tm$^{3+}$-DOTAM-Gly-Lys were produced with concentrations ranging from 5 to 20 mM. Finally, to study the effect of endogenous MT on the linewidth of the CEST effect, a series of pH 7 solutions (600 μL) of Tm$^{3+}$-DOTAM-Gly-Lys were produced with 10% bovine serum albumin (heated to cross-link) with concentrations ranging from 5 to 20 mM.

CEST spectra were acquired on a 9.4 T horizontal bore Agilent MRI scanner equipped with a 30 mm millipede radio frequency coil (Agilent, Palo Alto, CA). All *in vitro* measurements were made in triplicate unless stated otherwise. The pulse sequence
consisted of a continuous wave presaturation pulse ($B_1$) followed by a hard 90° pulse. Presaturation time ($TS = 3s$) and power ($B_1 = 14 \, \mu T$) were constant for all experiments, unless stated otherwise. Images were acquired using a two-dimensional fast spin echo (FSE) pulse sequence (FOV = 25.6 × 25.6 mm$^2$, data matrix: 64 × 64, TR = 500 ms, ESP = 10 ms, ETL = 16, ETE = 10 ms, 2 dummy scans, 3 averages) preceded by a continuous wave presaturation pulse. CEST spectra were obtained from the images by varying the frequency of the presaturation pulse from -60 to 60 ppm with 1-ppm steps. Temperature was controlled by blowing warm air over the phantoms using a Model 1025 Small Animal Monitoring and Gating System (SA Instruments Inc., Stony Brook, NY) and monitored using a fiber optic probe placed adjacent to the phantoms. A heating time of 15 minutes was allowed for the experimental temperature to be achieved.

Linewidth was studied as a function of several experimental conditions. To study the effect of temperature, CEST spectra from a series of 10 mM Tm$^{3+}$-DOTAM-Gly-Lys solutions (pH: 6.0, 6.5, 7.0, 7.5, 8.0) were obtained at 35, 36, 37, 38, and 39 °C. To study the effect of concentration, CEST spectra from a series of pH 7.0 Tm$^{3+}$-DOTAM-Gly-Lys solutions with concentrations of 5, 10, 15 and 20 mM were obtained at 37 °C. To study the influence of the inherent MT effect, the CEST spectra of a series of pH 7 Tm$^{3+}$-DOTAM-Gly-Lys solutions with 10% bovine serum albumin (BSA) and different concentrations (5-20 mM) were acquired at 37 °C. Relaxation rates were measured for both the aqueous and BSA phantoms to study the effects of relaxation on linewidth measurements. To study the effect of presaturation power, CEST spectra of a 10 mM pH 7 Tm$^{3+}$-DOTAM-Gly-Lys solution were acquired at 37 °C with different presaturation powers (8, 10, 12, and 14 µT). Finally, to study the effect of pH, CEST spectra of a series of 10 mM Tm$^{3+}$-DOTAM-Gly-Lys solutions (pH: 6.0, 6.5, 7.0, 7.5, 8.0) were acquired at 37 °C.

For each pixel, the MTR$_{asym}$ curve was fitted to a Lorentzian lineshape to measure the linewidth and chemical shift. An empirical exponential equation was derived to describe the relationship between linewidth and pH. The method used to produce temperature maps from the chemical shift of the MTR$_{asym}$ curve has been previously published [16, 17, 43]. Briefly, the chemical shift changes linearly with temperature (0.268°C/ppm)
However, the y-intercept of the linear relationship was observed to be pH-sensitive. Therefore, an empirical quadratic equation was derived to describe the relationship between the y-intercept of the chemical shift response and pH. The MTR$_{asym}$ curve was fitted to a Lorentzian lineshape, and the temperature was determined from the chemical shift of the peak using the chemical shift and the pH-calibrated linear relation.

The spin-lattice ($T_1$) and spin-spin ($T_2$) relaxation time constants were measured for each aqueous and BSA solution at 36 °C. The $T_1$ time constant was measured using an inversion recovery pulse sequence (repetition time (TR) = 10 s, inversion time (TI) = 0 to 3 seconds in 10 steps, 3 averages). The $T_2$ time constant was measured using a spin-echo sequence (TR = 10 s, TE = 5 to 60 ms in 5ms increments, 3 averages).

### 2.3.4 In vivo Experiments

For in vivo demonstration, C57BL/6 mice (8 months of age, weighing ~ 30 g, N=3) were anesthetized (induced using 4% isoflurane in oxygen and maintained using 1.5 - 2.5% isoflurane in oxygen). Each mouse was secured on a MRI-compatible stage. Temperature was monitored with a rectal temperature probe, and respiration was monitored with a respiratory sensor pad, which was connected to a pressure transducer, placed over the thoracic/abdominal region. Following the acquisition of pre-injection MR anatomical and CEST images, 50 µL of 100 mM Tm$^{3+}$-DOTAM-Gly-Lys dissolved in phosphate buffered saline (PBS) was injected directly into the left leg muscle [45], with the mouse secured to the MRI compatible stage. Using a 27-gauge needle, injection was performed at a rate of 12.5 µL/min at a tissue depth of 2mm. In vivo CEST image acquisition began within 15 minutes of injection. The animals were recovered following imaging. The animal procedure was performed according to a protocol approved by the Western University Animal Use Subcommittee.

In vivo imaging was performed on the same 9.4 T MRI scanner used for in vitro studies equipped with a custom-built 3 cm diameter radio frequency surface coil. Body temperature was maintained at 37 °C during the imaging procedure by blowing warm air over the animal using a model 1025 small-animal monitoring and gating system (SA Instruments Inc., Stony Brook, NY, USA). At baseline, a high-resolution $T_2$-weighted
image was acquired for localization using a fast imaging with steady state precession pulse sequence (FOV = 25.6 × 25.6 mm², data matrix = 256 × 256, TR = 6 ms, echo time = 3 ms, excitation flip angle = 70°, 1 slice, 1 mm thickness, 20 prescans, 1 average, total acquisition time = 1 min and 46 s). CEST images were acquired from the same slice pre- and post-injection. To maximize the signal to noise ratio in vivo, a fast spin echo pulse sequence was used for CEST imaging (FOV = 25.6 × 25.6 mm², data matrix = 64 × 64, TR = 5000 ms, ETL = 32, effective echo time = 10 ms, 2 mm thickness, and 1 average, total acquisition time = 10.2 s) preceded by a continuous wave presaturation pulse (B₁ = 14 µT, TS = 5s). For in vivo CEST images, presaturation pulse frequency was varied in ascending order beginning with -1000 ppm and included the following frequencies: ±1*(1000, 60 to 30ppm with 1-ppm steps along with 30, 20, 10, 5, 3, 2, 1ppm) and 0 ppm (total CEST acquisition time = 26 min 36s). All in vivo CEST images were spatially smoothed using a 2 x 2 filter. The temperature and pH maps were created using the CEST spectra on a pixel-by-pixel basis. For each pixel, the contrast to noise ratio was calculated. To achieve a 95% probability that a real CEST signal was observed, only pixels with contrast to noise ratio ≥ 2√2 were used to map temperature and pH [46, 47].

To correct B₀-inhomogeneity effects, the water peak of all in vitro and in vivo CEST spectra were fitted to a tenth-order polynomial on a pixel-by-pixel basis following interpolation of the spectrum to a resolution of 1 Hz. The water resonance was assumed to be at the frequency where the interpolated fitted polynomial was lowest. Each interpolated spectrum was shifted so that the fitted water resonance was positioned at 0ppm [5].

2.4 Results

Simulated CEST spectra for different pH values are plotted in Figure 2.2a. Corresponding linewidths are plotted with pH in Figure 2.2b with a fitted exponential function. The pH-dependence of simulated linewidths agrees well with the theoretical relation shown in equation [2.11].

63
Figure 2.2: a) Simulated MTR$_{\text{asym}}$ curves derived using the modified Bloch equations. Agent parameters used are listed in Table 1. The MRI parameters included $B_0 = 9.4$T, $B_1 = 14\mu$T, TS = 3 s. pH-dependent exchange rates ($k_{3W}$, $k_{\text{WS}}$) were calculated using Eq. 2.9 with $k_0 = 100$ Hz, $k_b = 200 \times 10^9$ Hz and $pK_W = 15.4$. b) Linewidths plotted with pH. The exponential function (Linewidth = $3.25 + 10^{0.99pH-7.44}$) is superimposed on the simulation linewidths ($R^2 = 0.9999$). The linewidths increase monotonically and logarithmically with pH as predicted by the steady state analytical solutions.

A typical CEST spectrum of a 10 mM pH 7 Tm$^{3+}$-DOTAM-Gly-Lys solution acquired at 37 °C is shown in Figure 2.3. The corresponding MTR$_{\text{asym}}$ curve is displayed on the baseline with the position of the linewidth measurement indicated. Both temperature and agent concentration had only a small effect on the CEST peak linewidth. The effect of temperature on the linewidth of the CEST peak is given for a series of 10mM Tm$^{3+}$-DOTAM-Gly-Lys aqueous solutions with different pH values (6.0, 6.5, 7.0, 7.5, and 8.0) in Figure 2.4a. The linewidths were constant from 36 to 38 °C for pH ≤ 7.5 solutions (pH: mean linewidth ± std. dev., 6.0: 4.3±0.2, 6.5: 4.6±0.1, 7.0: 5.5±0.2, 7.5: 8.9±0.6, 8.0: 14.1±0.7). The effect of temperature on the linewidth of the CEST peak is given for Tm$^{3+}$-DOTAM-Gly-Lys at pH 7.0 in aqueous solution in Figure 2.4b.

The effect of agent concentration on the linewidth of the CEST effect in aqueous solutions and in 10% BSA is shown in Figure 2.4c. BSA simulates the endogenous MT effect from protons bound to macromolecules in vivo. The linewidths (Aqueous: 5.3±0.2, BSA: 4.9±0.1) were independent of agent concentration within the concentration range
expected to be necessary for \textit{in vivo} PARACEST detection (5-20mM) [48]. Overall, the relative insensitivity of linewidth to temperature, agent concentration and MT effect suggests these conditions did not significantly modify the exchange rate between the amide and the bulk water protons. Relaxation rates of the aqueous and BSA solutions are listed in Table II. Figure 2.4c also demonstrates that linewidth is relatively independent of relaxation rates. The effect of saturation power ($\omega_1$) on the linewidth of the CEST peak is given for $\omega_1 = 8, 10, 12$ and $14 \mu$T values in Figure 2.4d. Linewidth increased linearly with power as expected from equation [2.8]. Specifically, at pH 7.0 and 37 °C, for $\omega_1 = 8$ to $14 \mu$T:

\begin{equation}
\text{Linewidth} = 0.19\omega_1 + 2.7
\end{equation}

MTR\textsubscript{asym} curves for a series of 10 mM Tm$^{3+}$-DOTAM-Gly-Lys solutions (pH: 6.0, 6.5, 7.0, 7.5, 8.0) at 37 °C are given in Figure 2.5a, and the dependence of the linewidth of the MTR asymmetry curve on pH is shown in Figure 2.5b. A transformation of the data to log-linear format is shown in Figure 2.5c. A linear fit of the log-linear data is superimposed. The linewidth of the CEST effect clearly increases with pH. The rate of increase is greater at pH ≥7 as expected since amide proton exchange is base-catalyzed [5]. pH can be determined uniquely from the linewidth of the CEST effect. The base-10 exponential relationship determined empirically between the MTR\textsubscript{asym} linewidth and the pH given in Figures 2.5b and 2.5c can be expressed as equations [2.13] and [2.14] respectively, and can be used for pH measurement.
Figure 2.3: A typical CEST spectrum of a 10 mM pH 7 solution of Tm$^{3+}$-DOTAM-Gly-Lys acquired on a 9.4 T MRI scanner at 37°C using a 14 µT 3 s saturation pulse. The linewidth of the CEST effect is defined as the full width at half maximum of the asymmetry curve generated by the subtraction of the halves of the CEST spectrum (pH = 7.0, $B_0 = 9.4$ T, $B_1 = 14$ µT, TS = 3 s, 37°C).
Figure 2.4: a) The dependence of MTR_{asym} linewidth on temperature for a series of 10 mM solution Tm^{3+}-DOTAM-Gly-Lys (pH: 6.0 (—), pH: 6.5 (---), pH: 7.0 (--.--), pH: 7.5 (.-.), pH: 8.0 (——)). B) Close-up of dependence of the MTR_{asym} linewidth on temperature for pH 7 solution shown in 4A. C) The dependence of the MTR_{asym} linewidth on concentration for the pH 7 solution of Tm^{3+}-DOTAM-Gly-Lys at 37°C in the absence and presence of MT effect. D) The dependence of the MTR_{asym} linewidth on saturation power for the pH 7 solution of Tm^{3+}-DOTAM-Gly-Lys at 37°C (B_0=9.4 T, B_1 = 14 μT, TS = 3 s for a,b,c,d). Error bars are equal to one standard deviation.

\[
\text{Linewidth} = 3.59 + 10^{0.69 \text{pH} - 4.49} \\
\text{pH} = \frac{\log(\text{Linewidth} - 3.59) + 4.49}{0.69}
\]

Figure 2.6 shows the \textit{in vitro} phantom pH distribution maps generated from each pixel’s MTR_{asym} linewidth and using equation 2.14. The measured pH within each 600 μL, 10 mM phantom was (\textbf{Phantom pH}: mean pH ± std. dev; 6.0: 6.2±0.2, 6.5: 6.5±0.1, 7.0: 6.9±0.04, 7.5: 7.6±0.03, 8.0: 8.0±0.03).
Similarly, the equation describing the relation between chemical shift and temperature was measured (data not shown) and is given by equations [2.15] and [2.16].

\[
\text{Chemical Shift} = 0.268 \times \text{Temperature} \left({}^\circ\text{C}\right) + y - \text{intercept} \quad [2.15]
\]

\[
y - \text{intercept} = 0.3738 \times \text{pH}^2 - 4.7824 \times \text{pH} - 40.679 \quad [2.16]
\]

Applying equations [2.15] and [2.16], Figure 2.7 shows the \textit{in vitro} temperature distribution maps generated using the chemical shift of the MTR\textsubscript{asym} curve from each pixel in a series of 600 µL, 10 mM phantoms with different pH at 37 °C. Average phantom temperature measurements were: (\textbf{Phantom pH}: mean temperature ± std. dev.) \textbf{6.0}: 37.2±0.2, \textbf{6.5}: 37.2±0.2, \textbf{7.0}: 37.3±0.2, \textbf{7.5}: 36.7±0.2, \textbf{8.0}: 37.3±0.3).

Figure 2.8a shows the average pre-injection and first post-injection CEST spectra obtained in a single mouse leg muscle, from pixels achieving 95% probability of CEST detection post-injection. Similar results were obtained in the other two mice. Both CEST spectra were normalized to the mean signal intensities measured following the ± 1000 ppm saturation pulses. From the average first post-injection spectrum, a clear CEST effect was observed along with slightly lower endogenous MT effect compared to pre-injection. The pH and temperature maps measured using PARACEST on a pixel-by-pixel basis are superimposed on the pre-injection high-resolution \textit{T}_2-weighted image in Figures 2.8b and 2.8c respectively. The mean (± std. dev.) \textit{in vivo} pH and temperature measured within each mouse (N=3) was: Mouse 1) pH=7.2 (± 0.2) and temperature=37.5 (± 1.1) °C (shown in Figure 8), Mouse 2) pH=7.3 (± 0.1) and temperature=36.9 (± 1.2) °C, and Mouse 3) pH=7.0 (± 0.2) and temperature=37.8 (± 0.5) °C.

The temperature and pH measurements were calculated using the first post-injection CEST spectrum for each mouse. The region exhibiting CEST effect was largest in the first post-injection spectra, with an average CEST effect of approximately 3%. The detected CEST region size decreased by more than half in the second CEST experiment and no CEST effect was detected in the third post-injection CEST experiment. The decreased CEST effect suggests that a large fraction of the agent had cleared the leg region within the imaging plane within the first hour following injection.
<table>
<thead>
<tr>
<th>Agent concentration</th>
<th>Aqueous solution</th>
<th>10% BSA solution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T₁ (ms)</td>
<td>T₂ (ms)</td>
</tr>
<tr>
<td>5 mM</td>
<td>1880 ± 10</td>
<td>38.0 ± 1.0</td>
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<td>10 mM</td>
<td>1180 ± 10</td>
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</tr>
<tr>
<td>20 mM</td>
<td>770 ± 10</td>
<td>10.0 ± 1.0</td>
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</tbody>
</table>

**Table 2.2:** Relaxation Rates of Aqueous and 10% BSA pH 7 Solutions of Different Concentrations of Tm³⁺-DOTAM-Gly-Lys (pH = 7.0, B₀ = 9.4 T, 37°C, Conc. = 5, 10, 15, 20 mM).
Figure 2.5: a) The dependence of MTR asym line shape on pH and b,c) the dependence of the MTR asym linewidth on pH of a 10 mM solution of Tm$^{3+}$-DOTAM-Gly-Lys at 37°C ($B_0 = 9.4$ T, $B_1 = 14$ µT, TS = 3 s, 37°C).

2.5 Discussion

A novel method is presented for in vivo extracellular pH measurement using only the amide proton site of PARACEST agent Tm$^{3+}$-DOTAM-Gly-Lys. Specifically, the linewidth of the CEST effect of the amide protons was uniquely related to pH. High-resolution in vitro pH maps were achieved with accuracy within 0.2 pH units of the expected value and with an inter-pixel standard deviation of less than 0.2 pH units in the range of pH 6 - 8 at a constant physiological temperature. In vitro temperature maps
were also achieved with accuracy within 0.3 °C of the expected value with an inter-pixel standard deviation of less than 0.3 °C. Following direct injection into the left mouse leg muscle, extracellular pH and temperature maps were produced in vivo.

![Figure 2.6: pH maps produced in phantom containing five tubes of solution of 10 mM Tm³⁺-DOTAM-Gly-Lys with different pH at 37°C (pH = 6.0, 6.5, 7.0, 7.5, 8.0, B₀ = 9.4 T, B₁ = 14 μT, TS = 3 s, 37°C). The pH maps calculated using the CEST peak linewidth agree well with the actual pH measurements (white labels).](image)

The use of the linewidth of the CEST effect represents a novel approach to pH measurement. The linewidth of the CEST effect is modulated by the proton exchange rate, which in turn depends on the sample temperature and pH. The current study demonstrated that the linewidth of the CEST effect for Tm³⁺-DOTAM-Gly-Lys was relatively constant around body temperature (36-38 °C). Linewidth was markedly more sensitive to temperature for pH 7.5 and pH 8.0 phantoms for temperatures above 37 °C. This temperature dependence can be predicted based on temperature-dependent increases
in the base-catalyzed exchange rate [5] and increased sensitivity as pH increases (equation [2.10]). Most clinical applications, including stroke and tumors, require measurement of extracellular pH between 6.0-7.5. Thus, our data suggests that temperature has negligible effects on exchange rate within the temperature range likely to be encountered \textit{in vivo} (36-38 °C) without deliberate tissue heating or cooling. The insensitivity of linewidth to temperature is important for measuring pH under physiological conditions where temperature may be altered.

![Temperature maps](image)

**Figure 2.7:** Temperature maps produced in phantom containing five tubes of solution of 10 mM Tm\(^{3+}\)-DOTAM-Gly-Lys with different pH at 37°C (pH = 6.0, 6.5, 7.0, 7.5, 8.0, \(B_0 = 9.4\) T, \(B_1 = 14\) μT, TS = 3 s, 37°C). The temperature maps calculated using the CEST peak linewidth and chemical shift agree well with the actual temperature measurements (white labels).

Additionally, the linewidth was insensitive to agent concentration in both aqueous solution and when mixed with 10% BSA, although the endogenous MT effect in the 10% BSA did reduce measured linewidths slightly. This result is expected as previous work has demonstrated the MTR\(_{\text{asym}}\) curve lineshape changes in the presence of MT effects.
However, within a constant medium, such as brain tissue, only subtle changes in MT effect are expected and would cause negligible effects on linewidth measurements. The differences in linewidth observed between aqueous and 10% BSA solutions are not expected within tissue regions in vivo. Different tissues may require separate calibration of linewidth and pH to account for differences in endogenous MT effects. Ratiometric methods proposed by Ward et. al. are also independent of concentration. However, the application of ratiometric methods to CEST, as proposed by Aime et. al. requires two experiments with different saturation powers and must account for endogenous MT effects under both conditions [34, 49, 50].

![Figure 2.8:](image)

**Figure 2.8:** a) Average preinjection and postinjection CEST spectra, MTR_{asym} curves on the x-axis. All curves and maps were generated using only pixels that exhibited significant PARACEST contrast (probability ≥ 95%) following injection. b) In vivo pH map superimposed onto a preinjection anatomical image. c) In vivo temperature map superimposed onto a preinjection anatomical image. The temperature and pH maps calculated in vivo agree well with normal temperature and pH measurements.

The linewidth of the CEST effect was dominated by the local pH and consequently, the linewidth of the CEST effect of the amide protons of Tm^{3+}-DOTAM-Gly-Lys was effective for pH mapping. The benefit of this method is that prior knowledge of in vivo tissue temperature or agent concentration is not necessary. The insensitivity of linewidth to temperature and concentration also means that the calibration of linewidth to pH established in vitro is directly applicable in vivo. The measured linewidth could be influenced by B_0-inhomogeneities. The current study fit a high-order polynomial to the
water peak to measure $B_0$ and correct CEST spectra on a pixel-by-pixel basis. However, this technique is susceptible to error for \textit{in vivo} experiments using large $B_1$ amplitudes due to the inherent asymmetry of the endogenous MT effect. Future \textit{in vivo} studies should employ the water saturation shift referencing (WASSR) technique to accurately measure $B_0$ in each pixel [51]. The modest sensitivity of linewidth to $\omega_1$ is another important consideration when using linewidth to measure the pH. Typically, $\omega_1$-inhomogeneities are on the order of 5-20\% \textit{in vivo} [52-54]. However, this sensitivity is not a significant limitation as \textit{in vivo} $\omega_1$ mapping is easily achieved [52-55] and must be used in combination with the linewidth response to $\omega_1$ (equation [2.12]) to correct pH maps. The greatest limitation of this method is that a significant portion of the CEST spectrum must be acquired to create an accurate asymmetry curve, which increases acquisition time. Assuming local $B_0$ variations of $\sim$1000 Hz, acquisition of approximately 50 saturation offsets points are required. Since numerous images must be acquired consecutively, the power and duration of the presaturation pulse must be minimized to avoid directly heating the sample. Such saturation pulse limits may make it difficult to achieve steady state conditions and detect the CEST contrast. In the current study, \textit{in vitro} experiments were preceded with a 3 second, 14 \mu T presaturation pulse to ensure that the results could be applied \textit{in vivo}. This saturation pulse nearly achieved a steady state condition resulting in the measurement of linewidth effects that agreed well with the theoretical model presented.

Within physiological ranges, the measurement of pH described in the current study using the MTR$_{\text{asym}}$ linewidth was independent of temperature. An alternative method proposed by Wegh \textit{et. al.} was independent of agent concentration, but sensitive to temperature fluctuations [27]. Specifically, the ratio of the CEST effect at the resonance frequency of the amide protons to the CEST effect at a frequency several ppm closer to the solvent water frequency was pH sensitive. This method does not require two different saturation experiments but is sensitive to temperature because of the dependence of amide proton chemical shift on temperature. The ratio of the CEST effects acquired at these two frequencies will also be different \textit{in vivo} due to the endogenous MT effect. So it is also difficult to use this method directly \textit{in vivo} even if the temperature is known. All
previous methods that use the CEST sensitivity of the PARACEST agent amide protons to measure pH must be carefully calibrated to ensure that the relationship established in \textit{vitro} between the pH and CEST effect is applicable in \textit{vivo}.

Noninvasive methods for temperature measurement using MRI have been developed recently [33, 56, 57]. The most common methods are based on the temperature dependence of the $T_1$ relaxation time, chemical shift, or diffusion coefficient of bulk water protons. However, the low temperature sensitivity (~ 0.01 ppm/$^{\circ}$C) of such methods limits their application to situations where a large temperature variation occurs. Previous studies have proposed MRI thermometry methods based on the measurement of the bound water chemical shift of PARACEST agents [16, 17]. Such methods provide the possibility for greater temperature sensitivity (~0.5 ppm/$^{\circ}$C or higher). Like the bound water associated with PARACEST agents, the chemical shift of amide protons within PARACEST agents is also temperature dependent and can be used for temperature measurement with similar temperature sensitivity. In the current study, it was shown that the amide proton chemical shift of Tm$^{3+}$-DOTAM-Gly-Lys could be used to accurately measure temperature in the pH range of 6.0-8.0, using a pH-calibrated linear relation between chemical shift and temperature.

The current approach improves on previous pH mapping methods using PARACEST agents by utilizing the linewidth of the CEST effect, which was shown to be insensitive to agent concentration and temperature (36-38 $^{\circ}$C). Using this approach pH and temperature measurement was achieved in the leg of a mouse around the site of intramuscular injection of Tm$^{3+}$-DOTAM-Gly-Lys. The average measured pH (7.2 ± 0.2 pH units) and temperature (37.4 ± 0.5 $^{\circ}$C) were both consistent with expected physiological values. Uncertainties of the empirical constants derived for equations [2.13-2.16] are expected to contribute some small error in measured pH and temperature maps.

The linewidth of the bulk water peak was narrower in post-injection CEST spectra and the MT effect was lower, compared to pre-injection spectra. Several factors may contribute to these changes including a decrease in the $T_1$ relaxation time constant of the
bulk water and/or bound protons, and an increase in local tissue water content following injection. Future work will investigate the role of these mechanisms in defining the shape of the CEST spectrum post injection. However, the linewidth measurements made in BSA phantoms with different agent concentrations (Figure 2.4c) indicate that both water content and $T_1$ relaxation have minimal impact on the linewidth of the CEST peak, which is required for pH mapping.

More in vivo work is needed to determine which tissues are amenable to pH mapping using this approach. However, the current study demonstrates in vivo pH and temperature mapping independent of agent concentration. This technique will likely be applicable to novel agents developed in the future with greater amide proton chemical shifts that reduce the loss in sensitivity from the endogenous MT effect.

2.6 Conclusion

A novel approach was developed to measure in vivo extracellular pH using the linewidth of the amide proton CEST effect of the PARACEST agent Tm$^{3+}$-DOTAM-Gly-Lys. Linewidth was found to be insensitive to agent concentration and changes in temperature between 36-38 °C. The amide proton chemical shift could also be used to measure temperature. High-resolution in vitro pH and temperature maps were successfully produced as well as in vivo maps of the mouse leg muscle. This work demonstrates the possibility for simultaneous measurement of in vivo temperature and pH using PARACEST agents.

2.7 Acknowledgements

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2.8 References


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Quantitative tissue pH measurement during cerebral ischemia using amine and amide concentration-independent detection (AACID) with MRI

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Abstract: Tissue pH is an indicator of altered cellular metabolism in diseases including stroke and cancer. Ischemic tissue often becomes acidic due to increased anaerobic respiration leading to irreversible cellular damage. Chemical exchange saturation transfer (CEST) effects can be used to generate pH-weighted MRI contrast, which has been used to delineate the ischemic penumbra after ischemic stroke. In the current study, a novel magnetic resonance imaging (MRI) ratiometric technique is presented to measure absolute pH using the ratio of CEST-mediated contrast from amine and amide protons: amine/amide concentration independent detection (AACID). CEST effects were observed at 2.75 ppm for amine protons and at 3.50 ppm for amide protons downfield (ie. higher frequency) from bulk water. Using numerical simulations and in vitro MRI experiments, we showed that pH measured using AACID was independent of tissue relaxation time constants, macromolecular magnetization transfer effects, protein concentration and temperature within the physiological range. Following in vivo pH-calibration using $^{31}$P-MRS, local acidosis is detected in mouse brain following focal permanent middle cerebral artery occlusion. In summary, our results suggest that AACID represents a noninvasive method to directly measure the spatial distribution of absolute pH in vivo using CEST MRI.
3.1 Introduction

Cerebral tissue pH is normally regulated at ~7.0-7.2 pH units by the cooperative action of a multitude of sensors and regulators within cellular compartments.[1] Deviation from pH-homeostasis is an indicator of disease and occurs in stroke when metabolic demand for oxygen exceeds supply. Lactic acidosis leads to irreversible cellular damage in ischemic tissue.[2] Therefore, pH is a potentially important and sensitive fundamental measure of metabolic state and disease progression. However, few methods measure absolute pH in vivo.[3] Even noninvasive magnetic resonance imaging (MRI) methods, which have had the greatest success to date, either suffer from low spatial and temporal resolution[4] or require assumptions that limit their clinical application.[5]

Several MRI observable parameters are sensitive to in vivo pH.[3] For example, the chemical shift between inorganic phosphate (P_i) and phosphocreatine (PCr) observed with phosphorus (31P) magnetic resonance spectroscopy (31P-MRS) is directly related to pH.[4] However 31P-MRS is limited by low signal to noise ratio resulting in low spatial and temporal resolution. Recently, Zhou et al. proposed a novel technique called amide proton transfer (APT) to measure pH using the chemical exchange saturation transfer (CEST) effects from amide protons associated with mobile peptides and proteins.[5] Amide protons freely exchange with bulk water protons at a rate that is pH-dependent. However, APT contrast is also affected by cellular water content, amide content, the bulk water spin-lattice (T_1) relaxation time constant, and macromolecular exchange-relayed nuclear Overhauser effects (NOEs).[5-7] Therefore, APT pH measurement is susceptible to error in conditions that cause appreciable changes in protein concentration and/or water content. Consequently, groups have successfully employed APT contrast to acquire pH-weighted MR images that correlate well with lactic acidosis.[8, 9] CEST effects derived from various metabolites including glutamate [10] and creatine [11] are also sensitive to pH however quantitative pH measurement using these CEST effects is limited by concurrent concentration and T_1 dependence.

To overcome limitations associated with concentration dependence, Ward and Balaban developed a ratiometric approach that was applied to exogenous compounds using the CEST effects at two distinct frequencies to measure quantitative pH independent of bulk
water $T_1$ and exchange site concentration.[12] Desmond et al. recently proposed a similar ratiometric approach to measure pH using endogenous CEST effects from amide (3.5 ppm) and amine (2.0 ppm) protons.[13] Here, we describe a novel method to measure absolute pH using the CEST effects of both amine (2.75 ppm) and amide (3.50 ppm) protons associated with endogenous mobile peptides and proteins: amine/amide concentration independent detection (AACID). AACID produces quantitative pH maps with markedly higher spatial resolution compared to $^{31}$P-MRS, and eliminates the assumptions of constant water content and protein concentration that limit APT. AACID is also independent of NOE and requires a simple pH-calibration step for in vivo application.

Amine and amide proton CEST effects are often considered to originate from mobile intracellular proteins and peptides.[5, 13-15] Recently, pH changes measured using endogenous amide CEST effects were considered to be of intracellular origin[5] because nearly 90% of total protein content exists in the intracellular space.[16] However, it is more accurate to consider the pH to be an average cerebral tissue pH that is heavily weighted to the intracellular compartment. Regardless, the pH resolution achieved using CEST effects (~0.2 pH units)[5] does not appear to be sufficient to distinguish the physiological differences between intracellular and extracellular spaces (~0.1 pH unit). Therefore, the measured pH is described as the cerebral tissue pH.

In the current study, numerical simulations were used to investigate the dependence of AACID measurements on pH, macromolecular magnetization transfer (MT), direct saturation and relaxation times. In addition, the pH, protein concentration and temperature dependencies of the AACID method were characterized in vitro. The AACID pH measurement was then calibrated in mouse brain using $^{31}$P-MRS pH as the standard measurement. Finally, high-resolution in vivo pH maps were acquired in the mouse brain following permanent middle cerebral artery occlusion (MCAO) and compared to cellular damage identified histologically. The assumptions required for absolute pH measurement using AACID, the impact of CEST effects from other metabolites on AACID, and potential applications of AACID at lower magnetic field strengths are discussed.
3.2 Methods and materials

3.2.1 CEST theory

CEST MRI contrast can be detected following the selective saturation of solute protons that exchange with bulk water protons. Solute protons resonate at specific frequencies that are chemically shifted ($\Delta \omega$) from bulk water. Figure 3.1a illustrates the distribution of different solute proton pools based on their resonant frequencies relative to bulk water. To observe CEST contrast, the chemical shift ($\Delta \omega$) of the exchanging solute protons must satisfy the slow-intermediate exchange condition ($\Delta \omega > k$), where $k$ is the exchange rate between solute and bulk water.[17] During frequency-selective saturation of the solute protons, a net transfer of irradiated high energy protons to the bulk water pool ensues by chemical exchange producing a reduction in the net magnetization of the bulk water leading to negative contrast in MRI. Figure 3.1b demonstrates the CEST effects on the bulk water following frequency selective RF saturation of the amide protons (3.50 ppm), and the amine protons (2.75 ppm). During saturation, longitudinal ($T_1$) relaxation simultaneously drives both pools toward their equilibrium population (Boltzmann) distribution leading to a steady state condition.[17] Following a long saturation pulse of frequency $\omega$, the bulk water steady state magnetization ($M_{ss}^w(\omega)$) is described by equation [3.1], where $M_0^w$ is the bulk water magnetization without saturation preparation, $k$ is the solute proton exchange rate, and $f$ is the mole fraction of solute protons as described by equation [3.2].[12, 17] A plot of $M_{ss}^w(\omega)/M_0^w$ as a function of the saturation pulse frequency is called a CEST spectrum (Figure 3.1c). CEST effects are often quantified using the magnetization transfer ratio (MTR) defined by equation [3.3].

$$M_{ss}^w(\omega)/M_0^w = 1/(1 + kfT_{1w})$$ \[3.1\]

$$f = \frac{\text{solute protons}}{\text{water protons}}$$ \[3.2\]

$$\text{CEST}(\omega) = 1 - M_{ss}^w(\omega)/M_0^w$$ \[3.3\]
The chemical exchange rate of protons from the solute pool to bulk water is both temperature- and pH-dependent. Within the narrow physiological temperature range (35-39 °C), the chemical exchange rate is dominated by modulations in pH.[5, 18] The pH-dependence of the solute proton exchange rate is described by equation [3.4] where $k_b$ is the base-catalyzed exchange rate, and $pK_W$ is the ionization constant of water ($pK_W=15.4$ at 37 °C).[19, 20]

$$k = k_b 10^{pH-pK_W}$$  \[3.4\]

Amide and amine protons associated with peptides have been shown to have different $k_b$ and therefore have a differential response to pH changes.[5, 13, 14] As pH increases, amine ($k_{amine} = 10^{pH-4.2}$) and amide ($k_{amide} = 5.57\times10^{pH-6.4}$) base-catalyzed exchange rates increase. As expected from equations [3.1] and [3.4], saturation of the amide proton pool (ie. $\omega = 3.50$ ppm) causes $M_{ss}^W(\omega)/M_0^W$ to decrease as pH increases (Figure 3.1c) producing an increase in the amide CEST effect (equation [3.3]). Paradoxically, the amine proton CEST effects appears to decrease as pH increases (Figure 3.1c), apparently defying equations [3.1] and [3.3]. However, the apparent decrease in CEST effect occurs because the amine proton exchange rate increases into the fast-exchange regime ($k > \Delta \omega$) when pH increases above ~6.5 pH units. Since the amide and amine CEST effects change in opposite directions with increasing pH, the ratio of these two effects is expected to be sensitive to pH.
Figure 3.1: Illustration of chemical exchange saturation transfer (CEST) principles and the effect of pH on amine and amide CEST signal: a) Schematic diagram of relevant solute proton pools that resonate at frequencies chemically-shifted by parts per million (ppm) from bulk water. b) Irradiation of amide (top) or amine (bottom) protons using a frequency-selective saturation pulse. Chemical exchange transfers amide (top) or amine (bottom) magnetization to bulk water, resulting in indirect saturation of the bulk water ($M_{SS}$). c) CEST spectra (vertically offset for clarity) measured at different pH (6.0, 6.5 and 7.0). The red and green portions of the curve illustrate the pH dependent response of the amide and amine protons.

3.2.2 AACID pH measurement

AACID is based on the CEST MRI experiment. Direct measurement of pH using the
CEST effect amplitude of a single solute proton pool is difficult due to the concomitant effects of solute concentration, saturation pulse amplitude and duration, and $T_1$ relaxation. Ward and Balaban first proposed a ratiometric approach to measure pH using the CEST effects from two different exchange sites on one CEST agent.[12] Briefly, a single solute may have two different proton exchange sites (i.e. Site 1 and Site 2). Rearranging equation [3.1] combined with equation [3.2] for both sites results in equation [3.4].

$$\frac{(M_0 - M_s)_{Site\,1,2}}{M_s^{Site\,1,2}} = k^{Site\,1,2}_{W} \left( \frac{\text{solute protons}}{\text{water protons}} \right)^{Site\,1,2}$$

[3.4]

Taking the ratio of equation [3.4] for two different sites produces equation [3.5],[12] where $T_{1W}$ effects and solute concentration effects cancel leaving only a ratio of pH-dependent exchange rates.

$$\frac{M_s^{Site\,2}(M_0 - M_s)^{Site\,1}}{M_s^{Site\,1}(M_0 - M_s)^{Site\,2}} = \frac{k^{Site\,1}_{W}T_{1W} \left( \frac{\text{solute protons}}{\text{water protons}} \right)^{Site\,1}}{k^{Site\,2}_{W}T_{1W} \left( \frac{\text{solute protons}}{\text{water protons}} \right)^{Site\,2}}$$

[3.5]

In the present study, amine protons resonating at 2.75 ppm and amide protons resonating at 3.50 ppm on endogenous proteins were considered to be Site 1 and Site 2 respectively. The bulk water magnetization following saturation at 6.0 ppm was used as a common reference point for both amine and amide CEST measurements (equation [3.6]). This approach minimizes endogenous macromolecule MT related signal loss while avoiding contamination from NOE effects, which occur between -2.0 and -4.0 ppm.[6, 7, 21] The common reference point also reduces the number of CEST images required for pH measurement.

$$\text{AACID} = \frac{M_s(3.50 \text{ ppm}) \times (M_s(6.0 \text{ ppm}) - M_s(2.75 \text{ ppm}))}{M_s(2.75 \text{ ppm}) \times (M_s(6.0 \text{ ppm}) - M_s(3.50 \text{ ppm}))}$$

[3.6]
The reference point was chosen to be 6.0 ppm because there are no observed CEST or NOE effects at 6.0 ppm in tissue or BSA phantoms and the 6.0 ppm CEST image is unaffected by CEST effects at 3.5 ppm and 2.75 ppm. The use of a single reference point for both CEST effects is justified when using a ratiometric method since any significant signal modulations at the reference frequency (ie. due to T2 relaxation or MT) will cancel.

All CEST contrast measurement methods are sensitive to inhomogeneities in the main magnetic field (B0) because the CEST effects are measured at specific frequencies. Therefore, it is essential that B0 maps are measured and a B0-correction is applied. The effects of the amplitude of the applied magnetic field (B1) on CEST measurement are complex due to the B1-dependence of saturation efficiency, direct saturation, and MT. Hence, it is also important to measure and correct for B1-inhomogeneities using B1 calibration curves.

3.2.3 Numerical Simulations

The Bloch-McConnell equations [22] were used to numerically simulate CEST spectra based on a four-pool exchanging system (bulk water, macromolecular, amine, and amide protons) using specific saturation pulse parameters. First, to simulate the relationship between AACID values and pH, CEST spectra were generated for different pH using previously reported pH-dependent base-catalyzed exchange rates of the amide and amine protons.[5, 13, 19] Ratiometric AACID values, calculated using equation [3.6] adopted from Ward and Balaban,[12] were measured directly from simulated spectra. In addition, macromolecular concentration was varied (9, 10 and 11%) to model potential changes in MT following ischemia.[23] Finally, normal tissue (NT) and ischemic tissue (IT) were modeled by incorporating tissue specific relaxation time constants and exchange rates for each pool (Table 3.1). AACID-pH relationships were simulated for both tissue types to study the cumulative effects of differences in water content, relaxation times and consequent changes in direct saturation.
Table 3.1: Simulation parameters used to simulate CEST spectra for normal (NT) and ischemic (IT) cerebral tissue.

### Simulation Parameters

<table>
<thead>
<tr>
<th>Proton Pools</th>
<th>Bulk water</th>
<th>Macromolecule</th>
<th>Amine</th>
<th>Amide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical Shift (ppm)</td>
<td>0</td>
<td>0</td>
<td>2.75</td>
<td>3.50</td>
</tr>
<tr>
<td>$T_1$ (s)</td>
<td>NT: 1.844$^a$</td>
<td>0.1$^b$</td>
<td>1.0$^c$</td>
<td>1.0$^c$</td>
</tr>
<tr>
<td></td>
<td>IT: 1.992$^a$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$T_2$ (s)</td>
<td>NT: 0.045$^a$</td>
<td>0.000015$^b$</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>IT: 0.054$^a$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concentration</td>
<td>NT: 39.1 M$^d$</td>
<td>0, 9, 10, 11 %-by-weight</td>
<td>10.0 mM</td>
<td>71.9 mM</td>
</tr>
<tr>
<td></td>
<td>IT: 51.8 M$^d$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exchange rate (s$^{-1}$)</td>
<td>-</td>
<td>50$^b$</td>
<td>$10^{pH-4.2e}$</td>
<td>$5.57x10^{pH-6.4f}$</td>
</tr>
</tbody>
</table>

$^a$ From ref. [24], $^b$ From ref. [22], $^c$ From ref. [10], $^d$ From ref. [25], $^e$ estimated from refs. [19] [13], and $^f$ From ref. [5].

3.2.4 In vitro solutions

To study the AACID pH measurement, a series of solutions containing 10% (by weight) bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, Missouri) dissolved in 600 µL of phosphate buffered saline (PBS) were produced with pH ranging from 6 to 8 pH units. Solution pH was adjusted using 1M HCl and 1M NaOH and confirmed with a standard pH electrode (Cole-Parmer, Montreal, Canada). To study the effect of protein concentration on AACID, a series of pH 7 solutions (600 µL) were produced with 6, 8, 10, 12 and 14% BSA. All phantoms were prepared in 5 mm NMR tubes. Physiological total protein content has been previously shown to be approximately 10% by weight in both rodent and human brains.[26] To measure potential contributions of CEST contrast from common brain metabolites, we prepared phantoms with each metabolite dissolved into pH 7.0 phosphate buffered saline (PBS) solutions at physiological concentrations (Sigma-Aldrich). We used the identical metabolites and concentrations as Cai et. al. to allow direct comparisons (Table 3.2).[10]
<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatine (Cr)</td>
<td>10</td>
</tr>
<tr>
<td>Myo-inositol (MI)</td>
<td>10</td>
</tr>
<tr>
<td>N-acetyl aspartate (NAA)</td>
<td>10</td>
</tr>
<tr>
<td>Glutamine (Gln)</td>
<td>2</td>
</tr>
<tr>
<td>Taurine (Tau)</td>
<td>2</td>
</tr>
<tr>
<td>Aspartate (Asp)</td>
<td>2</td>
</tr>
<tr>
<td>Glutamate (Glu)</td>
<td>10</td>
</tr>
<tr>
<td>γ-aminobutyric acid (GABA)</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 3.2: Metabolite concentrations used for *in vitro* measurement of potential contaminant CEST effects.

### 3.2.5 MR experiments

All phantom and animal MRI studies were performed in an Agilent 9.4T small animal MRI scanner (Agilent, Palo Alto, California). The bore of the magnet is 31 cm and the maximum gradient strength is 400 mT/m. A long 4s continuous wave radio frequency (RF) saturation pulse with a $B_1$ amplitude of 1.5 $\mu$T was introduced prior to a RF spoiled two-dimensional fast spin echo (FSE) pulse sequence for all phantom and animal CEST MRI studies.

#### 3.2.5.1 *In vitro* magnetic resonance imaging

All solutions were scanned using an Agilent 30 mm millipede volume coil (Agilent). All CEST images were acquired using the modified FSE pulse sequence (slice thickness = 4 mm, TR = 7000 ms, TE = 7 ms, ETL = 32, effective TE = 7 ms, 3 averages, 2 pre-scans, field of view = 25.6 x 25.6 mm$^2$, matrix size = 64 x 64, and a 4s long continuous wave RF saturation pulse with a $B_1$ amplitude of 1.5 $\mu$T). Total image acquisition time was 22s. CEST images were acquired with preceding saturation pulses at different frequencies. The saturation pulse frequencies, expressed in ppm relative to water, include -1000 ppm, -6.4 to -5.6 ppm (in 0.1 ppm steps), -3.9 to -1.6 ppm (in 0.1 ppm steps), -0.4 to 0.4 ppm (in 0.1 ppm steps), 1.6 to 3.9 ppm (in 0.1 ppm steps), 5.6 to 6.4 ppm (in 0.1 ppm steps), and 1000 ppm.
3.2.5.2 General animal procedures

All animal protocols conform to the guidelines established by the Canadian Council on Animal Care and were approved by The University of Western Ontario Animal Use Subcommittee. For in vivo studies, C57BL/6 male mice (3-5 months old, 24-28g) were used. Anesthesia was induced using 4% isoflurane in oxygen and maintained with 1.5%–2.5% isoflurane in oxygen. The mouse was secured on a custom built MRI-compatible stage. Temperature was monitored with a rectal temperature probe, and respiration was monitored with a respiratory sensor pad connected to a pressure transducer that was placed on the thoracic region. Body temperature was maintained at 37-37.5°C throughout imaging by blowing warm air over the animal using a model 1025 small-animal monitoring and gating system (SA Instruments Inc., Stony Brook, New York). Three animals were used for in vivo pH calibration and five different animals were used for MCAO demonstration. Two of the five animals used for MCAO demonstration were used for histology. Four different animals were used to measure the percent reduction in cerebral blood flow (CBF) obtained by permanent MCAO using laser Doppler flowmetry.[27]

3.2.5.3 In vivo proton magnetic resonance imaging animal procedures

All 1H MRI scans were performed with a custom-built 3.0-cm-diameter RF surface coil. CEST images were acquired using the modified FSE pulse sequence (slice thickness = 2 mm, TR = 7000 ms, TE = 7 ms, ETL = 32, effective TE = 7 ms, 3 averages, 2 pre-scans, field of view = 25.6 x 25.6 mm², matrix size = 64 x 64, and a 4s long continuous wave RF saturation pulse with a B₁ amplitude of 1.5 μT). Total image acquisition time was 22s.

CEST images were acquired with preceding saturation pulses at different frequencies. The saturation pulse frequencies, expressed in ppm relative to water, include -1000 ppm, -0.4 to 0.4 ppm (in 0.1 ppm steps), 1.6 to 4.2 ppm (in 0.1 ppm steps), 5.6 to 6.4 ppm (in 0.1 ppm steps), and 1000 ppm. For the pH calibration procedure, a larger slice thickness of 6 mm was used to measure whole brain average pHᵢ and match the 31P MRS field of
view. $T_1$-weighted images were acquired using a FSE pulse sequence (slice thickness = 2 mm, TR = 500 ms, TE = 10 ms, ETL = 4, effective TE = 10 ms, 4 averages, 2 pre-scans, field of view = 25.6 x 25.6 mm$^2$, matrix size = 128 x 128, total image acquisition time was 1 min 5s). $T_2$-weighted images were acquired using a FSE pulse sequence (slice thickness = 2 mm, TR = 3000 ms, TE = 10 ms, ETL = 4, effective TE = 40 ms, 1 average, 2 pre-scans, field of view = 25.6 x 25.6 mm$^2$, matrix size = 128 x 128, total image acquisition time was 1 min 42s). Diffusion-weighted images were acquired using a multislice spin-echo pulse sequence (slice thickness = 2 mm, TR = 1000 ms, TE = 35.49 ms, b-value = 1100 s/mm$^2$ in the readout direction, 4 averages, field of view = 25.6 x 25.6 mm$^2$, matrix size = 128 x 128, total image acquisition time was 8 min 32s).

3.2.5.4 In vivo phosphorus magnetic resonance spectroscopy

Similar to endogenous CEST pH measurement, $^{31}$P-MRS pH measurement mainly reflects intracellular pH because >75% of inorganic phosphate is intracellular.[28] Therefore, $^{31}$P-MRS provides an appropriate standard for pH-calibration of AACID measurements in vivo. All $^{31}$P-MRS data were acquired with a custom-built 2.5 cm diameter dual-tuned ($^{31}$P/$^1$H) RF surface coil. The 6 mm thick slices used for $^1$H CEST images contained a large portion of the brain. Following the acquisition of $^1$H CEST images in vivo, the $^1$H surface coil was replaced with a $^{31}$P/$^1$H dual-tuned surface coil without changing the animal’s position. Using the dual-tuned coil, anatomical $^1$H MRI scout images were acquired to confirm that the animal position was unchanged and to choose a 5 x 10 x 6 mm$^3$ voxel location. The $^{31}$P voxel location was chosen such that the 6mm thick voxel was centered on the 6mm thick $^1$H CEST slice in the z-direction. Furthermore, the 5 x 10 x 6 mm$^3$ voxel was placed inside the brain to produce negligible skull and skin contributions. After switching to the $^{31}$P frequency, the $^{31}$P MRS spectrum was acquired. Localized brain $^{31}$P spectra were acquired using image selected in vivo spectroscopy (ISIS, Excitation = 100 µs hard pulse, TR = 5s, spectral width = 12 kHz, 768 averages, 4 pre-scans, 512 complex points, field of view = 5 x 10 x 6 mm$^3$ - corresponding to 6 mm thick field of view used for $^1$H CEST images).[29] All $^{31}$P spectra were fitted using in-house software (fitMAN[30, 31]) to measure the chemical shift.
difference ($\delta$) between inorganic phosphate and phosphocreatine. pH$_i$ measurements were made using $\text{pH}_i = 6.75 + \log[(\delta-3.26)/(5.70-\delta)]$.[4, 32]

### 3.2.5.5 $B_0$ and $B_1$ corrections to CEST spectra

$B_0$ and $B_1$ field maps were generated for the image slice of interest prior to all CEST experiments. CEST data were frequency shifted to account for $B_0$ variations using the water saturation shift referencing (WASSR) technique.[33] Specifically, a $B_0$ field map was generated by acquiring a CEST spectrum using a FSE sequence (slice thickness = 2 mm, TR = 7000 ms, TE = 7 ms, ETL = 32, effective TE = 7 ms, 3 averages, 2 pre-scans, field of view = 25.6 x 25.6 mm$^2$, matrix size = 64 x 64, and a 100 ms long continuous wave RF saturation pulse with a $B_1$ amplitude of 0.2 $\mu$T, saturation frequencies = -0.5 to 0.5 ppm in 33 steps). The 33 WASSR points were interpolated to 401 points (1 Hz resolution) and fit to a 10$^{th}$ order polynomial. A $B_1$ field map was created using an actual flip-angle imaging (AFI) pulse sequence (TR = 20 ms, TE = 3.47 ms, # echoes = 2, flip-angle = 70$^\circ$, 1 average, field of view = 25.6 x 25.6 mm$^2$, matrix size = 64 x 64, total image acquisition time was 2 min 13 s).[34] For each pixel, $B_1$ was calculated using $B_1 = B_{1\text{ref}} \times \text{(actual flip-angle/70$^\circ$)}$ incorporating the flip angle map and $B_{1\text{ref}} = 1.5$ $\mu$T. Both $B_1$ and $B_0$ corrections were applied to all CEST images.[35]

### 3.2.5.6 CEST data processing

All acquired CEST MR data were loaded into MATLAB (version 7.5, R2007b) for analysis. Using custom MATLAB programs, $B_0$ and $B_1$ corrections were performed on a pixel-by-pixel basis and all CEST spectra were smoothed using the ‘smooth’ algorithm from the MATLAB curve fitting toolbox.

### 3.2.6 Middle cerebral artery occlusion

Focal cerebral ischemia was induced in 24-28g, C57BL/6 mice by middle cerebral artery occlusion (MCAO) using a filament technique as previously described.[36] Briefly, anesthesia was induced by inhalation of 4% isoflurane in a 100% O$_2$ mixture and maintained using 1.5% isoflurane. Under an operating microscope, the left common carotid was exposed through a midline neck incision. Then, a silicone rubber-coated
monofilament (701956PK; Doccol Company, Redlands, California) was inserted through the left common carotid artery into the internal carotid artery to occlude the middle cerebral artery (MCA). The monofilament remained in the MCA until sacrifice (permanent occlusion). Body temperature was maintained at 37±0.5°C by using a heating pad. MCAO induced ischemia was confirmed based on the T1, T2 and diffusion-weighted MR images following surgery, as well as histology. The percent reduction in cerebral blood flow (CBF) obtained by permanent MCAO was measured using laser Doppler flowmetry[37] in four different 24-28g, C57BL/6 mice.

3.2.7 Histology

Animals (N=2) were removed from the MRI scanner and sacrificed immediately after completion of MRI imaging. Animal brains were dissected and stained within 5 minutes after sacrifice. After decapitation, the brain was quickly removed and sectioned into 2 mm slices. The slices were immersed into 37 °C solutions of 2% TTC dissolved in normal saline.[27] After approximately 30 minutes, all brain slices were fixed in 10% phosphate-buffered formalin for photography. Brain slices corresponding to CEST imaging slices were identified using multi-slice scout MR images as a reference.

3.2.8 Statistical Analysis

Cerebral tissue pH measurements were made from pathological and contralateral regions within each mouse brain and expressed as mean ± s.d. Differences in mean pH between stroke and contralateral brain regions (n=5 mice) were tested using the Student’s t-test with p < 0.05 considered to be significant.

3.3 Results

3.3.1 Numerical Simulations

Bloch-McConnell simulations demonstrated that as pH increases within the physiological pH range (ie. pH = 6.0 to 8.0 pH units), the CEST contrast associated with amine protons and amide protons changes in opposite directions (Figure 3.2a-c). These changes are most easily observed in Figure 3.2c, where amide and amine CEST effects, as defined by equation [3.3] are displayed with varying pH. Specifically, as pH increases from 6.0 to
8.0, the CEST effect increases for the amide protons at 3.50 ppm. Conversely, the CEST effect associated with amine protons at 2.75 ppm decreases from pH 6.5 to ~7.5. The resulting AACID values decrease linearly as pH increases (Figure 3.2d). Numerical simulations also demonstrate that pH-dependent exchange rate effects dominate AACID measurements in brain tissue when considering expected variations in macromolecule concentration, water content and relaxation times in tissue following ischemia. First, varying macromolecular concentrations (9, 10 and 11 %) modeled potential changes in MT following ischemia[23] (Figure 3.2e) and confirmed negligible effects on the simulated AACID-pH relationship. Second, the AACID-pH relationship was simulated for both normal and ischemic brain tissue to study the cumulative effects of differences in water content, relaxation, and consequent changes in direct saturation. Known changes in water content, and T₁ and T₂ relaxation [24] also produced negligible changes in AACID measurement (Figure 3.2f).

### 3.3.1.1 In vitro studies

Solutions with varying pH were produced containing different concentrations of bovine serum albumin (BSA) dissolved in phosphate buffered saline (PBS). Although these solutions did not replicate all in vivo conditions (e.g. T₁ and T₂ relaxation time constants, and macromolecule MT), they provided a physical model of an exchanging system containing both amide and amine protons similar to that found in vivo. These solutions demonstrated the effects of changing pH, protein concentration, and temperature on CEST, and consequently AACID measurements. CEST spectra of 10% BSA dissolved in PBS solutions with varying pH demonstrate three distinct CEST effects (Figure 3.3a,b). Amide proton CEST effects are centered at ~3.50 ppm and two different amine proton CEST effects are centered at ~2.0 and ~2.75 ppm. Incrementally increasing solution pH from 6.0 to 8.0 caused the amide CEST effect to increase and the amine CEST effect at ~2.75 ppm to decrease. The amide and amine CEST effects at ~2.75 ppm are plotted separately with varying solution pH in Figure 3.3c. Ratiometric AACID values decreased linearly when increasing pH from 6.5 to 8.5 (Figure 3.3d) as predicted by numerical simulations. AACID measurements were nearly identical for pH 7.0 phantoms.
containing 6, 8, 10, 12 and 14% BSA (Figure 3.3e). AACID pH measurements obtained in a pH 7.0 10% BSA phantom were unaltered between 35-39 °C (Figure 3.3f).

Amine and amide CEST effects are also dependent on saturation pulse amplitude (B₁) and duration (t_{sat}) (Figure 3.4a,b). Similar to Cai et al., CEST effects were measured in vitro for eight metabolite solutions (Table 3.2) at 37 °C.[10] The CEST contrast produced by these brain metabolites was measured at 3.50 ppm and 2.75 ppm at physiological concentrations to determine the magnitude of contaminant contributions to AACID pH values (Figure 3.4c,d). Glutamate was the only metabolite to generate a significant contaminant CEST effect at 3.50 ppm (Glutamate CEST ≈ 2.3% compared to CEST from 10% BSA ≈ 9.2%) and 2.75 ppm (Glutamate CEST ≈ 3.0% compared to CEST from 10% BSA ≈ 19.4%). Note that the CEST effects from the amine protons associated with glutamate are optimal at ~1.0-3.0 ppm at physiological pH.[10] However, glutamate CEST contributions to AACID pH values are expected to be less than 5% due to ratiometric cancellation when using a 4s, 1.5 µT saturation pulse at 9.4T.
Figure 3.2: Numerical simulations of AACID Dependence on pH, Macromolecule Concentration, and Relaxation Time Constants in Brain: a) Simulated CEST-spectra for varying pH in normal brain tissue. b) Figure 3.1a magnified to highlight the amine and amide pH dependent CEST effects. Note: $M_z$ and $M_0$ represent the region of interest MRI signal acquired with and without a saturation pulse, respectively. c) Amine and amide CEST values measured directly from 2a using equation [3.3]. d) AACID values measured directly from 3.2a using equation [3.6]. e) AACID-pH relationships simulated for a range of macromolecular concentrations (% by-weight) using a 4s, 1.5 µT saturation pulse. f) AACID-pH relationships simulated for normal and ischemic brain tissue using a 4s, 1.5 µT saturation pulse. Numerical simulations predict that AACID values decrease approximately linearly with pH within the physiological pH and temperature range. Independence of physiological changes in MT and relaxation times during cerebral ischemia suggests that AACID can be used to calculate absolute pH in vivo.
3.3.2 In vivo AACID pH-calibration

Numerical simulations indicate that significant differences in macromolecular MT and proton exchange rates [5, 10, 22] between BSA solutions and brain tissue necessitated a separate in vivo AACID pH-calibration. $^{31}$P-MRS (Figure 3.5a) and CEST (Figure 3.5b)
spectra were acquired in vivo and postmortem in normal healthy mice (n=3). pH-calibration was achieved by fitting a linear model to mean whole brain in vivo and postmortem AACID values plotted against corresponding $^{31}$P-MRS pH measurements (Figure 3.5c). The calibration is valid assuming the $P_i$ and PCr metabolites used to measure pH by $^{31}$P-MRS exist in the same physical environment as the amide and amine protons used to produce the AACID value [5] and the pH-dependence of the AACID measurement is linear in vivo, as it was in both numerical simulations and in vitro. The resulting linear relation obtained by $^{31}$P-MRS calibration described in equation [3.7] was used to produce quantitative pH maps in vivo.

\[
pH = -4.0 \times \text{AACID} + 12.8
\]  

[3.7]

Figure 3.4: Amine and Amide CEST Dependence on Saturation Pulse Amplitude, Duration, and Metabolite Contributions: Amine and amide CEST effects from 10% (by weight) BSA dissolved in pH 7.0 PBS at 37 °C measured following a) a 4s continuous wave RF saturation pulse with amplitudes ranging from 0-3 $\mu$T, and b) a 1.5 $\mu$T continuous wave RF saturation pulse with duration ranging from 0-6s. CEST values measured at c) 2.75 ppm and d) 3.50 ppm for major brain metabolites at physiological concentrations (37 °C using a 4s, 1.5 $\mu$T continuous wave RF saturation pulse) compared to 10% BSA. Amide and amine CEST effects approach steady state after a 4s, 1.5 $\mu$T saturation pulse. Protein is shown to be the dominant contributor to AACID values compared to other brain metabolites, which suggests that AACID measurements are independent of changes in metabolite concentrations and can be used to calculate absolute pH in vivo.
Figure 3.5: AACID pH-Calibration *In vivo* using $^{31}$P-MRS: pH measurements and CEST spectra acquired using the standard $^{31}$P-MRS and $^1$H-MRI respectively *in vivo* and postmortem. a) *In vivo* (bottom) and postmortem (top) whole brain $^{31}$P-MRS spectra for one mouse. b) Corresponding CEST spectra. c) AACID and pH measurements for three different mice. *In vivo* AACID values decrease linearly with pH as predicted by numerical solutions and *in vitro* experiments. AACID pH-calibration data can now be used to calculate absolute pH *in vivo*.

### 3.3.3 Acute acidosis following MCAO

Following the pH-calibration of AACID measurements *in vivo*, high-resolution pH maps were produced in mice following permanent middle cerebral artery occlusion (MCAO). Several studies have reported local cellular acidosis after induction of ischemia.[5, 38, 39] Therefore, a MCAO mouse model[40] was used to establish the feasibility of *in vivo* pH measurement using AACID across the range of pH values encountered in ischemic conditions.

Following ischemia, anaerobic metabolism causes cellular acidosis.[5, 38, 41] Quantitative pH maps produced at two (Figure 3.6d) and five hours (Figure 3.6i) following permanent MCAO (n=5) showed a localized pH decrease that corresponded anatomically to changes observed in $T_1$-, $T_2$- and diffusion-weighted MR images (Figure 3.6a,b,c,f,g,h). Regions of interest (ROIs) containing the ischemic core and contralateral brain are shown in Figures 3.6d,i. Figure 3.6e shows the CEST spectrum from the contralateral brain ROI (blue) superimposed onto the spectrum from the ROI containing the ischemic core (red). At 3.50-3.70 ppm, the CEST spectra do no overlap, indicating a distinct difference in amide CEST effects (red arrow). Whereas, at 2.75 ppm, the CEST
spectra nearly overlap, indicating very little change in amine CEST effects (green arrow). This pattern of change was expected based on numerical and in vitro studies that also showed only small changes in amine CEST effect between pH 6.5-7.0 (Figures 3.2a,b and 3.3a,b). Regions of significant acidosis in pH maps were consistently larger 5 hours after surgery compared to 2 hours. For example, in the mouse shown in Figure 3.5, the region of significant acidosis (pH ≤ 6.6) within the imaging slice was approximately 15 mm$^3$ two hours after occlusion and increased to 29 mm$^3$ at five hours. At five hours post occlusion the pH values [mouse #: stroke mean ± s.d., contralateral tissue mean ± s.d.] calculated using the pH maps were: [mouse 1: 6.76 ± 0.19, 6.93 ± 0.14], [mouse 2: 6.57 ± 0.13, 6.90 ± 0.13], [mouse 3: 6.67 ± 0.22, 7.02 ± 0.13], [mouse 4: 6.64 ± 0.17, 7.04 ± 0.15], [mouse 5: 6.54 ± 0.16, 6.94 ± 0.16]. Ischemic and contralateral tissue pH mean values were calculated using all pixels in the entire ischemic and contralateral sides respectively. The average ischemic tissue pH (6.64 ± 0.09) was significantly lower (p<0.01 by t-test, n=5 mice) than the average contralateral tissue pH (6.97 ± 0.06). While pH maps were fairly consistent among different animals, specific quantitative measurements such as ischemic volumes and mean pH may vary with slice location. A series of molecular events initiated by cerebral ischemia lead to increased intracellular water content (i.e. edema) and consequently reduced water flow, leading to increased brain tissue water T$_1$ relaxation time constants.[24, 42] However, the numerical simulations and in vitro results from the current study, demonstrate that these physiological changes have negligible effect on the ratiometric AACID pH measurement. Following MR imaging animals were immediately sacrificed and histology was performed (N=2). Brain sections corresponding to the acquired pH maps were stained with 2,3,5-triphenyltetrazolium chloride (TTC) and demonstrated that regions of decreased pH corresponded to regions of low oxidative metabolism indicated by a lack of TTC staining (white, Figure 3.6j). The mean (± std. dev.) percent reduction in CBF measured with laser Doppler flowmetry [27] in healthy C57BL/6 mice was 87% ± 7% (N=4).
Figure 3.6: Cerebral Ischemia: Representative MR images of a mouse brain two hours (a-e) and five hours (f-j) following permanent middle cerebral artery occlusion (MCAO) in radiological orientation. a,f) T₁-weighted image, b,g) T₂-weighted image, c,h: diffusion-weighted image, d,i: pH map with ischemic (left) and contralateral (right) regions of interest (ROIs) drawn, and e) CEST-spectra measured from ischemic (red) and contralateral (blue) ROIs at 2 hours with CEST effects located at arrows (red arrow: amide CEST, green arrow: amine CEST), f) histology TTC-stained for mitochondrial cellular respiration (i.e. red stained tissue has functional mitochondrial and white unstained tissue does not).

3.4 Discussion

The current study demonstrates a novel method to noninvasively quantify in vivo pH using CEST-MRI contrast. The ratiometric approach called AACID is insensitive to variations in tissue T₁, T₂, macromolecule concentration, and temperature over the relevant physiological ranges following ischemia. Since the AACID measurement is a relative measure that compares two CEST effects, it is also insensitive to changes in the concentration of amine and amide protons relative to water protons that can be caused by changes in water content, protein content and diffusion.[12, 43] However, chemical shift corrections must be made to account for B₀ variation and CEST amplitude corrections must be made to account for B₁ variation on a pixel-by-pixel basis. Using AACID pH measurement, high-resolution maps of brain tissue pH were obtained in mice following permanent MCAO. Regions of significantly decreased pH at two and five hours after MCAO corresponded to regions of cellular damage.
Previous studies have shown that changes in pH can be measured by noninvasive MRI techniques.[5, 8, 9] Although the AACID technique avoids several sources of error associated with previous CEST-based methods to measure pH, three assumptions were required to measure quantitative pH using AACID. These assumptions were that: 1) the ratio of exchangeable amide and amine proton populations remain constant throughout the brain and during ischemic conditions, 2) the relation between AACID values and pH is linear in vivo, as it was found to be in numerical and in vitro studies, and 3) direct water saturation has a negligible effect on the AACID ratio. The validity of each assumption is discussed below.

First, the AACID technique assumes that the ratio of exchangeable amide to amine proton populations is constant throughout the brain, even in regions with different protein concentration. Consider that the measured CEST signals are from exchangeable amine and amide protons on mobile peptides and proteins, with the vast majority existing within the cell. There are 21 standard amino acids that constitute the structural units of peptides and proteins in eukaryotes,[44] along with many other less abundant amino acids that are formed by post-translation modifications. Of the 21 amino acids, only lysine has exchangeable amine protons on its side arm. Although each amino acid contains an amine functional group, when peptide bonds form between amino acids, the amine is transformed to an amide group. Therefore peptides and proteins may contain from one to hundreds of exchangeable amide protons, while only proteins with lysine-rich regions contain significant amounts of exchangeable amine groups. The average occurrence of lysine in proteins is ~6%.[44] In the current study, pH maps did not show any significant contrast between gray and white matter in mouse brain (Figure 3.6d) suggesting that the amine to amide ratio is the same in both gray and white matter. However, we cannot exclude the possibility that the ratio of amine and amide proton populations changes in disease, which would result in inaccurate pH measurements.

Accurate quantitative pH measurement also requires a linear relationship between AACID and pH in vivo. This assumption is reasonable considering a linear relationship was observed in both numerical simulations and in vitro. Numerical simulations provided a convenient theoretical model to investigate complex exchange systems.
Simulation of the AACID response to altered pH demonstrated a linear relationship under both aqueous (i.e. no endogenous macromolecule MT) and tissue (i.e. with endogenous macromolecule MT) conditions. However, the simulations also showed that endogenous macromolecule MT significantly shifted the AACID response to pH, necessitating a separate in vivo pH-calibration. Additional numerical simulations revealed that physiologically relevant regional changes in endogenous macromolecule MT effects (< 10%) do not significantly affect the linear AACID-pH relationship. AACID measurements were also insensitive to changes in water content, and variations in $T_1$ and $T_2$ relaxation, suggesting that direct saturation has negligible effects at 9.4T.

The CEST sensitivity of both the amide and amine protons depends on the chemical exchange rate. In order to detect CEST contrast, the exchange rate must be within the slow-to-intermediate exchange regime at 9.4T. At physiological pH and temperature, amide protons associated with endogenous protein have slow exchange rates of 10-300 s$^{-1}$ and APT contrast has been detected when using a low amplitude (~1.2 µT) saturation pulse.[5] Conversely, amine protons associated with free amino acids and metabolites have much faster exchange rates (500-10000 s$^{-1}$).[10, 19] However due to the significant CEST effects observed at 2.75 ppm from 10% BSA solutions (Figures 3.3a,b) using a 4s, 1.5 µT continuous wave RF saturation pulse, the authors suspect that amine protons associated with peptides and proteins are in the slow-to-intermediate exchange regime (e.g. 100-1500 s$^{-1}$) at pH ≤ 7.0.[17] In vitro results indicate that at 9.4T, amine proton exchange rates pass through the slow, intermediate and fast exchange conditions as pH increases from 6.0 to 8.0. Exchangeable amine protons associated with endogenous peptides and proteins resonating at ~2.75 ppm were also recently detected by spin-locking techniques using a 5s saturation pulse with amplitudes as low as ~2 µT.[45] Therefore, in the current experiment, a saturation pulse with a low amplitude (1.5 µT) and a long duration (4s) was used to selectively saturate the relatively slowly exchanging amide and amine protons and avoid contaminant CEST contrast from fast exchanging amine protons associated with common brain metabolites such as glutamate and creatine.[10, 11]
Since chemical exchange rate is sensitive to pH and temperature, the effects of temperature modulation of the system must also be considered. Zhou et al. recently showed an increase in amide proton exchange rate of ~1% per 1 °C compared to a ~76% increase in exchange rate following an increase in pH of only 0.4 pH units.[5] These results suggest that base-catalyzed exchange rate effects mediated by pH dominate temperature (Arrhenius) mediated exchange rate effects within the physiologically relevant range. Theoretical modeling of temperature effects is difficult,[5] therefore in vitro verification was performed in the current study (Figure 3.3e). Using in vitro protein solutions containing amine and amide protons, AACID values were measured at different temperatures (35, 36, 37, 38, 39 °C). As expected from Zhou et al., the amide and amine CEST effect is more sensitive to physiologically relevant changes in pH than to physiologically relevant changes in temperature and AACID values did not change significantly across the measured temperature range (Figure 3.3e). Within the range of temperature variation expected in cerebral ischemia,[46] amine and amide CEST effects were negligibly altered in comparison to the magnitude of the changes observed when pH was altered within the physiological range.

The use of CEST-MRI contrast to measure pH has evolved over the last several years. The first approach to measure CEST effect was to divide the NMR signal intensity following a saturation pulse by the signal intensity without saturation. However, this measurement is contaminated by endogenous macromolecule MT effects, direct water saturation, and blood oxygen level dependent (BOLD) effects.[5] Therefore Zhou et al. introduced the novel MTR_{asym} curve to maximize CEST sensitivity, while eliminating the mentioned interferences.[5] However when using MTR_{asym}, APT contrast is modulated by changes in tissue pH, as well as amide content, water content, T_1 relaxation time constants, and macromolecular NOEs; therefore, quantitative pH measurement using APT should be used cautiously.[5-7] AACID pH measurement avoids NOE contamination by normalizing the amide and amine signal intensities following saturation to the signal intensity at 6.0 ppm following saturation. Normalization to 6.0 ppm decreases CEST sensitivity at 2.75 and 3.50 ppm compared to MTR_{asym} methods because the endogenous macromolecule MT effect is not completely removed. However, normalizing both 2.75 and 3.50 ppm to 6.0 ppm allows the measurement of two different
CEST effects while acquiring only three images. The 6.0 ppm point is not influenced by amine or amide CEST effects, or NOE effects, and it provides a good indicator of local $T_1$ related signal loss, BOLD effects, and macromolecule MT for normalization. Similar results are possible when using any reference at $\Delta \omega > 6.0$ ppm, however CEST sensitivity decreases as $\Delta \omega$ increases away from the water resonance because contributions from macromolecule MT will begin to dominate measured AACID values. The current normalization at 6.0 ppm provided a linear AACID-pH relation that ensured a straightforward in vivo calibration.

While CEST effects have been detected at 3.50 ppm at clinical field strengths (1.5-3T), further work is required to assess the feasibility of measuring the 2.75 ppm amine CEST effect at these lower magnetic field strengths. The AACID method is expected to have lower sensitivity to pH at lower magnetic fields because the amine proton exchange likely approaches the fast exchange rate condition and because direct saturation of water will be increased. However, the method would remain independent of NOE and would require less saturation power than at higher magnetic field strengths. The AACID technique is immediately applicable in humans using current 7T and 9.4T human MRI systems, although an initial pH-calibration would be required. In addition, three dimensional AACID pH maps can be generated with significantly higher spatial and temporal resolution using steady state CEST acquisition methods. Application of the AACID technique to measure pH in other diseases such as cancer is possible with proper in vivo pH-calibration.

The quantitative resolution, or the uncertainty in a determined value (e.g. pH value) is a useful parameter when comparing different measurement techniques. Using methods described by Pardue et al., quantitative resolution is defined as the ratio of the uncertainty in measured response to the sensitivity of a measurement system. In the current study, the quantitative resolution is determined using the in vivo pH-calibration data (Figure 3.5). Results in Figure 3.5c, reveal a measured response uncertainty of 0.036 (i.e. standard deviation of AACID values from the linear fit in Figure 3.5c) and an AACID pH-sensitivity of 0.253 pH$^{-1}$. Therefore the quantitative resolution of pH measurements is
0.14 pH units. Zhou et al. report a slightly lower quantitative resolution, stating that pH changes of ~0.2 pH units or greater can be detected using amide proton CEST effects.[5]

3.5 Conclusion

AACID represents a novel technique to quantify in vivo pH using endogenous CEST contrast. Using AACID, CEST-MRI can provide noninvasive, high-resolution pH distribution maps within animal models and could be extended to human subjects. Quantitative pH measurement using AACID is independent of protein concentration, water content, temperature, and NOEs at 9.4T. In vitro results indicate that glutamate may contribute ~5% to the AACID measurement in vivo. Following in vivo pH-calibration, pH was measured by AACID in a mouse model of MCAO. Decreased pH was observed on the ischemic side of the brain at two and five hours after the start of cerebral ischemia. The tissue region showing decreased pH increased in size between two and five hours. Absolute in vivo pH measurement could provide an indicator of disease progression, severity and treatment response.

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Chapter 4

4 Imaging chemical exchange saturation transfer (CEST) effects following tumor-selective acidification using lonidamine

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Abstract:

Increased lactate production through glycolysis in aerobic conditions is a hallmark of cancer. Some anticancer drugs have been designed to exploit elevated glycolysis in cancer cells. For example, lonidamine (LND) inhibits lactate transport leading to intracellular acidification in cancer cells. Chemical exchange saturation transfer (CEST) is a novel MRI contrast mechanism that is dependent on intracellular pH. Amine and amide concentration-independent detection (AACID) and apparent amide proton transfer (APT*) represent two recently developed CEST contrast parameters that are sensitive to pH. The goal of this study was to compare the sensitivity of AACID and APT* for the detection of tumor-selective acidification after LND injection. Using a 9.4T MRI scanner, CEST data were acquired in mice approximately 14 days after implanting $10^5$ U87 human GBM cells in the brain, before and after administration of LND (dose: 50 mg/kg or 100 mg/kg). Significant dose dependent LND-induced changes in measured CEST parameters were detected in brain regions spatially correlated with implanted tumors. Importantly, no changes were observed in $T_1$- and $T_2$- weighted images acquired before and after LND treatment. The AACID and APT* contrast measured before and after LND-injection exhibited similar pH-sensitivity. Interestingly, LND-induced contrast maps showed increased heterogeneity compared to pre-injection CEST maps. These results demonstrate that CEST contrast changes after administration of LND could help localize brain cancer and monitor tumor response to chemotherapy within one hour of treatment. The LND-CEST experiment uses an anticancer drug to induce a metabolic
change detectable by endogenous MRI contrast and therefore represents a unique cancer detection paradigm that differs from other current molecular imaging techniques that require injection of an imaging contrast agent or tracer.

**Abbreviations used:** GBM, glioblastoma multiforme; pH, intracellular pH; CEST, chemical exchange saturation transfer; LND, lonidamine; RF, radiofrequency; MTR$_{\text{asym}}$, asymmetric magnetization transfer ratio; NOE, nuclear Overhauser effect; MT, magnetization transfer; AACID, amine and amide concentration-independent detection; APT*, apparent amide proton transfer; PBS, phosphate-buffered saline; FSE, fast spin-echo; ETL, echo train length; WASSR, water saturation shift referencing; AFI, actual flip-angle imaging; ROI, region of interest; H&E, hematoxylin and eosin;
4.1 Introduction

Primary brain glioma is a rare form of cancer accounting for < 2% of all cancers, however it represents the second leading cause of death among neurological diseases in the United States [1, 2]. Glioblastoma multiforme (GBM) is the most lethal and most common form of glioma [2-4]. The current standard of care of GBM tumors includes surgical resection of the primary tumor followed by aggressive concurrent radiation and chemotherapy [2-4]. Despite advances in therapeutic strategies coupled with neurological imaging throughout treatment, mean GBM patient survival time is approximately one year after diagnosis [1-4]. Over 90% of GBM tumors recur due to migratory cancer cells that evade treatment [4].

Currently, clinicians rely on standard anatomical $^1$H MRI to detect tumors and monitor treatment response [2, 4], yet morphological changes caused by treatment are often difficult to detect and quantify especially during radiation therapy when pseudo-progression markers may exist [3, 5]. Using anatomical MRI techniques, clinicians often require several weeks to conclude that a GBM tumor is unresponsive to therapy before prompting a change in treatment [4]. Since GBM tumors are among the most aggressive human tumors, there is a need to develop imaging techniques that are able to detect tumor response within days or even hours after initial treatment. For example, Zhou et al. recently showed that tumor response to radiation therapy (ie. radiation necrosis) could be detected within 3 days of treatment using a novel $^1$H MRI contrast mechanism called chemical exchange saturation transfer (CEST) [5]. The ability to quantify tumor response immediately after therapy will enable clinicians to quickly tune and optimize therapy, improving patient outcome.

Malignant cancer cells undergo genetic mutations that result in increased aerobic glycolysis, known as the Warburg effect [6]. The Warburg effect leads to higher lactic acid production but cancer cells adaptively increase expression of intracellular pH (pHi) regulators (e.g. ion transport channels and pHi-buffering enzymes) to maintain a relatively alkaline pHi [7]. Alkaline pHi enables increased proliferation and evasion of apoptosis by cancer cells [7]. A class of chemotherapy agents target increased glycolysis
in tumors by inhibiting key pH$_i$-buffers and/or glycolytic enzymes such as hexokinase or by blocking ion transport channels [6]. Consequently, many chemotherapy agents cause changes in physiological properties such as pH$_i$ almost immediately after starting treatment [8-11]. Monitoring immediate tumor pH$_i$ response to metabolic stressors could provide a measure of tumor aggressiveness and predict tumor response to treatment [12]. For example, Sagiyama et al. recently used a pH-sensitive CEST MRI contrast to monitor tumor response to chemotherapy with temozolomide [3]. It was found that CEST MRI contrast changed significantly in orthotopic GBM tumors 7 days after a single course of temozolomide treatment compared to untreated tumors. Since no changes in tumor volume, cell density or apoptosis were observed in treated tumors, Sagiyama and colleagues hypothesized that the observed changes in CEST contrast may be attributed to a decrease in pH$_i$ caused by temozolomide.

The purpose of the current study was to evaluate the sensitivity of pH-dependent CEST contrast for the detection of changes in tumor pH$_i$ induced by a single dose of the drug lonidamine (LND), which has been used to treat cancer in Europe and Canada [10]. LND is an anticancer agent that exploits the Warburg effect to target and destroy malignant cancer cells [6, 10]. More specifically, LND limits aerobic respiration in cancer cells by blocking pyruvate transport from the cytoplasm [13]. Due to the Warburg effect, cancer cells have a greater reliance on pyruvate produced by glycolysis in the cytoplasm compared to normal cells. Inhibition of pyruvate transport impedes oxidative phosphorylation in tumors leading to energy depletion [10, 14]. Furthermore, LND blocks lactate efflux from cells by inhibiting the monocarboxylic acid transporter [10]. Cancer cells produce significantly more lactate compared to normal cells [6] and consequently, we hypothesize that tumor-selective intracellular acidification occurs [10, 11, 14] following lonidamine treatment that will produce CEST MRI contrast.

4.2 Theory

CEST contrast is generated using protons associated with endogenous mobile proteins and peptides that are 1) chemically-shifted by $\Delta \omega$ from bulk water protons and 2) freely exchanging at a rate $k_{ex}$ with bulk water protons. To generate CEST contrast,
exchangeable protons associated with specific molecules can be saturated using a frequency-selective radiofrequency (RF) saturation pulse. The process of chemical exchange transfers saturated protons to the bulk water proton pool leading to a decrease (darkening) in MR image intensity. CEST effects from amide protons resonating at $\Delta \omega = 3.50$ parts per million (ppm) downfield (i.e. higher frequency) from bulk water protons (referenced to 0 ppm) or amine protons resonating at $\Delta \omega = 2.0$ and $\Delta \omega = 2.5$-$2.75$ ppm have been used by several groups to generate CEST contrast [15-19]. Amide and amine proton CEST effects are pH-dependent since their chemical exchange rates $k_{\text{ex}}$ are base-catalyzed [20]. The majority of mobile proteins and peptides reside in cells and therefore amide and amine CEST effects are considered to be $\text{pH}_i$-dependent. Cerebral ischemia animal models have been used to demonstrate a local decrease in amide CEST effects at 3.50 and increase in amine CEST effects at 2.5 ppm in regions of intracellular acidosis [15-17].

CEST effects may be observed using a z-spectrum. A z-spectrum is a plot of the normalized bulk water magnetization $M_{\text{sat}}/M_0$ as a function of the saturation pulse frequency, where $M_{\text{sat}}$ and $M_0$ are the bulk water magnetization measured after and without saturation respectively [21]. Traditionally, CEST effects are quantified using the asymmetric magnetization transfer ratio (MTR$_{\text{asym}}$) that was first introduced by Zhou et al [16]. MTR$_{\text{asym}}$ measurements are calculated using equation [4.1]:

$$\text{MTR}_{\text{asym}}(\Delta \omega) = \frac{M_Z(\Delta \omega) - M_Z(-\Delta \omega)}{M_Z(-\Delta \omega)}$$ [4.1]

where $M_Z(\Delta \omega)$ is the bulk water magnetization measured after saturation at the frequency $\Delta \omega$. However some groups have found that nuclear Overhauser effects (NOEs) at frequency offsets $\Delta \omega$ between -2.0 to -4.0 ppm and asymmetric macromolecular magnetization transfer (MT) effects can significantly contaminate MTR$_{\text{asym}}$ measurements [22, 23]. Therefore, it is difficult to quantify CEST effects using the MTR$_{\text{asym}}$ technique [22]. Recently, two novel techniques were introduced to enable quantification of amide and amine CEST effects in vivo while avoiding upfield NOEs and
asymmetric MT effects. Our group developed a CEST technique called amine and amide concentration-independent detection (AACID) that uses the ratio of CEST effects of amide and amine (Δω = 2.75ppm) protons to generate pH<sub>i</sub> dependent contrast independent of tissue macromolecule concentration and temperature [17]. Also, Jin et al. recently demonstrated a novel three offset measurement technique to measure a parameter named apparent amide proton transfer ratio (APT*) [23]. Both AACID and APT* techniques are sensitive to pH<sub>i</sub> and have been used in vivo to detect acidosis in cerebral ischemia models [17, 23]. In the current study, we compare the sensitivity of AACID and APT* for the detection of LND-induced pH<sub>i</sub> changes in tumors. The magnitude of single-dose LND-induced CEST contrast could help predict tumor response to treatment.

4.3 Experimental

4.3.1 Subjects

Nine mice were included in the current study including six NU/NU mice with U87 GBM brain tumors that were used to evaluate the effect of injecting 50 mg/kg (N=3) and 100 mg/kg (N=3) of LND. C57BL/6 male mice (N=3) were used to study the effects of LND on normal brain tissue. All animal procedures were performed according to a protocol that was consistent with guidelines established by the Canadian Council on Animal Care and was approved by the University of Western Ontario Animal Use Subcommittee.

4.3.2 Lonidamine

LND (C<sub>13</sub>H<sub>10</sub>C<sub>12</sub>N<sub>2</sub>O<sub>2</sub>) was purchased from Lancrix Chemicals (Shanghai, China). The drug (37.5 mg) was dissolved in 3 mL of pure dimethyl sulfoxide (DMSO) and vortexed until the solution was clear. LND was administered intraperitoneally (i.p.) at a dose of 50 mg/kg or 100 mg/kg with a syringe pump (Harvard Apparatus, Holliston, MA, USA) over the course of 2 min.
4.3.3 *Animal tumor preparation*

GBM brain tumors were induced in 21-25g, NU/NU mice (N=6) using U87 GBM cells established from a human GBM (ATCC; Rockville, MD, USA) as described previously [24]. Briefly, U87 GBM cells were grown in Dulbecco’s modified Eagles’s medium supplemented with 10% fetal bovine serum (Wisent Inc., St-Jean-Baptiste, QC, Canada) at 37 °C in a humidified incubator with 5% CO₂ and passaged twice a week. On the day of injection, U87 GBM cells were washed and dissociated with versene solution (phosphate-buffered saline (PBS) plus 0.5 mM EDTA), then washed twice with PBS, counted and re-suspended to a final concentration of 1 x 10⁵ cells in 2 µL PBS. Prior to injection, mice were anesthetized by inhalation of 4% isoflurane and maintained using 1.5% isoflurane. The mouse was placed in a stereotactic head frame (Stoelting instruments, Wood Dale, IL, USA). The scalp was swabbed with betadine and an incision was made in the scalp to expose the bregma. A 1 mm diameter hole was drilled at coordinates measured from the bregma (1 mm anterior and 2 mm lateral). U87 GBM cells (2 ml) were injected at a rate of 0.5 µL/min, at a position 3 mm deep from the bregma into the right frontal lobe using a Hamilton (Reno, NV, USA) syringe with a 27-gauge needle attached.

4.3.4 *General mouse preparation for in vivo imaging*

Anesthesia was induced using 4% isoflurane in oxygen and maintained with 1.5%–2.5% isoflurane in oxygen. The mouse was secured on a custom-built MRI-compatible stage and the head was secured using surgical tape to limit motion due to respiration. Temperature was monitored with a rectal temperature probe, and respiration was monitored with a respiratory sensor pad connected to a pressure transducer that was placed on the thoracic region. Body temperature was maintained at 36.9-37.1°C throughout imaging by blowing warm air over the animal using a model 1025 small-animal monitoring and gating system (SA Instruments Inc., Stony Brook, NY, USA). Following pre-injection imaging, the MRI-compatible stage was removed from the scanner without altering the animal position with respect to the stage. The mouse was
replaced in the scanner immediately after LND injection. Animals were sacrificed immediately after MR imaging.

4.3.5 *In vivo imaging parameters*

All animals were scanned using an Agilent (Palo Alto, CA, USA) 9.4 T small animal (31 cm horizontal bore) MRI scanner using an Agilent 30 mm millipede volume coil. The maximum gradient strength is 40 mT/m. Tumors were initially detected using multi-slice standard anatomical $T_1$-, $T_2$- and diffusion-weighted MRI. Upon tumor detection, a slice of interest was selected with maximal tumor coverage and single-slice $T_1$-, $T_2$- and diffusion-weighted MR images were acquired. $T_1$-weighted images were acquired using a standard fast spin-echo (FSE) pulse sequence (TR/TE = 500/10 ms, echo train length (ETL) = 4, the time between excitation and the echo at the center of $k$-space (effective TE) = 10 ms, 4 averages, 2 pre-scans, FOV = 19.2 x 19.2 mm$^2$, matrix size = 128 x 128, slice thickness = 2 mm, TA was 1.1 min). $T_2$-weighted images were acquired using a FSE pulse sequence (TR/TE = 3000/10 ms, ETL = 4, effective TE = 40 ms, 1 average, 2 pre-scans, FOV = 19.2 x 19.2 mm$^2$, matrix size = 128 x 128, slice thickness = 2 mm, TA = 1.7 min). Diffusion-weighted images were acquired using a multislice spin-echo pulse sequence (TR/TE = 1000/35 ms, $b$-value = 1100 s/mm$^2$ in the readout direction, 4 averages, FOV = 19.2 x 19.2 mm$^2$, matrix size = 128 x 128, slice thickness = 2 mm, TA = 8.5 min). CEST MR images were acquired for the slice of interest using a FSE pulse sequence (TR/TE = 7000/7 ms, ETL = 32, effective TE = 7 ms, 3 averages, 2 pre-scans, FOV = 19.2 x 19.2 mm$^2$, matrix size = 64 x 64, slice thickness = 2 mm, TA = 0.4 min, preceded by a 4 s long continuous wave RF saturation pulse with a $B_1$ amplitude of 1.5 $\mu$T). Z-spectra were obtained by acquiring 57 CEST MR images, each with different saturation frequencies (in ppm) including -0.6 to 0.6 ($\Delta = 0.1$), 1.6 to 4.5 ($\Delta = 0.1$), and 5.4 to 6.6 ($\Delta = 0.1$) and 1000. In each animal, a $B_0$ field map was generated for the image slice of interest immediately prior to each CEST experiment. A separate $B_0$ map was acquired pre- and post-injection. $B_0$ distribution maps were measured using the water saturation shift referencing (WASSR) technique [25]. Specifically, a WASSR spectrum was acquired using a FSE sequence (TR/TE = 7000/7 ms, ETL = 32, effective TE = 7 ms, 3 averages, 2 pre-scans, FOV = 19.2 x 19.2 mm$^2$, matrix size = 64 x 64, slice thickness =
2 mm, TA = 0.4 min and a 100 ms long continuous wave RF saturation pulse with a $B_1$ amplitude of 0.2 $\mu$T, saturation frequencies = -0.5 to 0.5 ppm in 16 linearly spaced steps). The 16-point WASSR spectrum was interpolated to 401 points (1 Hz resolution) and fit to a $10^{th}$ order polynomial. Z-spectra data were frequency shifted to account for $B_0$ variations. A $B_1$ field map was created using an actual flip-angle imaging (AFI) pulse sequence (TR = 20 ms, TE = 3.47 ms, # echoes = 2, flip-angle = 70°, 1 average, FOV = 19.2 x 19.2 mm$^2$, matrix size = 64 x 64, TA = 2.22 min) [26]. For each pixel, $B_1$ was calculated using $B_1 = B_{1\text{ref}} \times (\text{actual flip-angle/70°})$ incorporating the flip angle map and $B_{1\text{ref}} = 1.5 \mu$T. $B_1$ variation was less than 5% throughout the brain and no $B_1$ correction was necessary.

4.3.6 CEST data processing

All acquired CEST MR data were loaded into MATLAB (Mathworks, Natick, MA, USA) for analysis. Using custom MATLAB programs, $B_0$ corrections were performed on a pixel-by-pixel basis and all z-spectra were smoothed using the ‘smooth’ algorithm from the MATLAB curve fitting toolbox. Pre- and post-injection images were co-registered using custom MATLAB programs.

4.3.7 CEST and contrast calculations

Using $B_0$-corrected and smoothed z-spectra, AACID values were measured on a voxel-by-voxel basis. AACID represent the ratio of CEST effects of amine protons resonating at 2.75 ppm and amide protons at 3.50 ppm, normalized by MT effects measured after saturation at 6.0 ppm and is calculated using equation [4.2] [17].

$$AACID = \frac{M_z(3.50 \text{ ppm}) \times (M_z(6.0 \text{ ppm}) - M_z(2.75 \text{ ppm}))}{M_z(2.75 \text{ ppm}) \times (M_z(6.0 \text{ ppm}) - M_z(3.50 \text{ ppm}))}$$

[4.2]

The bulk water magnetization following saturation at 6.0 ppm (ie. $M_z(6.0 \text{ ppm})$) serves as a common reference point for both amine and amide CEST effects. As previously described in detail, the 6.0 ppm normalization point may slightly reduce CEST sensitivity compared to asymmetry methods, however it avoids contamination from NOEs that occur
between −2.0 to −4.0 ppm and it reduces the total number of MR images required [17].
Furthermore, the common reference point at 6.0 ppm represents a good estimate of the endogenous MT contribution.

APT* measurement of CEST effects from the amide protons resonating at 3.50 ppm were also quantified using the simple three offset measurement technique according to equation [4.3].

\[
APT^* = \left( \frac{M_z(3.0 \text{ ppm}) - M_z(4.0 \text{ ppm})}{2.0} - M_z(3.50 \text{ ppm}) \right) / M_z(1000 \text{ ppm}) \tag{4.3}
\]

Contrast to noise ratio (CNR) was measured as described by equation [4.4] where \( \text{CEST}_{\text{Baseline}} \) represents the CEST parameters (AACID or APT*) measured at baseline and \( \text{CEST}_{\text{LND}} \) represents the same parameter after LND treatment. The background noise (\( \sigma_{\text{Background}} \)) was approximated as the standard deviation of the CEST parameters within normal tissue at baseline.

\[
\text{CNR} = \frac{\text{CEST}_{\text{LND}} - \text{CEST}_{\text{Baseline}}}{\sigma_{\text{Background}}} \tag{4.4}
\]

4.3.8 Statistical Analysis

Mean AACID and APT* measurements were made from tumor and contralateral regions of interest (ROIs) for each mouse brain in quantitative CEST maps acquired before and after LND injection as well as LND-induced contrast maps. Differences in mean CEST values measured in tumor ROIs before and after LND injection were tested using Student’s paired t-test with MATLAB statistics toolbox with \( p < 0.05 \) considered to be statistically significant.
4.4 Results

4.4.1 CEST imaging of normal brain tissue after LND treatment

A representative set of CEST images acquired from a healthy control animal (C57BL/6 male mouse) is presented in Figure 4.1. For each control mouse, CEST data were collected at baseline (ie. immediately before LND injection) (Fig. 4.1a,d) and ~50 min after i.p. bolus administration of 100 mg/kg LND (Fig. 4.1b,e). LND-induced contrast maps (Fig. 4.1c,f) show the spatial distribution of CNR calculated from the AACID (Fig. 4.1c) and APT* (Fig. 4.1f) distributions maps using equation [4.4]. Some small variations in LND-induced CNR is visible in the brain in both AACID and APT* contrast maps. Much of the variation in the contrast maps is spatially correlated to fine brain structures such as ventricles and may be attributed to motion between acquisitions. Some heterogeneity may also be attributed to variations in LND effects and delivery within normal brain tissue. Mean CEST values measured before and after injection of 100 mg/kg LND treatment and LND-induced contrast are summarized for all control mice (N=3) in Figure 4.2. There was no significant difference between AACID whole brain mean values acquired at baseline and after LND treatment. Similarly, no significant LND-induced change was measured using APT*.
Figure 4.1: Representative set of CEST data from a healthy C57BL/6 mouse brain: CEST images acquired immediately before (a, d) and 50 min after (b, e) i.p. injection of 100 mg/kg LND. AACID and APT* values were calculated from the same z-spectra. Contrast maps (c, f) were calculated using CEST maps acquired at baseline and after LND treatment using equation [4.4].

Figure 4.2: Summary of CEST and contrast values from healthy C57BL/6 mice (N=3): a) Mean AACID and b) APT* values acquired at baseline and ~50 min after 100 mg/kg LND treatment. No significant differences were observed before and after LND treatment in both the AACID and APT* values. As a result the average LND-induced contrast was equal to background noise.
4.4.2 CEST imaging of U87 GBM brain tissue after LND treatment

Figure 4.3 presents a set of anatomical MR images for a representative NU/NU mouse 14 days after U87 GBM cancer cell implantation. For all brain tumor mice, regions of interest (ROIs) containing tumor (dashed line) and normal contralateral (solid line) tissue were drawn based on anatomical changes observed in T2-weighted images as shown in Figure 4.3a. The relatively low tumor contrast observed in the T2-weighted anatomical MR images (Figure 3) is attributed to a combination of partial volume effects associated with the large (2 mm) slice thickness used and the expected similarity of the T2 relaxation time constants in the tumor and contralateral brain tissue [27, 28]. NU/NU mice with U87 GBM brain tumors were treated with 100 mg/kg (N=3) or 50 mg/kg (N=3) of LND. CEST MRI data are presented in Figures 4.3-4.7 for a representative NU/NU mouse with U87 GBM brain tumor that was treated with 100 mg/kg LND. Figure 4 shows average baseline (blue) and post-injection (red) B0-corrected z-spectra obtained using all pixels in the normal contralateral tissue-containing ROI (Fig. 4.4a) and tumor tissue-containing ROI (Fig. 4.4b). Baseline amide CEST peaks consistently had larger amplitudes in tumors compared to contralateral tissue. Although amine protons resonating between 2.50 ppm and 3.0 ppm did not generate clear CEST peaks, they also consistently generated larger CEST effects in tumor tissue at baseline. This effect is more easily visualized using the reference line superimposed on each baseline z-spectrum in Figure 4.4 with endpoints empirically set to Mz/M0 values at 1.6 ppm and 4.6 ppm. Furthermore, in Figure 4.4a, normal contralateral tissue z-spectra showed no significant change in amine CEST effect at 2.0 ppm and 2.75 ppm or amide CEST effect at 3.50 ppm (ie. both spectra overlap) following LND injection. A small vertical offset is visible between spectra at saturation frequencies greater than ~2.50 ppm that may be due to a minor change in macromolecular MT effects. Conversely, more distinct changes were observed in the z-spectrum of the tumor ROI after LND injection (Figure 4.4b). Figure 4.4b shows a marked decrease in the amide CEST effect between 3.0 and 4.0 ppm following LND injection. This change strongly suggests LND-induced tumor acidification as amide CEST effects decrease in tissue upon acidosis. In tumor ROIs, CEST spectra demonstrated a very small change in amine CEST effect at 2.0 ppm and no
change in amine CEST effect at 2.75 ppm as expected from previous work [17]. Notably, the minor vertical offset noted above in normal tissue spectra was not observed in tumor spectra.

Figure 4.3: Standard anatomical MR imaging of U87 GBM brain tumor before injection of lonidamine: a) T2-weighted image with tumor (dashed line) and contralateral (solid line) regions of interests (ROIs) drawn. b) T1-weighted and c) diffusion-weighted images were also acquired using the identical slice of interest.

Figure 4.4: Chemical exchange saturation transfer (CEST) z-spectra collected at baseline and ~50 min after administration of lonidamine (LND): Average B0-corrected z-spectra calculated from a) normal contralateral tissue-containing region of interest (ROI) and b) tumor tissue-containing ROI with error bars equal to one standard deviation. A reference line (gray dashed) was empirically superimposed onto baseline z-spectra to aid visualization of differences between z-spectra in contralateral and tumor tissue. These zoomed Z-spectra highlight the significant changes in the 1.6 to 4.5 ppm frequency range.
Figure 4.5: CEST data from a U87 GBM brain tumor model treated with 100 mg/kg LND: CEST images acquired immediately before (a, d) and 50 min after (b, e) i.p. injection of 100 mg/kg LND. AACID and APT* values were calculated from the same z-spectra. Contrast to noise ratio maps show the change in CEST effects caused by tumor acidification following administration of LND normalized to the background noise (c, f).
Figure 4.6: Summary of CEST and contrast values from NU/NU mice with U87 GBM brain tumors (N=3): a,b) Mean AACID and c,d) APT* values acquired at baseline and ~50 min after 100 mg/kg LND treatment in tumor regions of interest (ROIs) (a,c) and contralateral ROIs (b,d) with error bars equal to one standard deviation. Mean LND-induced contrast values are shown with error bars equal to one standard error.

Figure 4.5 shows quantitative CEST distribution maps measured on a voxel by voxel basis using CEST data measured before (Fig. 4.5a,d) and after (Fig. 4.5b,f) injection of 100 mg/kg LND. At baseline, tumor regions consistently presented lower AACID and elevated APT* measurements (Fig. 4.5a,d respectively) compared to normal tissue. The average tumor CNR values (N=6) for each CEST parameter [CEST parameter: tumor mean CNR ± standard error] calculated using CEST contrast maps were: [AACID: 4.99 ± 1.11] and [APT*: 5.12 ± 0.82]. Approximately 50 min after LND injection, a marked increase in AACID and reduction of APT* values was observed in tumor regions only (Fig. 4.5b,e respectively). Conversely, tissue relaxation time constants and/or tissue
water content appeared unaffected by LND treatment because no difference was observed between $T_1$-, $T_2$- and diffusion-weighted images acquired before and after injection. Contrast maps (e.g. Fig 4.5c, e) were used to locate and quantify changes in AACID and APT* caused by 100 mg/kg LND treatment. A marked increase in spatial heterogeneity appears in LND-induced contrast maps generated using both APT* and AACID maps within tumor regions, which was not observed in the pre-injection CEST maps. Figure 4.6 summarizes CEST data from all brain tumor animals treated with 100 mg/kg LND (N=3) calculated using each pixel in tumor and contralateral tissue-containing ROIs from baseline and post-injection images as well as contrast maps. In tumor ROIs, AACID values were consistently and significantly higher after LND injection, compared to baseline ($p<0.05$ by $t$-test, N=3). Similarly, APT* values showed a trend toward lower values in tumor ROIs after LND injection, compared to baseline APT* ($p=0.078$ by $t$-test, N=3). The LND-induced signal change was significantly higher in tumor regions compared to normal tissue using either CEST measure (Fig. 4.6). Therefore, both AACID and APT* contrast maps produced high tumor contrast and could potentially be used to detect tumor-specific effects of LND injection. The LND-induced CNR generated using AACID and APT* values were not significantly different. At ~50 minutes after 100 mg/kg LND treatment, the average CNR values (N=3) for each CEST parameter [CEST parameter: tumor mean CNR ± standard error, contralateral mean CNR ± standard error] calculated using CEST contrast maps were: [AACID: $4.54 ± 1.79, 1.41 ± 1.06$] and [APT*: $4.82 ± 2.51, 1.81 ± 0.51$].

To investigate the dose-dependence of LND-induced AACID and APT* contrast, a set of NU/NU U87 GBM brain tumor mice (N=3) were treated with 50 mg/kg LND. The LND-induced contrast in tumors generated with 50 mg/kg was consistently lower compared to animals treated with 100 mg/kg LND for both AACID and APT*. No significant difference was observed between AACID and APT* LND-induced contrast maps in animals treated with 50 mg/kg LND. Figure 4.7 summarizes the dose-dependence of the mean contrast values generated using each pixel in tumor ROIs in AACID and APT* maps following LND treatment using 50 mg/kg (N=3) and 100 mg/kg (N=3) doses. At ~50 minutes after 50 mg/kg LND treatment, the average CNR values (N=3) for each CEST parameter [CEST parameter: tumor mean CNR ± standard error,
contralateral mean CNR ± standard error] calculated using CEST contrast maps were: [AACID: 3.24 ± 0.66, 1.21 ± 1.34] and [APT*: 3.96 ± 1.94, 1.82 ± 1.25].

Figure 4.7: Dose dependence of mean LND-induced contrast values calculated using each pixel in tumor regions of interest (ROIs): Mean LND-induced contrast values generated using a) AACID and b) APT* values in tumor ROIs are shown with error bars equal to one standard error for different LND doses.

4.4.3 Histological analysis of U87 GBM tumors after LND treatment

Following MRI experiments, animals were euthanized and two brains were excised for histology. Brain sections (5 µm thick) corresponding to the selected slice of interest were stained for morphological identification using hematoxylin and eosin (H&E). For the representative animal presented in Figures 4.3-4.5, H&E stained brain sections confirmed that regions of high LND-induced CNR within CEST contrast maps corresponded to U87 GBM tumor regions, as shown in Figure 4.8. Two regions of heterogeneous H&E stain uptake that were observed within the tumor (labeled with boxes b and c in Figure 8a) are attributed to vasculature and/or inflammatory cells. Interestingly, regions b and c appear to correspond spatially to regions of heterogeneity observed in the LND-induced CNR.
maps (Figure 4.5c,e). However, robust registration of the LND-induced CNR map with the brain section is required to conclude that the regions are correlated. Some signal variation could also be attributed to motion, as was observed in the AACID and APT* contrast maps obtained in healthy mice (Figure 4.1).

**Figure 4.8: H&E stained brain section from representative U87 GBM brain tumor model:** a) Brain section corresponding to the slice of interest used for CEST MR images in Figure 3 with two regions of interest that display stain heterogeneity attributed to vasculature and/or inflammatory cells labeled b and c. b-c) Magnified regions of interest b and c respectively.

### 4.5 Discussion

The goal of this study was to determine whether CEST MRI contrast changes in response to single-dose LND treatment could be used to detect treatment response in orthotopic GBM tumor. LND is an anticancer agent that alters metabolism and pH. The results indicate that both the AACID and APT* CEST parameters studied are sensitive to LND-induced pH changes in tumors within 1 hour of treatment and that the changes observed
in tumors are roughly 4-fold higher in tumors than in surrounding brain tissue. Both AACID and APT* provided similar dose dependent CNR.

The use of CEST contrast has many potential clinical applications. Zhou et al. were the first to show that exchanging protons associated with endogenous mobile protons and peptides could be used to detect changes in pH, using amide CEST contrast [16]. Endogenous amide and amine CEST contrast has since been used to detect decreased pH in the ischemic penumbra after cerebral ischemia [16, 23]. Amide CEST contrast in tumor regions has also been used to detect tumors [29]. Recently, Zhou et al. discovered that differences in amide CEST contrast could be used to differentiate recurrent glioma from radiation necrotic tissue [5]. Some groups have also found that amide and amine CEST effects are sensitive to necrotic regions within tumors [18, 30]. In addition, as already mentioned, Sagiyama et al. discovered that amide proton CEST effects could detect temozolomide treatment effects one week after treatment [3].

Amide CEST peak amplitude is consistently larger in tumors compared to normal tissue. Large amide CEST peak amplitudes in tumors are typically attributed to several characteristics of tumor tissue including longer $T_1$ and $T_2$ relaxation time constants, a more dilute macromolecular pool with lower magnetization transfer effects, slightly alkaline pH and increased mobile cytosolic protein concentration [15, 18]. Amide protons originate from the amide groups in the backbone of endogenous proteins and peptides. Similarly, relatively slow-exchanging amine protons resonating between 2.0-3.0 ppm originate from lysine amino acids in proteins and peptides.

In the current study, LND treatment caused the amide proton CEST peak to decrease significantly in tumor regions only. Base-catalyzed amide proton exchange rates decrease exponentially with a decrease in pH and therefore local acidosis results in reduced CEST effects in vivo [16, 17, 23]. Amine protons resonating at 2.0 and 2.75 ppm also have base-catalyzed exchange, however LND treatment induced only small changes in CEST at 2.0 ppm and no change at 2.75 ppm. The difference in pH-sensitivity is attributed to the unique ionization constants ($pK_a$) of the different proton pools [20]. Our group recently showed that amine protons associated with proteins and peptides that
resonated at 2.75 ppm exchanged in the slow-to-intermediate regime at neutral pH in-vitro [17]. However in vivo, consistent with the observations in the current study, we were previously unable to detect pH$_i$-dependent changes in CEST effects at 2.75 ppm in acidic tissue regions after stroke [17]. The insensitivity of amine CEST effect to changes in pH$_i$ could be predicted based on the relatively low power saturation scheme used (ie. 4 s, 1.5 $\mu$T continuous wave saturation) that is not optimally tuned to faster exchanging amine protons (17). As Woods et al. demonstrated using numerical simulations [31], CEST peak amplitudes measured using a non-optimal saturation scheme is less sensitive to changes in exchange rate. Increased amine CEST contrast in tumor regions could be attributed to increased cytosolic protein concentration in tumors.

Ward and Balaban first demonstrated that a ratiometric approach was valuable because the ratio of two CEST contrasts is independent of exchange site concentration and bulk water T$_1$ [32]. Ratiometric techniques may offer improved pH-sensitivity compared to single CEST effect approaches in cases where both CEST effects change with pH in opposite directions. Ali et al. described another case where one pH-dependent CEST effect is normalized by a pH-independent CEST effect that acts as an unresponsive local control [33]. In such a case, the ratio is pH-dependent and independent of exchange site concentration and bulk water T$_1$ [32-34]. Based on the observed pH$_i$-sensitive amide and pH$_i$-insensitive amine CEST effects at 3.50 ppm and 2.75 ppm respectively, AACID ratios should be sensitive to changes in pH$_i$ and insensitive to local variations in protein content, and T$_1$ and T$_2$ relaxation time constants, assuming that the ratio of amine and amide groups is the same in all tissue types and does not change after LND injection [17]. Lower AACID values in GBM tumor tissue before LND treatment indicate a slightly alkaline tumor pH$_i$ compared to normal tissue. However, further work is required to directly relate the AACID ratio to absolute pH$_i$ within tumor tissues. Previous work by Desmond et. al. [35] suggests that using the amine CEST effect at 2.0 ppm rather than 2.75 ppm could improve the performance of the AACID technique. In the current study, the z-spectrum was not sufficiently sampled around 2.0 ppm to permit further investigation of this potential alternative, however future work should compare the pH-sensitivity of the AACID ratio using the different amine CEST effects at 2.0 and 2.75 ppm.
APT* values are sensitive to pH, but unlike AACID are also sensitive to protein content, magnetization transfer effects from endogenous macromolecules as well as T_1 and T_2 relaxation time constants. Following injection of LND, T_1-, T_2- and diffusion-weighted MR images did not change indicating that the observed changes in both AACID and APT* maps are dominated by changes in pH. The current results suggest that AACID and APT* are equally sensitive to changes in pH caused by LND. However, the AACID ratiometric approach is expected to reflect changes in pH more specifically than APT* in applications where changes in pH are accompanied by changes in tissue relaxation and MT effects.

LND administration causes intracellular acidification in cancer cells by inhibiting efflux of lactic acid. Upon LND injection, the Warburg effect leads to tumor-selective acidification. LND treatment did not induce significant changes in CEST values in normal tissue regions. This result agrees with recent work by Nath et al. who used $^{31}$P MRS to confirm that LND caused significant acidification within tumor tissue only [10]. Although several studies have confirmed that LND induces tumor acidification, this work is the first application of CEST imaging to spatially map LND-induced pH changes. In agreement with previous results, we showed that LND-induced changes in tumor pH depend on LND dosage. Ben-Yoseph and colleagues used $^{31}$P MRS to show that tumor pH decreased 0.45 pH units after injection of 100 mg/kg LND and tumor pH decreased approximately 0.25 pH units after injection of 50 mg/kg LND [11]. Assuming that LND injections in the current study generated similar changes in tumor pH per LND dose, our results indicate that both APT* and AACID parameters are sufficiently pH-sensitive to detect pH changes of ~0.25 pH units as was predicted in previous work [16, 17].

The key finding of this study is that amide proton-mediated CEST effects decreased significantly in GBM tumor regions immediately after LND treatment. Consequently, post-injection quantitative APT* and AACID distribution maps showed differences compared to baseline maps in tumor regions only. Tumor-specific drug effects were evident approximately 1 hour after dosing. LND-induced contrast maps offered high tumor contrast, consistently enabling accurate tumor detection and boundary delineation. Primary results presented here suggest the paradigm of CEST imaging before and after
application of a physiological stress could offer a novel technique tumor detection. To be clinically relevant, this paradigm must be operationalized using a physiologic stress that is well tolerated by the patient and must offer improved tumor contrast compared to baseline images. In the current study, the LND/DMSO injection was not well tolerated by the mice. However in some mice, LND-induced tumor contrast was higher than the contrast between contralateral and tumor tissue in the baseline CEST maps. Further work is required to evaluate the tolerability of alternative physiological stressors that could further improve tumor contrast. No significant differences were found between APT* and AACID parameters in terms of LND-induced tumor contrast. Heterogeneity observed in tumor regions of the APT* and AACID contrast maps correlated with morphological variations observed in H&E stained histology images (Figure 4.8). Morphological variations may be attributed to vasculature and/or inflammatory cells, which should be further investigated, however this was beyond the scope of the current work. Importantly, H&E stained histology slices consistently demonstrated minimal necrosis throughout the solid tumors, as seen in Figure 4.8. However, since necrotic tissue is common in humans with GBM [36], LND-induced changes in CEST effects should also be measured in necrotic tissue. If necrotic tissue is unresponsive to LND, the use of a physiologic stress combined with CEST imaging may offer a new approach to differentiate necrotic tissue from tumor and normal tissue.

The single-dose LND-CEST method could be translated to lower field strength clinical MRI scanners, which are routinely used to produce APT contrast in humans [37-39]. Several clinical trials investigating cancer treatment using LND in U.S., Canada and across Europe reached phase II and III status [40, 41], however, high liver toxicity at 3 months of dosing led to a hold on all clinical trials in the United States in 2006 [42]. Recently, cancer-targeted LND-containing liposomes were developed and were shown to improve adjunct chemotherapy in a small animal tumor model [42]. Importantly, the dose of LND used in the current study was 100 mg/kg, which is considerably higher than that used clinically in the treatment of cancer in adult humans, where the highest reported total single-day dose was 900 mg. The main side effects associated with LND treatment are typically mild and include myalgia, asthenia, and testicular pain [41, 43]. The dose in the current study was chosen to replicate previous studies in the MR literature [9-11, 14].
Similar studies [9, 10] have reported high toxicity when using high LND doses (ie. > 50 mg/kg), however Nath et al. found mortality to be completely avoided when dissolving LND in a Tris/glycine buffer [10, 11]. In the current study, the authors were unable to completely dissolve LND unless using pure DMSO as a solvent. DMSO is toxic at high dosages {Kelava, 2011 #215} and future studies should aim to limit the amount DMSO used.

The small sample size represents a limitation of the present study. In addition, absolute pH$_i$ was not measured because the calibration between AACID CEST and absolute pH has not been established in U87 GBM. Finally, this study was not designed to evaluate the predictive value of LND-induced pH-dependent changes in CEST contrast with a therapeutic outcome. Future longitudinal studies will be designed to follow animals following treatment.

Further work is required to investigate whether other clinically approved therapeutic agents can alter tumor pH$_i$ after single-dose administration and whether these changes are detectable by CEST MRI. In addition, future investigations must determine whether acute pharmacologically induced CEST contrast predicts treatment outcomes. Finally, tumor-selective LND effects were so clear that safer pharmacological stressors should be investigated in combination with CEST MRI for applications in cancer detection, differentiation, and grading.

4.6 Acknowledgements

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4.7 References


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Chapter 5

5 Discussion and Future Work

The aim of this thesis was to optimize the application of endogenous CEST and PARACEST contrast to measure temperature and pH in vivo. CEST experiments involve labeling of a very small pool of solute protons using a RF saturation pulse. CEST contrast is achieved if the labeled (or saturated) solute protons freely exchange with bulk water protons. Many proton exchange events involving the transfer of RF labeled protons to the bulk water pool (and vice versa) eventually leads to a reduction in $^1$H MR image signal intensity (ie. negative contrast). In biological systems, CEST contrast may be used to probe local temperature and pH since chemical exchange is sensitive to temperature and pH. If CEST contrast only depended on chemical exchange rate, then it would be relatively straightforward to measure absolute pH and/or temperature. However, CEST contrast depends on several other factors including solute concentration, tissue water content, local magnetic relaxation time constants, and MT effects from protons associated with endogenous macromolecules such as cell membranes. Therefore, isolation and quantification of pH-dependent exchange rate influences on CEST contrast presents a significant obstacle to measuring absolute pH in vivo. Experiments presented in this thesis led to the discovery of a novel technique to measure absolute temperature and pH using exogenous PARACEST MRI contrast as well as a new technique to measure absolute pH using endogenous CEST MRI contrast. Both new approaches were shown to be independent of solute concentration, magnetic relaxation, and MT effects in vitro. Quantitative pH and temperature was mapped regionally in vivo using mouse models. All MRI experiments were performed using an ultra high magnetic field (9.4 T) MRI scanner. In the first study (Chapter 2), the linewidth (ie. full width at the half maximum, FWHM) of the CEST peak corresponding to the amide proton associated with a PARACEST agent ($\text{Tm}^{3+}$-DOTAM-Gly-Lys) was shown to be sensitive to RF saturation pulse amplitude and exchange rate only using theoretical models and in vitro PARACEST solutions. An empirical equation was derived to describe the relation between the amide proton CEST peak linewidth and pH and a separate set of equations were calculated to describe the amide proton CEST peak chemical shift dependence on
pH and temperature. Absolute pH and temperature maps were calculated in *in vitro* using PARACEST solutions and *in vivo* immediately after direct injection into a healthy mouse leg based on *in vitro* calibration curves. In the second study (Chapter 3), a novel ratiometric approach named amine and amide concentration-independent detection (AACID) was developed to measure absolute pH. AACID represents a ratio of two different endogenous CEST effects originating from amide protons and amine protons associated with proteins and peptides resonating at 3.50 ppm and 2.75 ppm respectively. Again, numerical simulations showed that the AACID ratio is dependent on exchange rate only for a given RF saturation pulse. Quantitative pH maps were used to detect local acidosis *in vivo* using a mouse model of cerebral ischemia based on an AACID-pH calibration curve. In the third study (Chapter 4), we investigated the sensitivity of two endogenous CEST metrics to tumor-selective acidification using an anticancer drug called lonidamine (LND). Specifically, the LND-induced contrast was compared between a novel CEST parameter called apparent amide proton transfer (APT*) and AACID. LND treatment effects on APT* and AACID were investigated in healthy mouse brain tissue as well as glioblastoma tumor tissue. Endogenous CEST effects originating from amide protons associated with proteins and peptides decreased significantly in tumor regions only after LND injection. Both CEST metrics, AACID and APT*, were equally sensitive to LND treatment and produced equal LND-induced contrast to noise ratio.

5.1 Measuring extracellular pH and temperature using PARACEST peak lineshape

Chapter 2 focused on the application of CEST effects originating from amide protons associated with Tm$^{3+}$-DOTAM-Gly-Lys that resonate around -46 ppm upfield (ie. lower frequency) from bulk water protons. The chemical shift and the linewidth of the amide CEST peak were both used to simultaneously measure tissue temperature and pH$_{e}$. This study presented a novel application of the CEST peak linewidth that was defined as the full width at half the maximum (FWHM). Analytical steady state solutions derived using a weak saturation pulse (WSP) approximation (described in Section 1.5.2) were simplified and rearranged to show that the CEST peak linewidth is linearly related to RF
saturation pulse amplitude $B_1$ and amide proton exchange rate. A linear relation between the CEST peak linewidth with both the $B_1$ and amide proton exchange rate was confirmed using numerically simulated CEST spectra, which do not require any assumptions. Hence for a given saturation pulse (ie. fixed $B_1$) the CEST peak linewidth depends on amide proton exchange rate only. The amide proton exchange rate is pH-dependent since it is base-catalyzed and therefore amide CEST peak linewidth is pH-dependent. Amide proton exchange rate is also temperature-dependent however temperature effects on exchange rate caused negligible change in linewidth within the narrow physiological range.

As predicted by the Bloch-McConnell equations and base-catalyzed exchange rate kinetics, the amide CEST peak linewidth increased exponentially with increasing pH in vitro. CEST data derived from in vitro PARACEST solutions was used to show that the amide proton CEST peak was independent of PARACEST agent concentration, magnetic relaxation time constants and temperature within experimentally relevant ranges. In vitro experiments also demonstrated that the CEST peak linewidth was insensitive to physiologically relevant changes in endogenous MT effects. The addition of tissue-like semi-solid component to in vitro solutions introduced significant magnetization transfer (MT) effects that mimic inherent macromolecular MT effects observed in vivo. In the presence of MT effects, the linewidths were consistently reduced by a constant offset. However, the MT effects on linewidth are not expected to introduce significant error since the magnitude of inherent MT effects is nearly constant throughout normal tissue. It is important to note that in the presented study, pH maps were calculated using the linewidth-pH relation derived in aqueous solutions (i.e. in the absence of MT effects). Therefore, future applications of the linewidth to measure absolute pH should derive empirical linewidth-pH calibration curves in vivo or using in vitro phantoms that mimic physiological MT effects. Furthermore, future experiments should correct CEST peak linewidth measurements for $B_1$–inhomogeneity effects using a quantitative $B_1$ map and a separate in vitro calibration to determine the linewidth dependence on $B_1$.

After injection of PARACEST agents into biological systems, the precise agent concentration is unknown. Similarly, tissue temperature is rarely known in tissue
affected by stroke or cancer. Therefore, the linewidth technique overcomes two major obstacles in absolute pH measurement using PARACEST agents since it is independent of agent concentration and tissue temperature. A major drawback of the linewidth technique is that it requires acquisition of the entire CEST peak lineshape for accurate pH measurement. However, CEST peak acquisition enables simultaneous measurement of the chemical shift. Using the mathematical relation between pH, temperature and chemical shift, it is possible to measure pH and temperature simultaneously.

Tm$^{3+}$-DOTAM-Gly-Lys was injected directly into a healthy mouse leg and PARACEST contrast was detected in vivo. Our group is among the first groups to detect PARACEST contrast in vivo. Quantitative pH maps were acquired using the linewidth-pH calibration curves derived using aqueous PARACEST solutions. Absolute temperature and pH measurements agreed well with values in the literature. The CEST spectra in regions of high PARACEST contrast (i.e. large CEST peak amplitude) demonstrated significantly less endogenous MT effects compared to normal tissue regions. The reduced MT effects suggest that direct injection of PARACEST agent may result in a pocket of PARACEST fluid at the injection site. Further investigation is ongoing to determine if the PARACEST contrast observed in vivo is from PARACEST agent within a fluid pocket or diffused throughout the tissue.

Linewidth analysis may be applied to any CEST peak originating from solute protons with pH-dependent exchange rate. Proper in vitro calibration is necessary since most amide proton pools have unique spontaneous and base-catalyzed exchange rate constants (equation [2.10]). Development of PARACEST agents with greater detection sensitivity would improve the overall accuracy and utility of the linewidth technique. Ongoing experiments are focused on determining which agent characteristics offer the best contrast to noise ratio in vivo. Ideally, new agents will generate sufficient contrast to enable detection after intravenous (i.v.) injection in order to measure pH and temperature across the entire tissue of interest. Two strategies to optimize PARACEST agents for in vivo pH measurement using the linewidth technique are; 1) modify the agent such that the amide protons resonate at very large (> 100 ppm) chemical shifts to avoid endogenous MT effects [1] and 2) modify the agent such that the water molecule bound to the
paramagnetic metal exhibits non-optimal exchange leading to less T2-shortening and subsequent tissue signal loss in the presence of the agent [2, 3]. Recently, our group developed an agent with minimal T2-shortening effects, which led to improved pH measurement accuracy [2].

5.2 Measuring intracellular pH using AACID

The study presented in Chapter 3 focused on the development of a novel technique called amine and amide concentration-independent detection (AACID). AACID represents a ratio of CEST effects originating from amine and amide protons associated with endogenous proteins and peptides. The AACID ratio is linearly dependent on pH under physiological conditions. The vast majority of proteins and peptides originate in the intracellular space and therefore AACID pH measurements are heavily weighted to the intracellular compartment. The AACID ratio was theoretically derived using the analytical steady state solutions derived using the WSP approximation. Analytical steady state solutions predict that the AACID ratio depends on the pH-dependent exchange rate only. Furthermore, CEST spectra were numerically simulated using a four-pool version of the Bloch-McConnell equations to simulate AACID ratios in the presence of endogenous MT effects. Introduction of physiological MT effects induced a significant change in the numerically simulated AACID-pH relation.

The AACID ratio was linearly dependent on pH for in vitro protein aqueous solutions. In agreement with numerical simulations, in vitro AACID values were independent of protein concentration, magnetic relaxation time constants and temperature. Initial pilot studies concluded that a 4 s, 1.5 µT RF saturation pulse generated AACID values with the greatest sensitivity to pH (unpublished results). The AACID-pH calibration curve generated in vitro could not be applied to in vivo AACID measurements to calculate absolute pH since the BSA protein solutions did not precisely mimic endogenous MT, magnetic relaxation time constants and chemical exchange properties encountered in vivo. Therefore, an in vivo AACID-pH calibration curve was calculated using 31P MRS pH measurements. The AACID-pH calibration relies on a constant pHi during collection of the AACID values and 31P MRS data. This assumption may be susceptible to small error in pH-calibration since the overall experiment takes approximately 2 hours for the
in vivo measurements alone. However, AACID and $^{31}\text{P}$ MRS results were consistent among different animals suggesting that this assumption is valid.

Importantly, AACID measurements required $B_1$-correction since the use of a surface RF coil resulted in significant $B_1$-inhomogeneity across the mouse brain. Quantitative $B_1$ maps consistently showed that most MRI voxels had actual $B_1$ values that were below the prescribed $B_1$ value. Plots of CEST spectra values (i.e., $M_x(\Delta \omega)/M_0$) at $\Delta \omega = 2.75, 3.50$ and 6.0 ppm with the measured $B_1$ values showed that CEST values were linearly dependent on $B_1$ within the range of $B_1$ values encountered for in vivo experiments (1.0 – 1.5 µT). Therefore, $B_1$-calibration curves were derived in vivo and CEST spectra values were corrected on a pixel-by-pixel basis using $B_1$-maps. AACID values were then calculated using $B_1$-corrected CEST spectra values. The effects of $B_1$-inhomogeneities on CEST data were successfully accounted for and corrected in this study, however, $B_1$-correction is difficult and relies on accurate $B_1$ measurement as well as consistent $B_1$-calibration curves among different experiments with slightly different RF coil setup. For example, differences in the distance between the surface RF coil and the mouse brain may significantly alter the range of $B_1$ values throughout the brain. Therefore, future applications involving the $B_1$-correction of AACID CEST data require a more extensive investigation of the $B_1$-dependence of CEST spectra values (at 2.75, 3.50 and 6.0 ppm) across a larger range of $B_1$ values. The $B_1$-correction step was avoided in the study presented in Chapter 4 by using a volume RF coil that provides high $B_1$-homogeneity.

Application of the AACID technique in humans is possible. AACID is immediately applicable for ultra-high field strength (≥ 7T) human MRI scanners. Further investigation is required to determine the utility of AACID for absolute pH measurements using clinical field strength (≤ 3T) human scanners. The major obstacle at lower field strength will be to detect any amine CEST effects at 2.75 ppm. The amine protons are expected to approach the fast-exchange regime at clinical field strengths. Human AACID-pH calibration represents another obstacle to overcome before the AACID technique is available in the clinic. However, high-resolution $^{31}\text{P}$ MRS using chemical shift imaging approaches may represent a feasible approach to registering AACID ratios.
with absolute pH$_i$ measurements. Furthermore, the conventional fast spin-echo (FSE) pulse sequence requires long acquisition times. However, several fast pulse sequences that are modified for CEST have recently been described in the literature [4-6]. Such rapid CEST pulse sequences are needed to enable rapid AACID-pH measurements in the clinic.

A key assumption of the AACID technique is that the ratio of amine and amide proton concentrations is constant throughout the tissue. This assumption may limit oncology applications of AACID if amine/amide proton concentration ratio changes in tumor tissue. However, ongoing experiments indicate that the AACID-pH relation is identical in tumor tissue and normal tissue. Therefore, the AACID pH measurement technique appears to be valid for investigating both stroke and cancer patients.

5.3 Measuring tumor acidosis following chemotherapy using APT* and AACID

The study presented in Chapter 4 investigated the application of CEST MRI for monitoring tumor response to a chemotherapy agent immediately after treatment. A chemotherapy agent called lonidamine (LND) is known to cause tumor intracellular acidification. Therefore, we compared the sensitivity of AACID and APT* to LND treatment. The most important finding in this study was that CEST imaging is capable of monitoring changes in pH$_i$ due to pharmacologically altered metabolism. Significant changes were observed in tumor regions in both the AACID and APT* maps, while no changes were observed in the $T_1$-, $T_2$-, and diffusion-weighted MR images. Overall, no significant difference in sensitivity to LND treatment was measured between AACID and APT*.

One drawback of the LND-CEST experiments is the duration of the experiment. The experiment requires both pre- and post-injection CEST maps, which takes approximately 2 hours of MR imaging. In addition, LND effects are not observed until approximately 50 minutes post-injection. This would be an expensive protocol in the clinic and most cancer patients would not be fit to remain in the scanner for such a long time. Therefore, future experiments should be optimized using more modern and faster pulse sequences.
It should be noted that clinical protocols for metabolic imaging using positron emission tomography (PET) also require several hours. Another obstacle of the LND-CEST experiment is the susceptibility to motion artifacts. When subtracting pre- and post-injection CEST maps, it is assumed that the maps were acquired from identical slices. The most efficient solution to eliminate any motion artifacts would be to acquire three-dimensional CEST maps and if necessary, employ an image registration protocol prior to CEST data processing. One modern CEST pulse sequence is capable of acquiring a three-dimensional whole brain volume in just over 10 seconds per saturation frequency ([4]). Finally, the LND-CEST experiment is currently not clinically translatable since the LND proved to be fatal to all of the animals. The toxic effects of LND have led our group to begin investigating other chemotherapy agents that known to alter tumor metabolism.

This study demonstrates that CEST imaging can detect tumor response to treatment within one hour of LND treatment. Interestingly, LND causes tumor acidification by blocking lactate efflux from the cell. Therefore, the level of LND-induced tumor acidosis is thought to be dependent on the rate of glycolysis (i.e. lactate production). Further experiments are required to investigate whether the LND-induced CEST contrast could be used to probe tumor metabolic activity. In this case, LND-induced contrast maps could be used to differentiate necrosis from recurrent tumor tissue. Furthermore, LND-induced contrast maps (both AACID and APT*) proved to be effective in localizing tumor tissue. In the case that LND-toxicity can be avoided either using a different solvent solution [7, 8] or using liposomes [9], the LND-CEST experiment potentially offers several exciting clinical applications.

5.4 Future Work

Each of the three studies presented in this thesis offer a novel technique to measure temperature, pH, or treatment-induced pH changes. Future work will focus on optimizing and employing the presented techniques to answer the central questions regarding the role to pH and temperature in diseases such as cancer and stroke.
New PARACEST agents are currently being strategically synthesized to have greater detection sensitivity in vivo. Using optimized PARACEST agents, the linewidth technique will be calibrated using either in vivo or in vitro phantoms with physiological MT effects to improve linewidth-pH calibration. Animal models will be used to investigate potential clinical applications of PARACEST agents that would motivate potential clinical trials. For applications in cancer, the linewidth technique will be used to investigate pH_e and temperature signatures throughout the growth period (approximately 2-3 weeks in mice) of different tumor types (i.e. low- and high-grade gliomas). Further analysis would evaluate if the tumor pH_e and/or temperature could be used to predict tumor outcomes such as tumor growth rate, volume, metastatic migration, and necrosis. Furthermore, the linewidth technique will be applied to study the effect of different anti-cancer treatment routines on pH_e and temperature. For example, different subsets of animals will undergo specific protocols involving chemotherapy and/or radiation therapy. Tumor temperature and pH_e maps acquired before, during and after therapy could be used to monitor tumor response. Again, retrospective analysis would evaluate whether pre-treatment maps could be used to predict treatment response. Another interesting application would be to monitor tumor pH_e after treatment with sodium bicarbonate (NaHCO_3) [10]. NaHCO_3 is an alkaline compound that has been hypothesized to neutralize the acidic extracellular tumor microenvironment. pH mapping using PARACEST MRI presents a novel noninvasive technique to test this hypothesis by monitoring tumor pH_e throughout NaHCO_3 treatment.

Future experiments to measure absolute pH_i in small animals will aim to investigate pH_i in cancer and stroke. For example, in stroke, the hypothesis that a pH_i threshold exists that can be used to predict areas of irreversible neuronal damage. To test this hypothesis, focal ischemia will be induced by middle cerebral artery occlusion (MCAO) for a set of occlusion times followed by reperfusion. Occlusion times should range from 0 to 2 hours in order to achieve varying sizes of infarct lesions [11]. Quantitative pH_i maps will be acquired immediately after MCAO surgery. Animals will be sacrificed immediately after pH_i map acquisition and processed for histology. A spatial analysis of pH_i maps and histology stained for neuronal damage would enable evaluation of a pH_i threshold that may predict neuronal death. If a pH_i threshold is calculated, then further studies should
be developed to employ pHᵢ maps to guide administration of tissue plasminogen activator (tPA) in mice after stroke.

Similar to pHₑ maps derived using the linewidth technique, pHᵢ maps should be measured in animals with different types of tumors throughout the tumor growth period. Quantitative analysis should investigate whether tumor pHᵢ can act as a biomarker to predict any clinical parameters such as metastasis, treatment response, and survival. Furthermore, the clinical significance of pHᵢ heterogeneity throughout tumors should also be investigated. In all cases, histology should be performed to spatially correlate tumor pHᵢ distribution with morphological markers such as necrosis, vasculature and inflammation. In addition, the intracellular to extracellular tumor pH gradient can be investigated by applying the AACID (pHᵢ) and linewidth (pHₑ) techniques in the same experiment. Tumor pH gradient must be investigated for different tumor types and treatment strategies to investigate whether any clinical advantage exists when acquiring both pHᵢ and pHₑ compared to only pHᵢ or pHₑ.

Future application of the LND-CEST experiment must first eliminate the lethal toxicity in order to ensure clinical utility. Pilot experiments must test different solvents [7-9] and/or different tumor-specific metabolism altering drugs [7, 12]. To study the hypothesis that LND-induced contrast is a biomarker of glucose metabolic activity, the LND-induced contrast maps should be compared to radiolabeled ¹⁸F deoxyglucose (FDG) uptake measured using PET imaging in mouse brain tumors. Further investigation should determine if LND-induced contrast signifies tumor grade and growth rate. Finally, the sensitivity of LND-induced contrast maps for localizing small tumors should be tested.
5.5 References


Curriculum Vitae

Nevin McVicar, BSc.

EDUCATION

2009 –2014  PhD in Medical Biophysics, Western University
- Researching in vivo applications of chemical exchange saturation transfer (CEST) MRI contrast to measure temperature and pH

Clinical MSc in Medical Biophysics, Western University
CAMPEP-Accredited – Radiation oncology stream

2005 - 2009  BSc in Mathematics and Physiology, McGill University

SCHOLARSHIPS & AWARDS

2013-present  Natural Sciences and Engineering Research Council of Canada (NSERC)
Postgraduate scholarship (PGS) Doctoral - $42 000/2 yrs

2013  Ontario Graduate Scholarship (OGS) - declined
Queen Elizabeth II Scholarship - $15 000/1 yr.

2013  Magna Cum Laude Merit Award
International Society of Magnetic Resonance in Medicine (ISMRM)

2012–present  Canadian Institutes of Health Research (CIHR)
Trainee in Cancer Research and Technology Transfer (CaRTT) – $19 100/yr.

2009-present  Western Graduate Research Scholarship (WGRS)
The University of Western Ontario, London, Ontario - $9 000/yr.

2009 – 2012  Graduate Research Stipend
The University of Western Ontario, London, Ontario - $20 000/yr.

2008  Natural Sciences and Engineering Research Council of Canada (NSERC)
Undergraduate Student Research Award (USRA) – $4500

PEER-REVIEWED PUBLICATIONS

McVicar, N., Li, AX., Meakin, Bartha, R., Imaging of chemical exchange saturation transfer (CEST) effects following tumor-selective acidification using lonidamine. Submitted to NMR in Biomedicine on June 25, 2014.


**WORK EXPERIENCE**

2013  **Linear Accelerator Quality Assurance Internship**
London Regional Cancer Program (LRCP), London, Ontario
- Performed weekly mechanical, safety and radiation dosimetry tests (~100 hours)

2011 - 2013  **Graduate Teaching Assistant**
Western University, London, Ontario
- Performed weekly tutorials and graded assignments (~55 hours per term)

**CONFERENCES & WORKSHOPS**


2012  **Nevin McVicar**, Li, AX., Suchy, M., Hudson, R., Menon, R., Bartha, R. “*Simultaneous In vivo Temperature and pH Mapping Using a PARACEST-MRI*”. *London*
Oncology Research Day (Regional conference, Abstract and Poster Presentation); International CEST Imaging Workshop (International conference, Abstract and Poster Presentation, PhD work)


ADDITIONAL SKILLS

Languages - Fluent in English (fluent), French (intermediate) and Portuguese (beginner)
Computer programming – Proficient in MATLAB programming
Comprehensive WHMIS Certification - Laboratory and Chemical Safety Training
Basic Mouse Training, Gas Anesthesia Certification - UWO Animal Care Training
Basic Mouse Handling Certification - UWO Animal Care Training