DEVELOPMENT AND APPLICATION OF acidoCEST MRI
FOR EVALUATING TUMOR ACIDOSIS IN PRE-CLINICAL CANCER MODELS

by

Liu Qi Chen

____________________

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SIGNED: Liu Qi Chen
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Last but not least, I want to thank my parents for their unwavering support and trust in me for pursuing overseas graduate studies, and my husband for his unconditional love that knitted us much closer together.
DEDICATION

Dedicated to my loving husband, Yoon Kah, and my adorable children, Hong Wen, and Hong Jun.

P/s: acidoCEST MRI is developed in conjunction with the creation of Hong Wen and Hong Jun.
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<td>APT</td>
<td>Amide Proton Transfer</td>
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<tr>
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<td>Amide Proton Transfer Ratio</td>
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<td>ATP</td>
<td>Adenosine TriPhosphate</td>
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<td>AUC</td>
<td>Area Under the Curve</td>
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<td>Carbonic Anhydrase-IX</td>
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<td>CA</td>
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<td>FISP</td>
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<td>FLASH</td>
<td>Fast Low-Angle SHot</td>
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<td>FLEX</td>
<td>Frequency-Labeled EXchange</td>
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<tr>
<td>GE</td>
<td>Gradient Echo</td>
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<td>H&amp;E</td>
<td>Hematoxylin and Eosin</td>
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<td>HIF-1α</td>
<td>Hypoxia Inducible Transcription Factor-1 Alpha</td>
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<td>i.v.</td>
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<td>NMR</td>
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<td>M₀</td>
<td>Magnetization without saturation</td>
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<td>MRI</td>
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<td>NHE</td>
<td>Na⁺/H⁺ Exchanger</td>
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<td>NIR</td>
<td>Near Infra Red</td>
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<tr>
<td>PARACEST</td>
<td>PARAmagnetic Chemical Exchange Saturation Transfer</td>
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<td>PET</td>
<td>Positron Emission Tomography</td>
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<td>PK</td>
<td>Pharmacokinetic</td>
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<td>pHLIP</td>
<td>pH Low Insertion Peptide</td>
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<td>pHe</td>
<td>Extracellular pH</td>
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<tr>
<td>pH i</td>
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<td>Point RESolved Spectroscopy</td>
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<td>VEGF-A</td>
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ABSTRACT

Tumor acidosis is an important biomarker in cancer. We have developed a noninvasive imaging method, termed acidosis Chemical Exchange Saturation Transfer (acidoCEST) MRI to measure extracellular pH (pHe) in the tumor microenvironment. Chapter 1 introduces the importance of measuring tumor acidosis and presents various imaging modalities and their shortcoming to measure pHe. Appendix A describes the optimization of acidoCEST MRI for \textit{in vivo} pHe measurement. The acidoCEST MRI protocol consists of a CEST-FISP acquisition and Lorentzian line shape fittings. We determined the optimal saturation time, saturation power and bandwidth, 5 sec, 2.8 µT and 90 Hz respectively. We also tried various routes of administration to increase contrast agent uptake in the tumor. We decided upon 200 µL bolus followed by 150 µL/hr infusion. The optimized acidoCEST MRI protocol was tested on a mammary carcinoma mouse model of MDA-MB-231. Our method can detect an increase in pHe in the bladder and tumor of the mice treated with bicarbonate. We used this optimized acidoCEST MRI method to measure pHe in lymphoma tumor model of Raji, Ramos and Granta 519 as described in Appendix B. Pixel-wise pHe maps showed tumor heterogeneity. The pHe of Raji, Ramos and Granta 519 were determined to be mildly acidic with no significant difference. Appendix C describes the evolution of pixel-wise analysis in more detail. Besides the pHe map and spatial heterogeneity, we were able to determine the % contrast agent uptake. We monitored these biomarkers in two different mammary carcinoma mouse models, MDA-MB-231 and MCF-7 longitudinally and made comparisons between the different tumor models: MCF-7 were more acidic, more heterogeneous and faster growing than MDA-MB-231.
CHAPTER 1

IMAGING THE pH OF THE

EXTRACELLULAR TUMOR MICROENVIRONMENT

PUBLICATIONS

1.1 IMPORTANCE OF MEASURING TUMOR ACIDOSIS

1.1.1 Tumor Acidosis

An emerging hallmark of cancer is the disregulation of cellular energetics, which involves reprogramming cellular energy metabolism to most effectively support neoplastic proliferation [1]. Under aerobic conditions, normal cells convert glucose to pyruvate via glycolysis in the cytoplasm, and thereafter dispatch the pyruvate to the oxygen-consuming mitochondria to produce carbon dioxide and ATP (Figure 1.1) [2]. Under anaerobic conditions, pyruvate is converted to lactic acid in the cytosol. This anaerobic metabolism is inefficient as it produces ~18 fold fewer ATP molecules relative to mitochondrial oxidative phosphorylation. Otto Warburg first reported that cancer cells limit their energy metabolism largely to glycolysis, even in the presence of oxygen [3]. Despite lower energy production efficiency, increased glycolysis allows the diversion of glycolytic intermediates into various biosynthetic pathways, including those generating nucleosides and amino acids [4]. This in turn facilitates the biosynthesis of the macromolecules and organelles required for assembling new cells, supporting the active cell proliferation in neoplastic disease.

The consequence of increased intracellular production of lactic acid is extracellular tumor acidosis. To maintain an intracellular pH (pHi) that is slightly alkaline (~pH 7.4), tumor cells up-regulate several proton extrusion mechanisms such as the Na\(^+\)/H\(^+\) exchanger (NHE), HCO\(_3\)\(^-\) transporter, carbonic anhydrase IX, vacuolar-ATPase, and the H\(^+\)/K\(^+\) ATPase [5,6]. Excess protons are excreted into the extracellular matrix, causing the extracellular pH (pHe) of the tumor microenvironment to become acidic. In certain tumor
types such as human MCF-7 mammary carcinoma, the pH has been measured to be as low as pH 6.44 [6]. Chronic exposure to acidic pH has been reported to promote invasiveness and metastatic behavior in several tumor types [7-9].

Figure 1.1. Schematic of glycolysis and associated metabolic pathways that create biomolecules for tumor growth. Adapted from reference [2].

1.1.2 Tumor Acidosis Affects Chemotherapy

Most chemotherapeutic agents enter cancer cells via passive diffusion across the cell membrane, which requires the agent to be in a non-ionized form. Thus, the cell toxicities of weak base drugs such as daunorubicin, doxorubicin and mitoxantrone, are greatly reduced under acidic conditions [10-12] (Figure 1.2). When the pH drops below the pKa of these weak bases, they become predominantly protonated and positively charged, and are therefore less permeable to cell membranes, resulting in cellular drug resistance.
Conversely, weak acid chemotherapeutic drugs such as chlorambucil, cyclophosphamide and 5-fluorouracil have higher cytotoxicity at lower pH [11]. Hence, knowing the pH of the tumor microenvironment can enable physicians to select the best chemotherapy based on tumor pH, and provide personalized chemotherapy for each individual patient.

Figure 1.2. Tumor acidosis causes chemoresistance against weak-base drugs. A) Uptake and retention of mitoxantrone, a weak-base drug, was greater in CH3 tumor tissue that was neutralized with sodium bicarbonate. B) Mitoxantrone treatment (1 dose of 12 mg/kg or 2 doses of 6 mg/kg) caused a greater growth delay when sodium bicarbonate neutralized the tumor acidosis. C) Sodium bicarbonate also improved survival with mitoxantrone treatment. Reproduced with permission from reference [12].
1.1.3 Alkalinizing Therapy to Counter Tumor Acidosis

Research has been conducted to investigate alkalinizing therapy as an option to increase tumor pH that can enhance chemotherapy and counteract acid-mediated invasion and metastasis. In vitro results have shown that the cytotoxicity of doxorubicin has 2.25-fold enhancement when the pH of the cell culture media was raised from 6.8 to 7.4 [13]. In the same study, 200 mM bicarbonate in drinking water was introduced ad libitum to a mouse model of MCF-7 mammary carcinoma. In vivo results showed that the tumor volume of the MCF-7 tumors in mice treated with both bicarbonate and doxorubicin were significantly smaller than mice treated with only doxorubicin (p<0.03). In another study, bicarbonate treatment was also shown to reduce the formation of spontaneous metastases, with fewer metastatic lung lesions and longer survival times in mouse models of MDA-MB-231 metastatic breast cancer [14] (Figure 1.3). Computer simulations showed that the addition of a moderate amount of bicarbonate in blood that is ~40% higher than normal serum concentration can reduce the amount of intratumoral and peritumoral acidosis and can almost completely eliminate tumor invasion [15].

![Figure 1.3](image.png)

Figure 1.3. Treatment of a mouse model of β-galactosidase-labeled MDA-MB-231 mammary carcinoma with NaHCO₃. The mouse were treated with 200 mM NaHCO₃ ad libitum for 60 days resulted in lower lung metastases as evidence by A) β-galactosidase-induced fluorescence staining of lung lesions and B) the number of lesion pixels per animal. Reproduced with permission from reference [14].
However, alkalinizing treatments remain controversial because the duration of alkalinization to cause an effective outcome is unknown, and the detriments to normal tissues such as kidney and liver are also unknown. Furthermore, the pKa of bicarbonate is ~6.9. Hence introducing bicarbonate to a tumor with pH > 6.9 will ultimately lead to unnecessary alkalinization. Therefore, being able to accurately monitor pH in tumors and normal tissues will greatly aid in evaluating the utility of alkalinizing treatment for cancer care.

1.2 IMAGING METHODS TO MEASURE IN VIVO PH

A variety of biomedical imaging methods have been developed in an attempt to measure tumor pH. Each method has shown a remarkable ability to produce quantitative measurements during in vivo studies, although the accuracies of these quantitative measurements have been difficult to confirm, due to a lack of a “gold standard.” Perhaps more importantly, most of these methods have disadvantages which complicate or eliminate the possibility that the method can be translated to the radiology clinic to diagnose cancer patients. The following descriptions provide a summary of each method, including advantages and disadvantages.

1.2.1 pH measurements with methods other than MRI

1.2.1.1 pH electrode

The traditional ion-selective glass electrode is one of the first types of instrumentation that was used to measure pH in a human tumor. The pH of surface tumors such as malignant melanomatosis can be measured using a pH electrode because these tumors are
easily accessible to the percutaneous technique [16]. At the completion of each procedure, the nodule has to be excised to avoid the possibility of tumor fungation. More recently, a needle-shaped pH electrode has been developed to minimize the invasiveness and improve the speed of this technique (Figure 1.4). The pH measurement is assumed to be accurate because this microelectrode can be accurately calibrated with external buffer solutions. The spatial resolution depends on the number of insertions and the spacing between each insertion into the solid tumor, which has ranged between 4 – 20 insertions at 0.5 – 1.0 cm intervals in tumor studies with canines [17]. When the tumor is not on the surface, an additional localizer step such as MRI is required to locate the tumor and assist the placement of the pH electrode into the tumor, and to direct the electrodes away from necrotic areas in the tumor. Delineation of pH\textit{i} from pH\textit{e} is impossible with electrode-based techniques due to the size of the electrode compared with the size of cells.

Figure 1.4. A pH microelectrode for measuring \textit{in vivo} tumor pH.
A) A photo of an angled glass electrode with a platinum wire and 0.1 N hydrochloric acid electrolyte. B) The microelectrode was inserted into a melanoma nodule of a patient, stabilized with sponge rubber between the skin and electrode shaft, and secured with adhesive tape. A calomel reference electrode was secured to the skin. Adapted from reference [16] with permission.
1.2.1.2 Optical Imaging with Fluorescence

Optical imaging with a fluorescence dye is a low cost imaging tool with great versatility. A fluorescence agent can be engineered to couple with a peptide, antibody, or other biomolecule, which can bind to a tumor biomarker and enhance selectivity for tumor tissue [18]. The pH can be measured independent of concentration by assessing the ratio of fluorescence signals at different emission wavelengths (Figure 1.5) [19-22], or at different fluorescence lifetimes [23]. Near-infrared (NIR) light at 700 – 900 nm wavelength is preferred for in vivo imaging because NIR light can propagate through tissue to a depth of approximately one centimeter and still be adequately detected relative to background signals, due to lower tissue absorption and autofluorescence in this wavelength regime [18]. Optical imaging has high sensitivity and can detect nanomolar concentrations of agents. It also has good temporal resolution on the order of seconds [14,19,24,25]. Surface-accessible tumors can be imaged at excellent micron-scale spatial resolution. Based on these merits, pre-clinical optical imaging has been applied to surface-accessible tumors such as the rabbit ear chamber [25] and window chamber models of mammary carcinoma [14], and optical imaging has been used during image-guided surgery that provides access to the surface of tumors [26]. However, optical imaging cannot interrogate tumors that are located in deep tissues, which severely limits the utility of this imaging method for measuring tumor pH.
1.2.1.3 Positron Emission Tomography

Positron emission tomography (PET) is a widely used molecular imaging modality in both clinical and research settings. Whole-body PET imaging of mice can be performed in less than 10 minutes. To image tumor pH with PET, a $^{64}$Cu radioactive nuclide has been conjugated to the pH low insertion peptide (pHLIP), which folds to form an α-helix and inserts itself into cell membranes when the tumor pH is acidic (Figure 1.6) [27-30]. This method has high sensitivity, and therefore can produce images with an administration of agent as low as ~0.01 ng/kg of mouse weight. Higher retention of the agent within the tumor has been shown to correlate with lower acidity [27]. However, this method depends on an equilibrium between the peptide conformations that can and cannot insert into a cell membrane, and therefore the fraction of membrane-inserted
peptide has a sigmoidal relationship with pH. This causes the pH measurement to be semi-qualitative instead of quantitative. Furthermore, PET has spatial resolution of ~2 mm [31] and may not be as applicable to measure pH in small tumors such as tumors in lymph nodes, and may not be able to adequately assess tumor heterogeneity.

Figure 1.6. PET imaging of tumor pH. A) A pHLIP peptide inserts into cell membranes in low pH conditions. B) A membrane-insertion of a variation of pHLIP shows a sigmoidal dependence on pH. C) PET imaging of $^{64}$Cu-DOTA-pHLIP showed higher uptake and retention in a subcutaneous mouse model of LNCaP relative to PC-3, indicating that the LNCaP tumor model had lower pH. MR spectroscopy confirmed that the average pH values of LNCaP and PC-3 tumors were 6.78 ± 0.29 and 7.32 ± 0.10, respectively. Reproduced with permission from references [27-29].

1.2.1.4 Electron Paramagnetic Resonance

Electron paramagnetic resonance (EPR) spectroscopy is a technique for studying material with unpaired electrons. Because most stable molecules have all of their electrons paired, only a few molecules are sensitive to EPR. This limitation also means that EPR offers great specificity and there is no competing background signal [32]. The use of pH-
sensitive nitroxides offers a unique opportunity for non-invasive assessment of pH values from 0 -14 in living animals with sensitivity in the µM regime [33]. The pH can be determined by measuring the frequency separation of the spectral peaks, or by quenching the EPR signal within a pH-dependent polymer (Figure 1.7) [34]. Unfortunately, tissues must be irradiated with high power to perform EPR studies, which limits this technique to the study of small animal models and peripheral human tissues.

![Figure 1.7. EPR imaging of pH.](image)

The nitroxide radical TEMPO shows strong EPR signals in solution, which are quenched when TEMPO is encapsulated in a nanoparticle. A) Degradation of the nanoparticle at low pH de-quenches the EPR signals from TEMPO. B) No change in the nanoparticle at neutral pH retains the EPR-quenched state. C) Phantom images demonstrate that the difference in pH can be spatially localized. Reproduced with permission from reference [34].

1.2.2 Magnetic resonance based methods

Magnetic resonance imaging (MRI) and MR spectroscopy (MRS) are excellent whole-body imaging tools that provide excellent soft tissue contrast with little radiation exposure. Beyond anatomical information, recent MRI and MRS developments have focused on providing environmental biomarker evaluations (pH, temperature, and oxygen) and molecular information (proteins, enzyme activity, gene expression,
metabolites, and metal ions) [35]. In recent years, innovations in MR instrumentation have drastically improved spatial and temporal resolution, and it is now possible to image \textit{in vivo} tissues with \textasciitilde0.1 mm spatial resolution.

1.2.2.1 Magnetic Resonance Spectroscopy

Magnetic resonance spectroscopy (MRS) has been used for more than three decades to examine metabolite distributions among living cells and tissues. The most common applications of \textit{in vivo} MRS are used to detect endogenous signals from $^1$H, $^{31}$P or $^{23}$Na [36]. $^1$H MRS is the most favorable spectroscopic method because $^1$H has the highest inherent sensitivity with 99.98% natural abundance and the highest gyromagnetic ratio of stable isotopes [37], and most clinical MR instruments have the capability to detect $^1$H MR signals. Furthermore, the most atoms in the human body are hydrogen atoms, 67% by atomic percent. Tumor pHe can be measured by detecting endogenous metabolites that have chemical shifts that are sensitive to pH. For example, pH is correlated with the chemical shift difference between the two protons on histidine, both of which are pH-sensitive. Such analyses do not require known concentrations of histidine [38]. Another example is (±)2-imidazole-1-yl-3-ethoxycarbonylpropionic acid (IEPA), which can measure pH by comparing the pH-dependent chemical shift of one proton with the pH-independent chemical shift of a second proton in the same molecule (Figure 1.8) [39]. Thus a ratio of the two chemical shifts correlates with pH in a concentration independent manner. However, the use of IEPA to measure pHe is questionable, because IEPA is a pH buffer and can alter the pHe by interfering with the clearance of extracellular proton to the blood stream.
Figure 1.8. $^1$H MR spectroscopy imaging of tumor pHe.
A) The $^1$H spectrum from within a glioma showed the chemical shift of the IEPA H2 resonance (arrow), B) which is correlated with pH as shown by an *in vitro* titration. C) A parametric map of IEPA signal amplitudes showed accumulation of the agent in the glioma. D) A parametric map of pHe was determined from the chemical shift of the IEPA H2 resonance and the correlation shown in panel B. Reproduced with permission from reference [39].

$^{31}$P MRS has been used to measure the chemical shift of endogenous inorganic phosphate (Pᵢ). However, this chemical shift is weighted to reporting the pHᵢ rather than the pHe, because ~2-3 mM of Pᵢ is in the intracellular compartment and only ~1 mM is in the extracellular compartment [37]. The exogenous contrast agent 3-aminopropylphosphonate (3-APP) can be used to measure pHe via $^{31}$P MRS (Figure 1.9) [40]. The chemical shift of 3-APP is referenced to the chemical shift of Pᵢ and therefore information about the concentration of 3-APP is not required. Furthermore, 3-APP has low toxicity and can measure pHe within a physiological range of pH 6 to 8. This method
of analysis allows simultaneous pHe and pHi measurements with 3-APP and P$_i$ \cite{41,42}, and has been used to study alkalosis and acidification of tumor models \cite{43,44}. This method has been a popular choice as a cross-reference when developing new methods to measure pHe. \cite{27,45,46}.

![Figure 1.9. 31P MR spectroscopy of tumor pHe.](image)

A) The 31P spectrum from mouse leg muscle injected with 3-APP can measure pHe (pH$^{ex}$) from the 3-APP chemical shift, and can measure pHi (pH$^{in}$) from the chemical shift of inorganic phosphate (P$_i$). B) The 31P chemical shift of 3-APP is correlated with pH as shown by an in vitro titration. C) The pHe and pHi measured with 3-APP and P$_i$ in a tumor before (bottom) and after (middle) injection of 3-APP, and after euthanasia (top). Reproduced with permission from reference \cite{40}.

Even though 31P MRI has 100% natural abundance, its signal sensitivity relative to 1H MRI signals is only 6.6%. For comparison, 19F is 100% naturally abundant and has an MR sensitivity of 83% relative to 1H \cite{37}. Furthermore 19F MRS has a relatively large chemical shift difference, and an almost lack of endogenous MR signal in normal tissues.
The fluorinated compound ZK-150471 emits two $^{19}$F MR signals that have a frequency difference which is dependent on pH (Figure 1.10) [47]. This $^{19}$F agent has been used to detect changes in tumor pH in response to treatment with hydralazine and/or heating. In a study that compared $^{19}$F and $^{31}$P MRS at a 1 cm$^{3}$ spatial resolution, $^{31}$P MRS required ~40 min of acquisition time, while its counterpart $^{19}$F MRS only required ~5 min [45]. However, pH-sensitive $^{19}$F contrast agents have practical problems that limit in vivo use, such as the instability of some fluorinated compounds and non-specific accumulation in normal tissues, which can result in low sensitivity in the tumor tissue. To improve stability and specificity, $^{19}$F contrast agents can be encapsulated into nanogels that specifically target a tumor. The diameter of the nanogel is pH-sensitive, and indirectly measuring a change in diameter via $^{19}$F MRS provides a pH measurement [48].

Figure 1.10. $^{19}$F MR spectroscopy of tumor pH.
A) The $^{19}$F MR spectrum of ZK-150471 shows two peaks. B) The chemical shift difference of these two peaks is correlated with pH. C) The average pH measured with $^{19}$F MRS showed a decrease after heating with and without 5 mg/kg hydralazine. Reproduced with permission from reference [47].

Despite the promise of $^{31}$P and $^{19}$F MRS for measuring tumor pH, these MRS methods have many detriments for clinical use. Most clinical MRI instruments do not have the capability to measure isotopes other than $^1$H. A typical $^1$H MRS result requires long acquisition time greater than 30 min, and provides only coarse resolution of
approximately 1 cm³. Due to the low signal to noise ratio (SNR), MRS require high degree of magnetic field homogeneity and minimal movement [37]. Over the years, strategies have been developed to reduce acquisition time and/or to increase spatial resolution. For example point resolved spectroscopy (PRESS) [49] and stimulated echo acquisition mode (STEAM) [50] have been developed to enable simultaneous acquisition of spectra from multiple volumes 1 mm³ spatial resolution. However, the relationship between SNR, spatial resolution, and acquisition time are dictated by the physics of the nuclear spins and electronics. Hence, improved resolution or reduced acquisition time is always associated with reductions in SNR. For example, a 40 mL voxel interrogated with a clinical 1.5 T MRI instrument requires 30 min acquisition time, while a 105 mL voxel requires only 6 min of acquisition time with a 1.5 T MRI instrument [38].

1.2.2.2 Hyperpolarized ¹³C Spectroscopy
The ¹³C isotope has 1.1% natural abundance and a MRS sensitivity of 0.016% relative to ¹H MRS at 37°C. Dynamic nuclear polarization (DNP) can be used to increase the sensitivity of ¹³C MRS [51]. This technique involves cooling a ¹³C labeled molecule to ~1 K and then transferring polarization from electron spins to ¹³C nuclei with microwave irradiation. The sample is then warmed rapidly to body temperature while retaining a high level of nuclear spin polarization. A ratio of H¹³CO₃⁻ and ¹³CO₂ from injected ¹³C labeled bicarbonate is correlated with pH (Figure 1.11) [46]. Each acquisition is ~ 5 sec and yields a resolution of 6.14 cm³ per voxel. Despite the ultrafast acquisition, hyperpolarized ¹³C has a rapid decay of ~20 sec and requires a special transceiver coil
and hyperpolarizer. Furthermore, $^{13}$C labeled bicarbonate can only measure a weighted average of pHi and pHe.

![Figure 1.11](image)

Figure 1.11. Hyperpolarized $^{13}$C MR spectroscopic imaging of tumor pH. A) $^{13}$C MR spectroscopy was used to measure the ratio of $[^{13}CO_3]/[^{13}CO_2]$, which was used to calculate pH based on the Henderson-Hasselbalch equation, $pH = pK_a + \log_{10}(^{13}CO_3/^{13}CO_2)$. B) A solid line shows the correlation of the pH values determined from $^{13}$C MRS and an electrode. The dashed line representing equal measurements if provided as a visual reference. C) A subcutaneously implanted EL4 tumor in a mouse is outlined in red in an axial MR image. D) The pH map of the same mouse calculated from $^{13}$C MR spectroscopic imaging. The tumor margin is outlined in white. Reproduced with permissions from reference [46].

1.2.2.3 $T_1$ relaxivity MRI

The use of a paramagnetic metal complex as a MRI contrast agent is now widely accepted in diagnostic radiology. Agents currently approved for clinical use are based on low-molecular weight chelates of gadolinium that partition throughout all extracellular space and enhance the $T_1$ MR relaxation of nearby water protons. The $T_1$ relaxivities of some MRI contrast agents are pH-dependent, such as Gd(III) chelates that have a pH-
sensitive ligand, which can block water from accessing the Gd(III) ion only under certain pH conditions [35, 52-60]. Other agents currently approved for clinical use are based on superparamagnetic iron oxide nanoparticles that enhance the T₂ MR relaxation of nearby water protons. For example, iron oxide nanoparticles encapsulated within pH-responsive nanocapsules [61] and pH-sensitive hydrogels [62] exhibit different T₂ relaxation properties under different pH conditions.

Among these relaxation-based MRI contrast agents, Gd-DOTA-4AmP has been applied to measure in vivo pHe with the greatest success [63]. Knowledge on the concentration of Gd-DOTA-4AmP is required to convert the T₁-weighted MRI signal to a pHe measurement, because the MRI signal is dependent on both concentration and the T₁ relaxation effect of the agent. A pH-unresponsive contrast agent with an analogous chemical structure can be used to account for the concentration of pH-responsive agent [35]. For example, two serial boluses of Gd-DOTA-4AmP and Gd-DOTP have been used to measure tumor pHe in C6 glioma and renal carcinoma [64-66]. The T₁-weighted MRI signal from Gd-DOTP was used to determine the temporally dynamic concentration of this agent in the tumor, which was assumed to be identical to the temporally dynamic concentration of Gd-DOTA-4AmP. Based on this concentration, the T₁-weighted MRI signal of Gd-DOTA-4AmP was used to determine the pHe. However, this method with serial injections makes the risky assumption that the biodistributions are identical for two MRI contrast agents that are administered at different times. To avoid this assumption, a single cocktail of Gd-DOTA-4AmP and Dy-DOTP has been administered to a mouse model of C6 glioma (Figure 1.12) [67]. The Gd-DOTA-4AmP dominated the T₁
relaxation process generated by the agents, whereas Dy-DOTP dominated the $T_2^*$ relaxation process of the agents. The $T_2^*$ relaxation effect was then used to determine the pixel-wise concentration of Dy(III), which was assumed to be identical to Gd(III). This value was used to convert the pixel-wise $T_1$-weighted MRI signals into pH$_e$ maps. In a similar *in vitro* study, the ratio of $T_1$ and $T_2$ relaxation processes of a single agent, [GdDOTAam)$_{33}$ – Orn$_{205}$], was shown to be correlated with pH [68]. However, this large-molecule contrast agent has not been demonstrated with *in vivo* studies, and may not be clinically translatable because large-molecule Gd(III) chelates are potentially toxic.

![Figure 1.12](image)

**Figure 1.12.** Relaxation-based MRI of Gd-DOTA-4Amp and DyDOTP can measure tumor pH$_e$.

A) The change in water linewidth before injection (left) and after injection (right) is used to estimate the concentration of the agent. B) A parametric map of the $r_1$ relaxivity of the agent in a glioma model is obtained from a $T_1$-weighted map and the concentration of the agent. C) The $r_1$ relaxivity of the agent is pH-dependent, D) which can be used to convert the $r_1$ relaxivity map to a pH map (color scale bar shows pH units). Reproduced from reference [67] with permission.
1.2.2.4 Chemical Exchange Saturation Transfer MRI

A chemical exchange saturation transfer (CEST) MRI experiment can selectively detect multiple proton exchanging sites within the same contrast agent. This provides a great advantage relative to relaxivity-based MRI methods, because a ratio of CEST effects from the same CEST agent is independent of concentration. If one or both of the CEST effects is dependent on pH, then this ratio can be used to accurately measure pH. CEST MRI has advantages relative to other imaging methods that measure pH. CEST MRI does not require special instrumentation such as hyperpolarizer for $^{13}$C MRS, or a detector coil for $^{19}$F, $^{31}$P or $^{13}$C MRS. CEST MRI allows full body imaging and its not limited by depth of view as with fluorescence imaging. CEST MRI uses relatively low energy radio frequency and does not raise safety concerns like EPR or PET imaging. Most importantly, CEST MRI is non-invasive, which is a great improvement relative to invasive electrodes and microsensors.
Table 1.1. Summary of pH measurements for various instrumentations

<table>
<thead>
<tr>
<th>Method</th>
<th>Sampling time</th>
<th>Spatial resolution</th>
<th>Agent [C]</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microelectrode</td>
<td>Fast, sec</td>
<td>Localized measurement</td>
<td>No agent required</td>
<td>fast; accurate if calibrated with an external buffer; limited to surface-accessible tumors; requires MRI to guide electrodes into tumors</td>
<td>[16,17]</td>
<td></td>
</tr>
<tr>
<td>Fluorescence imaging</td>
<td>Fast, sec</td>
<td>5 µm</td>
<td>µM – nM</td>
<td>sensitive; low cost; can be used during clinical fluorescence guided surgery; limited to surface-accessible tumors</td>
<td>[14, 18-25]</td>
<td></td>
</tr>
<tr>
<td>PET</td>
<td>10 min</td>
<td>2 mm</td>
<td>nM</td>
<td>fast; whole body imaging; requires radioactive isotope; coarse resolution; limited accuracy</td>
<td>[27-30]</td>
<td></td>
</tr>
<tr>
<td>¹H MRS</td>
<td>&gt; 30 min</td>
<td>1 mm³</td>
<td>mM</td>
<td>simultaneous measurement of pH and detection of metabolites; poor sensitivity; some agents are pH buffers that change tissue pHe</td>
<td>[37-39]</td>
<td></td>
</tr>
<tr>
<td>³¹P MRS</td>
<td>40 min</td>
<td>1 cm³</td>
<td>mM</td>
<td>Can simultaneously measure pHi and pH; Requires a ³¹P MRI transceiver coil</td>
<td>[40-44]</td>
<td></td>
</tr>
<tr>
<td>¹⁹F MRS</td>
<td>5 min</td>
<td>1 cm³</td>
<td>µM – mM</td>
<td>fast; good sensitivity; Requires a ¹⁹F MRI transceiver coil</td>
<td>[45, 47, 48]</td>
<td></td>
</tr>
<tr>
<td>Hyperpolarized ¹³C MRS</td>
<td>5 sec</td>
<td>6.14 cm³</td>
<td>mM</td>
<td>very fast; Requires a ¹³C MRI transceiver coil; requires a hyperpolarizer instrument; Short hyperpolarized ¹³C life time; Measures pH and pHe</td>
<td>[46]</td>
<td></td>
</tr>
<tr>
<td>pH dependent T₁ relaxation</td>
<td>&lt; 1 min</td>
<td>0.1 cm³</td>
<td>mM</td>
<td>fast; high resolution; Requires a cocktail of contrast agents</td>
<td>[64-67]</td>
<td></td>
</tr>
<tr>
<td>CEST MRI</td>
<td>~5 min</td>
<td>µm – mm</td>
<td>mM</td>
<td>good specificity; poor sensitivity</td>
<td>[35]</td>
<td></td>
</tr>
</tbody>
</table>
1.3 CEST

1.3.1 CEST Definition

CEST MRI employs an endogenous or exogenous chemical agent that has a proton which can exchange with water at a slow-to-moderate rate of 100 – 5000 Hz (Figure 1.13A). The protocol for CEST MRI starts with saturating a specific resonance frequency of an exchangeable proton on the CEST agent (Figure 1.13B). Saturation is a condition wherein the number of nuclear spin magnetic moments (“spins”) aligned against the field is increased at the expense of spins aligned with an external magnetic field. This soft irradiation pulse temporary disrupts the Boltzmann distribution of spins and leads to a decrease in MRI signal amplitude of the exchangeable proton in a NMR spectra, which depends on the net difference of spins aligned with and against the magnetic field [69]. Due to the natural process of chemical exchange of labile protons between molecules, this proton on the CEST agent is transferred to a near-by water molecule *(Error! Reference source not found. 3C)*. The spin aligned against the field from the exchangeable proton is transferred to the population of spins for water, in exchange for a spin aligned with the field that is transferred to the population of spins for the agent. This transfer of saturation from the exchangeable proton to the water proton reduces the MRI signal amplitude of water. In practice, the CEST MRI protocol starts with a series of selective saturation RF pulses (Figure 1.13D), followed by a standard MRI acquisition sequence that can measure the amplitude of water signal throughout the image (Figure 1.13D,E). A CEST spectrum is obtained by iterating a series of saturation frequencies and recording the normalized water signal amplitudes (Figure 1.13E,F).
Figure 1.13. Chemical exchange saturation transfer.
A) The number of magnetic moments aligned with the $B_0$ static magnetic field is greater than the number aligned against the $B_0$ field for n amide proton and a water proton. B) Selective saturation of the MR frequency of the amide proton causes the magnetic moments to equilibrate between states. C) Subsequent chemical exchange of the amide proton and water proton transfers some of the saturation to the water protons, causing a partial equilibration of the states for the water protons. D) The CEST-FISP MRI protocol consists of a series of Gaussian-shaped saturation pulses repeated “m” times, followed by a FISP MRI acquisition sequence. The entire process is repeated for a series of “n” saturation frequencies. E) Fourier transformation of the frequency-domain signals creates a series of “n” MR images at each saturation frequency. F) The integral of the MR signal of the tumor is plotted as a function of saturation frequency for the “n” images, creating a CEST spectrum. A sum of three Lorentzian line shapes was fitted to the experimental CEST spectrum to quantify the CEST effects at 5.6 and 4.2 ppm, and also account for the direct saturation of water at 0.0 ppm (black line, raised 10% above the CEST spectral peaks to improve the view).

The CEST agent typically has a low concentration in the 1 to 10 mM range. The MR spectroscopic resonances of the agent are not directly observable during a standard MRS acquisition due to the low concentration of agent relative to the concentration of water.
However, with continuous RF irradiation (Figure 1.13D), the saturation from the agent is continuously transferred to the bulk water (Figure 1.13C), resulting in a reduction of water intensity that is observable by MRI (Figure 1.13E,F). Hence, this continuous transfer of saturation serves as amplification for the agent and allows for indirect observation of the CEST agent at low mM concentrations [70].

1.3.2 The CEST MRI Pulse Sequence

The general scheme of a CEST experiment is saturation followed by MRI acquisition (Figure 13D,E). The RF saturation is characterized by its total duration ($T_{sat}$), amplitude ($\omega_1 = \gamma B_1$) and off-resonance frequency ($\Delta_{RF}$). The effectiveness of spin saturation depends upon an ability to selectively irradiate at the specific proton frequency. Due to hardware limitations, most imaging applications employ a train of shaped RF pulses to simulate a continuous wave function (Figure 1.13D). Typically, Gaussian or Half-Sinc shaped pulses are interleaved with delays and crusher gradients. Other saturation techniques, such as on-resonance, positive CEST, FLEX, dual saturation methods, DQCEST, use other selective saturation methods that are nicely described in another review [70].

The CEST effect approaches steady state when the saturation pulses are applied for a time longer than the $T_1$ relaxation time of the bulk water [71]. During the earliest CEST studies performed in solution, up to 15 seconds (> 3 $T_1$) of saturation time was used to ensure steady-state saturation, because the $T_1$ relaxation times of chemical solutions are typically 3-5 seconds [72]. During more recent in vivo studies with mouse models, the
saturation time is usually set to 3 to 5 seconds to provide a practical time frame with anesthetized mice, and because the $T_1$ relaxation time is typically 1-2 seconds for most tissues. Although complete saturation is not achieved with this shorter saturation time, the incomplete saturation can be accounted for during the analysis of the results, as long as steady state is still achieved [73,74]. Following saturation, a short acquisition time is required because the altered spin population of the water that generates CEST sensitivity can be lost during a longer acquisition time.

Spin echo (SE) [72,75] and gradient echo (GE) [76] MRI acquisition sequences were initially coupled with the saturation period to create a CEST MRI protocol. The long repetition time (TR) in SE acquisition (typically TR > 3 $T_1$) allows complete longitudinal relaxation and provides the highest possible CEST contrast for a given CEST preparation and is considered as the “gold standard” [77]. GE acquisition is a faster acquisition method, but still requires considerable time. These slow CEST-SE and CEST-GE methods are acceptable for in vivo studies of exogenous CEST agents only when two saturation frequencies are used, because studies with more saturation frequencies simply require too much time for anesthetized mice.

In practice, the acquisitions of CEST images with a series of saturation frequencies (Figure 1.13E) provide a more robust CEST spectrum, (Figure 1.13F) which may be used to correct for $B_0$ magnetic field inhomogeneity, and other effects that compete with CEST such as the direct saturation of water and the Magnetization Transfer (MT) effect.
Therefore, studies have been conducted that use short MRI acquisition following the long (~5 sec) CEST saturation.

Fast SE methods or the Rapid Acquisition with Relaxation Enhancement (RARE) method can reduce the number of acquisitions by collecting a train of spin echoes [77]. These signals can be acquired in ways that fill a large portion of the matrix of MR frequencies used to construct the image (also known as k-space) with just one saturation-excitation event. For example, a RARE acquisition method with a factor of 16 [78-80] and 64 [81,82] have been used to acquire a matrix of 128 k-space lines, which only required 8 (128/16) and 2 (128/64) saturation-excitation events, reducing the total acquisition time by 16- and 64-fold. Although higher RARE factors equate to shorter acquisition time, there is also increased radiofrequency deposition in tissues (measured as the specific absorption rate) and the potential for unintentional CEST or MT effects from multiple refocusing pulses [83].

Spoiled Gradient Echo (SGE) or Fast Low Angle SHot (FLASH) MRI have a very rapid acquisition time, typically less than 1 sec [84]. A small excitation angle allows for faster recovery of longitudinal magnetization that permits a shorter TR and minimizes T1-weighting in the image. CEST-FLASH has been shown to be useful for measuring CEST effects in samples with a short T1 relaxation time [77]. Echo Planar Imaging (EPI), which is currently one of the fastest MRI acquisition methods, uses a single excitation pulse followed by a continuous signal acquisition in the form of a gradient echo train [85,86]. CEST-EPI has been performed with MRI scanners that have lower magnetic field
strengths [85]. However a low excitation angle during EPI increases sensitivity to magnetic susceptibility artifacts. EPI is especially prone to ghost images due to gradient imperfections that perturb spatial encoding, and chemical shift artifacts due to narrow readout bandwidth in the phase-encoding direction [83]. Therefore, CEST-EPI has been more problematic to implement at higher magnetic field strengths.

Fast Imaging with Steady-state free Precession (FISP), also known as steady-state GE, is a fast imaging technique introduced to compensate for the shortcomings of CEST-RARE and CEST-EPI. In an FISP acquisition, the residual transverse magnetization after an RF pulse is conserved and will participate in the subsequent signal until steady state is achieved. CEST-FISP has a 4-fold reduction in SNR as compared to CEST-SE, but the decrease in SNR is compensated by a 50-fold reduction in acquisition time [87]. Therefore, rapid FISP acquisitions allow a more sensitive image analysis to quantify both physical and physiological parameters. In fact, CEST-FISP allowed the detection of pH in the tumor region while CEST-RARE did not when using exogenous CEST agents [74,81].

In an alternative approach, a long pre-saturation pulse can be split into a multitude of shorter pre-saturation pulses that are interleaved with an ordinary two-dimensional EPI acquisition for multi-slice CEST imaging [88]. Spins in a given slice have a longer time to relax before the next observation pulse occurs at their resonance frequency. Hence the CEST effect accumulates with an increasing number of image slices. Multislice CEST-imaging has been applied to in vivo amide proton transfer (APT) imaging [89,90].
However, the pulse designed to excite spins in one slice may excite the CEST agent in another slice. To combat this issue, the RF irradiation can be divided into two uneven segments: a long primary RF pulse that introduces the steady state CEST contrast, and a repetitive short secondary RF irradiation module to lock the CEST contrast. Such methods have been applied to the in vivo imaging of acute stroke animals [91].

Table 1.2. Summary of MRI acquisition method for CEST imaging

<table>
<thead>
<tr>
<th>Method</th>
<th>Typical Sat. time</th>
<th>Acq. time</th>
<th>Total time</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEST-SE</td>
<td>5 sec</td>
<td>25.6 min</td>
<td>25.7 min</td>
<td>[87]</td>
</tr>
<tr>
<td>CEST-GE</td>
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<td>15 min</td>
<td>15.1 min</td>
<td>[76]</td>
</tr>
<tr>
<td>CEST-FLASH</td>
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<td>0.64 sec</td>
<td>3.64 sec</td>
<td>[84]</td>
</tr>
<tr>
<td>CEST-RARE(^2)</td>
<td>5 sec</td>
<td>48 sec</td>
<td>53 sec</td>
<td>[80]</td>
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<td></td>
<td></td>
<td>12 sec</td>
<td>17 sec</td>
<td></td>
</tr>
<tr>
<td>CEST-EPI</td>
<td>3 sec</td>
<td>6 sec</td>
<td>9 sec</td>
<td>[85]</td>
</tr>
<tr>
<td>CEST-FISP</td>
<td>5 sec</td>
<td>0.4 sec</td>
<td>5.4 sec</td>
<td>[87]</td>
</tr>
</tbody>
</table>

1. MRI acquisition time for acquiring 128 k-space lines. This time does not include the CEST saturation pulse.
2. The CEST-RARE acquisition method is listed with RARE factors of 16 and 64.

1.3.3 CEST Spectrum Analysis

The most common and most simplistic method for analyzing a CEST spectrum is to evaluate magnetization transfer asymmetry (Figure 1.14A and Equation 1.1). This evaluation method compares the amplitude of the water signal upon saturation at the MR frequency of the exchangeable proton of the contrast agent (\(\omega\)) with the water signal upon saturation at the contralateral frequency (\(-\omega\)) [92]. This difference is normalized to the total water signal in the presence of saturation, \(M(-\omega)\). Some analysis methods substitute the denominator with \(M_0\), which represents water signal without saturation.

\[
\%CEST = \frac{M(-\omega) - M(\omega)}{M_0} \tag{1.1}
\]
Figure 1.14. Graphical illustration of CEST spectrum analysis.
A) MTR asymmetry analysis [72], B) integral of signal area and is complementary analysis [95], C) linewidth analysis [96], and D) Lorentzian line shape analysis [97].

MTR asymmetry analysis is a simple and powerful tool when: 1) the background CEST spectrum is symmetrical, and 2) the resonance frequency of the bulk water proton is exactly at 0.0 ppm throughout the entire image (i.e., the system has perfect $B_0$ homogeneity). In an in vivo setting, these two conditions are rarely met. First and foremost, exchangeable hydroxyl, amide and amine groups on mobile proteins and peptides contribute to an endogenous MT effect that ranges from 0.5 to 120 ppm, with the maximum at ~3.5 ppm [93,94]. This leads to considerable background noise especially for applications with endogenous exogenous diamagnetic CEST agents that have a $\omega$ MR frequency near water (Section 1.4.2 and 1.4.4). Furthermore, tissue
heterogeneity and non-optimal sample shimming contribute to the deviation of bulk water chemical shift from 0.0 ppm. Such imperfections in CEST spectra limit our ability to detect CEST contrast from noisy \textit{in vivo} data. In recent years, many studies have been performed to tackle these limitations and improve the detection of CEST, which are outlined below.

1.3.3.1 Non-punctual analyses

Instead of evaluating the CEST effect from two punctual intensities, an integral of the signal area from a given range of frequencies has also been assessed (Figure 1.14B and Equation 1.2) \cite{95}. This integral approach is more robust and less sensitive to noisy CEST spectra because it contains information from a range of frequencies in the neighborhood of the offset frequency where the CEST effect is at the maximum. In an empirical observation, the integral approach is a more accurate evaluation of CEST contrast than the traditional punctual analysis for a paramagnetic CEST (PARACEST) agent (Section 1.4.3), while the complementary integral approach (Equation 1.3) is more accurate for evaluating a diamagnetic CEST (DIACEST) agent. A detriment to the integral approach is the influence of $T_2$ relaxation on the evaluation of the CEST effect. Furthermore, the Bloch-McConnell equations modified for chemical exchange only assess the effects of a single saturation frequency. Thus, the integral approach cannot be directly linked to these equations, which limits theoretical analysis of this integral approach.

\begin{equation}
    CEST = \frac{A^{\text{OFF}} - A^{\text{ON}}}{A^{\text{OFF}}} \tag{1.2}
\end{equation}
Another approach is linewidth analysis of a single CEST peak (Figure 1.14C) [96]. The linewidth is defined as the full width at half maximum of the CEST peak. Although the linewidth analysis does not directly measure CEST effects, the width of peak is proportional to pH (Equation 1.4, where $pK_W$ is the ionization constant of water). The linewidth analysis is independent of agent concentration for a given saturation pulse shape, duration and power. It has been demonstrated that the linewidth analysis is less sensitive to temperature fluctuation within the physiological pH range of 6.0 to 7.5, than pH > 7.5. These features are important in an in vivo setting where fluctuations in concentration and temperature can be an issue. However, linewidth analysis is not suitable for evaluating overlapping CEST effects.

\[ \text{Linewidth} \propto 10^{PH-pK_W} \quad (1.4) \]

The CEST effect has been modeled to be a Lorentzian line shape for exchangeable protons in a liquid-like state, and a Gaussian line shape for exchangeable protons in a semi-solid state (e.g., with a $T_2$ relaxation time of 13 µs) [97,98]. Lorentzian line shapes can be used to deconvolute the CEST effects of multiple exchangeable protons in a liquid-like state, such as the CEST effects of two amides on a single agent [97]. Lorentzian line shape fitting can also separate the CEST effect from MT and direct saturation of bulk water [99] and account for variance in the chemical shifts of bulk water due to $B_1$ magnetic field inhomogeneity. Due to these advantages, we chose to use Lorentzian line shape analysis for our studies.
1.3.3.2 Smoothing Function

A smoothing function can be applied to correct noisy CEST spectra, to compensate for low SNR without any a priori model. For example, a $12^{\text{th}}$-order polynomial function can be used to improve the analysis of the CEST effects of endogenous proteins in the brain [100,101]. The water resonance is assumed to be at the frequency with the lowest signal intensity of the interpolated fitted curve. After fitting, the measured curve minima are shifted to 0.0 ppm to account for $B_0$ magnetic field inhomogeneity. This high-order polynomial interpolation is more suitable for endogenous CEST and DIACEST agents, which mainly have CEST effects concentrated at low frequency ranges around the bulk water resonance. When considering high-frequency signals, a high-order interpolating polynomial can create large oscillations, resulting in high interpolation error. For PARACEST agents that have a higher frequency offset, a $3^{\text{rd}}$-order polynomial smoothing spline is more applicable [102]. This low order polynomial smoothing has also been applied to the analysis of a DIACEST agent for \textit{in vivo} imaging [81,103].

1.3.3.3 Contrast to Noise Threshold

To ensure that the calculated CEST contrast does not arise from noise within the \textit{in vivo} CEST spectra, a contrast-to-noise (CNR) threshold filter can be included in the evaluation. For example, additional filtering criteria such as a high coefficient of determination for the interpolating curve ($R^2 > 0.99$) can be applied to ensure the validity of CEST contrast. This is because noisier CEST spectra results in lower $R^2$ values [104]. A threshold for the minimum CEST amplitude can also be set. Some studies of DIACEST agents have applied an arbitrary threshold for the CEST amplitude [81,104].
In another example, the threshold for a PARACEST agent Yb-HPDO3A was set at above the average CEST value obtained from the pre-contrast image [82]. A more analytical approach sets the minimum threshold to $2\sqrt{2}$ times the standard deviation of the image noise which represents a 95% probability that the CEST amplitude is real [74]. Alternatively, a minimum threshold of $3\sqrt{2}$ times the standard deviation of the image noise represents a 99% probably of detecting a real CEST signal [73,77]. We have selected this more analytical approach for our studies and set a CNR threshold of $2\sqrt{2} \times$ noise for a 95% probability of determination.

1.3.3.4 Numerical Approach

The well-established Bloch-McConnell equations (Equation 1.5) can be modified to include parameters that model chemical exchange [104], such as the water exchange rates, chemical shifts of the agent, relaxation rates and applied $B_1$ field [71]. In its simplest form, a set of six simultaneous equations have been used to model two exchanging pools for endogenous amide proton transfer that represent an amide proton and bulk water [106]. For an agent with two exchanging protons, such as Eu-DOTAM$^{3+}$ that generates CEST effects from the labile amide proton and a bound water molecule, nine simultaneous equations have been proposed for a three-exchange pool model [71]. To more accurately navigate the in vivo environment, a four-pool model has been proposed to include additional information about relaxation times, proton exchange rates and concentrations associated with macromolecules in tissue [106]. This model has also been elegantly extended for an arbitrary number of pools by using empirical coupling terms to link a matrix of pools [107]. The accuracies of applications of such numerical
approaches depend on the accuracy of parameter estimates. For example, the chemical
shift of the agent-bound water and the $T_2$ of the macromolecular protons can be measured
accurately, while the exchange rates and the $T_1$ and $T_2$ relaxation time constants of bulk
water can only be approximated. This is particularly relevant when many parameters are
unknown or difficult to measure, such as $T_1$ and $T_2$ in vivo, and the concentration of the
mobile protons of the CEST agent. Another apparent disadvantage is the lack of an
algebraic simplification for more complex multi-pool systems, and solving 6, 9 and 12
simultaneous equations for two-, three- and four-pool model can be a daunting and
computer intensive task [71].

$$\frac{dM_x(t)}{dt} = \gamma (M(t) \times B(t)) - \frac{M_x(t)}{T_2}$$ (1.5a)

$$\frac{dM_y(t)}{dt} = \gamma (M(t) \times B(t)) - \frac{M_y(t)}{T_2}$$ (1.5b)

$$\frac{dM_z(t)}{dt} = \gamma (M(t) \times B(t)) - \frac{M_z(t)-M_0}{T_2}$$ (1.5c)

1.3.3.5 Summary

There are numerous post-acquisition data analysis methods that evaluate the CEST effect
from noisy in vivo imaging results. Different research groups use their own unique set of
techniques to perform CEST spectrum analysis. Some of these techniques are unique to
an individual group, while others are common among various groups. Typically, a set of
techniques is required to analyze in vivo CEST spectra. For example, our research
approach used Gaussian filtering or 3 x 3 binning, cubic spline smoothing, Lorentzian
line shape fitting, followed by $2\sqrt{2}$ x noise thresholding for in vivo pH measurements
with iopromide [74, unpublished work]. For its analogue iopamidol, Aime and colleagues used spline smoothing, interpolation, interpolation thresholding, and a noise threshold [81,103].

1.4 CEST MRI CONTRAST AGENTS

1.4.1 The characteristics of a CEST MRI contrast agent

For in vivo applications, MRI contrast agents must have high water solubility with low osmolality and low biochemical toxicity. The CEST effect from the agent is governed by Equation 1.1 and 1.6 [97].

\[
\frac{M_s}{M_0} = \frac{1}{1 + T_{1\text{sat}} k_{CA} \left( \frac{n_{CA}[CA]}{n_{H_2O}[H_2O]} \right)}
\]

(1.6)

where:

- \(M_s, M_0\): water magnetization with and without selective saturation of the agent
- \(T_{1\text{sat}}\): \(T_1\) relaxation time in the presence of selective saturation
- \(k_{CA}\): chemical exchange rate of a proton from the agent to water
- \(n_{CA}, n_{H_2O}\): the number of magnetically equivalent exchangeable protons on the contrast agent and water molecule
- \([CA], [H_2O]\): the concentration of contrast agent and water

As depicted by Equation 1.6, increasing the number of equivalent protons on the agent will increase the CEST effect. There are numerous contrast agents that exploit this approach, such as Yb-DOTAM with 8 exchangeable protons [108], homomeric polypeptides [109], and dendrimers of Yb(III)-DOTAM [110]. These approaches may
decrease the minimum concentration of CEST agent that is needed to create a detectable CEST effect. Notably, the CEST effect achieved by a 5 mM concentration of a single monomer can also be achieved by a 0.3 mM concentration of a dendrimer that carries 16 monomers, which shows that the CEST effect scales with the number of monomers in a small polymeric system. Nonetheless, the effective concentration of monomers within the polymer is still 5 mM in this example [110]. Therefore, if the monomer is potentially toxic, such as a monomer that contains a lanthanide ion, then polymerization does not adequately address this toxicity problem. Furthermore, the larger diameter of a polymer may affect delivery of the agent into tumor tissues. For these reasons, we elected to use a nontoxic small molecule CEST MRI contrast agent for our studies.

For CEST to be successfully detected, the chemical exchange rate of the labile proton needs to be in the slow to intermediate regime. More specifically, the chemical shift difference between bulk water and agent (\(\omega\)) has to be greater than the exchange rate, \(k_{CA}\) (\(\omega > k_{CA}\)). The chemical shift of DIACEST agents is typically within a range of 0.5 to 5 ppm from water, and the exchange rates of these DIACEST agents are on the order of ~2 x 10\(^3\) s\(^{-1}\) or slower. These ranges encompass the exchange lifetimes of amides and hydroxyl groups. PARACEST agents can exhibit large pseudocontact shift up to 700 ppm, hence the exchange rates can range up to 21,000 Hz for studies performed with a 300 MHz, 7T MRI scanner. Faster exchange rates theoretically allow detection of much lower concentrations, because the CEST effect can be quickly transferred to water relative to the slower T1 relaxation rate. In addition, the large chemical shift (\(\omega\)) of most PARACEST agents enables the selective RF pulse to be applied far from the bulk water
resonance, which reduces direct water saturation. For in vivo applications, a large chemical shift of PARACEST agents beyond ±120 ppm also avoids the overlap with magnetization transfer contrast that arises from background endogenous semisolid macromolecules.

1.4.2 Endogenous CEST
An ingenious application of CEST is to detect exchanging groups that are already present in the tissue, such as hydroxyl, amide and amine groups in proteins and peptides. Endogenous CEST avoids the need of an exogenous contrast agent, resulting in high impact in both in vivo applications and clinical translatability. For example, glycoCEST detects hydroxyls (0.5 – 1.5 ppm) from glycogen in liver and muscle [111], gagCEST detects hydroxyls (0.9 – 1.5 ppm) from glycosaminoglycan in cartilage [112], gluCEST detects amines (3.0 ppm) from glutamate in brain [113] and amide proton transfer (APT) detects amides (3.5 ppm) from proteins and peptides in brain [100].

Among the established endogenous CEST methods studied, only APT can measure differences in pH. The chemical exchange between the amide protons and the bulk water is base-catalyzed, so that the exchange rate decreases with decreasing pH. The pH-weighted APT imaging method has been widely used to study acute ischemic stroke in an animal model (Figure 1.15) [101,114-118].
Figure 1.15. Endogenous CEST MRI of tumor pHi.
A) The asymmetry of the magnetization transfer ratio (MTR$_{\text{asym}}$), a measure of CEST, decreases after ischemia is induced in a rat model. B) This decrease in CEST, also represented as a change in the amide proton transfer ratio ($\Delta$APTR), has been correlated with pHi as measured with $^{31}$P MR spectroscopy. C) The MTR$_{\text{asym}}$ can be mapped in a rat model of ischemia, D) which can be converted into a pH map. Reproduce with permission from references [100, 114, 117].

When using APT to evaluate pH, the generated contrast depends on many parameters which compromises the measurement of pH. These other parameters include water proton concentration, amide proton concentration, spin-lattice relaxation rate and saturation time when measuring CEST via MTR$_{\text{asym}}$ at 3.5ppm (Equation 1.7) [100]. For this reason, the pH-weighted APT image can measure relative changes in pH, but no absolute pH value can be calculated. In the past, pH-weighted APT images have been correlated with apparent diffusion coefficient (ADC) images [116-118], isotropic diffusion-weighted
images [100,105], and lactic acid content using non-imaging methods [114,116].

Alternative quantification methods have been applied, such as calibrating with various pH solutions of creatine [85], cross reference with $^{31}$P MRS [100], and employing QUEST/QUESP to establish a relationship between the amide proton transfer ratio (APTR) and pH [119].

$$
MTR_{\text{sym}}(3.5\text{ppm}) = MTR_{\text{sym}}(3.5\text{ppm}) + \frac{[\text{amide proton}]}{[\text{water proton}]} \cdot \left(1 - e^{-\frac{R_{1w}}{R_{1w}^{\text{sat}}}}\right) \cdot k_{\text{base}} \cdot 10^{\text{pH-pK}_w} \quad (1.7)
$$

Due to the high concentration of mobile proteins and peptides in the cytoplasm, APT mainly measures intracellular pH [100]. Furthermore, as shown in Equation 1.7, an increased in APTR can potentially be caused by an increase in cytosolic protein and peptide content rather than intracellular pH. In fact, the increased protein and peptide content in the tumor is the most likely explanation for changes in APT contrast in tumors, because the intracellular pH is highly regulated by active proton exporting systems [5] and there is usually only a small difference (< 0.1 pH unit) between a malignant tumor and normal tissue. Thus, APT imaging would be a more appropriate tool to provide visual information about the presence and grade of tumor based on increased content of mobile proteins and peptides, as shown in past clinical studies [85,91].

1.4.3 Exogenous PARACEST agents

Paramagnetic Gd(III) is the most commonly used contrast agent for MRI because it is the most efficient at relaxing bulk water protons, providing excellent contrast in a $T_1$ weighted image. However, having such a rapid water exchange effectively reduces water proton pseudocontact shifts to near zero, which makes it unsuitable for CEST detection.
On the contrary, other lanthanide ions have slower exchange rates and are more suitable as PARACEST agents [120].

PARACEST agents contain a lanthanide ion that greatly shifts the MR resonance frequency of the exchangeable amide protons from the MR frequency of water, which expands the range of MR frequencies that can generate a CEST effect, away from the direct saturation of water experienced in the APT experiment. This expanded frequency range facilitates the development of an agent with two CEST effects that have different MR frequencies. Due to the variety of the chelation chemistry, PARACEST agents can be designed to possess both pH-responsive and pH-unresponsive CEST effects within a single agent. A ratio of the two CEST effects can then be used to measure pHe without complications of the concentration term (equation 1.6 and 1.8) [97], unlike approaches with Gd(III) pH-responsive relaxivity agent or APT.

\[
\frac{[M_0-M_s]}{[M_s]}_{CA1} = \frac{\nu_{CA2}K_{CA2}}{\nu_{CA1}K_{CA1}} \tag{1.8}
\]

Aime and colleagues were the first to design a series of pH-responsive ratiometric PARACEST agents (Ln-DOTAM-Gly, Ln = Pr, Nd, Eu) [121-123]. The CEST effect from an amide is pH-responsive while the metal-bound water is pH-unresponsive. The ratio of CEST effects from Pr-DOTAM-Gly gave the most sensitive pH response over a range of pH 5.5 – 7.5. Unfortunately, owing to fast exchange rate of metal-bound water at physiologic temperature, high saturation of 87.6 µT power was required [123]. Such high saturation power exceeds the specific absorption rate (SAR) and limits the agent’s applicability for in vivo tumor pH_e measurements.
To avoid detecting the CEST effect from a metal-bound water, the same group measured the CEST effects of hydroxyl groups in Yb-HPDO3A, an analogue to FDA approved ProHance (Gd-HPDO3A) (Figure 1.16) [82,124]. The ratio of hydroxyl CEST effects arises from the two isomeric forms, and was linearly correlated within the pH range of 5.2 – 6.7. Using this agent, the pHe of early and late stage murine melanoma flank tumors were measured to be pH 6.1 and 5.8, respectively. Although this approach is promising, these pHe values are far below the typical range of acidic tumors, which ranges from pH 6.4 to 7.2. Therefore, this result may have a systematic error that produces pH measurements that are too low. Another limitation is that Yb-HPDO3A can only measure pH below pH 6.7, which is less than the physiologic pH range for tumors and normal tissues.

Our research group has previously developed Yb-DO3A-oAA that has an aryl amine and amide (Figure 1.17) [80,97]. The chemical exchange rate of an aryl amine is sufficiently slower due to the hydrogen bonding to proximal carboxylates. This allows the use of lower saturation powers (20 µT [73]) that are safer for in vivo preclinical measurements of tumor pH. In addition, the base-catalyzed chemical exchange rates of an amide and aryl amine were different, and a ratio of these CEST effects is linearly correlated to pH within the range of 6.35 – 7.57. Using this agent, the pHe measured in a mammary carcinoma flank tumor was pH 6.8 ± 0.21, which is well within the physiologic pH range [73]. Nonetheless, the use of the lanthanide ion and 20 µT saturation power are not consider safe for clinical translatability.
Figure 1.16. CEST MRI of tumor pH with Yb-HPDO3A.
A) An *in vivo* CEST spectrum of the tumor mass before and after i.v. injection of the agent into a mouse model of B16-F10 melanoma. B) A ratio of the two CEST effects is calibrated with pH at 33°C. Although the calibration is dependent on temperature, the temperature can be determined from the chemical shifts of the CEST effects. C) An anatomical image shows the location of the subcutaneous tumor. D) The pixel-wise pH map of the tumor shows a heterogeneous distribution of pH values with an average pH of 5.8. Reproduced with permission from reference [82].
Figure 1.17. CEST MRI of tumor pHe with Yb-DO3A-oAA.
A) The CEST spectrum of Yb-DO3A-oAA was fitted with Lorentzian line shapes to measure two CEST effects. B) A ratio of the CEST effects was linearly correlated with pH. C) The same CEST ratio was measured in a MCF-7 mammary carcinoma model after direct injection of the agent into the tumor tissue. D) A map of pHe was determined from the map of the CEST ratio. E) The pHe map was filtered to only retain results from pixels that had two statistically significant CEST amplitudes, resulting in a pHe map of the tumor and tube containing the agent (the other tube contained only water). Reproduced with permission from reference [80].

An alternative to measuring the magnitude of two CEST peaks is measuring a ratio of CEST at two frequencies. Wu and colleagues took a ratio of CEST signals at 55 and 49 ppm of Eu-DO3A-tris(amide), that only has one CEST effect [126]. A ratio of activation frequencies at 55 and 49 ppm is linearly correlated to pH within the range of 6.0 and 7.6 and is independent of agent concentration.
An alternative to ratiometric PARACEST is the use of linewidth analysis of a single CEST peak (Figure 1.18) [96]. The linewidth of the CEST effect of Tm-DOTAM-Gly-Lys is independent of agent concentration for a given saturation pulse conditions. Within the physiological range of pH 6.0 – 7.5, linewidth analysis is less sensitive to temperature. The agent was directly injected into mouse’s leg muscle and a pH value of 7.2 ± 0.2 was measured. This example also provides the possibility of conjugating DOTAM with peptides to improve pharmacokinetic properties such as high cellular uptake and longer intracellular retention [126,127]. However, this method of analysis is not suitable for overlapping CEST effects.

Figure 1.18. CEST MRI of leg pHe with Tm-DOTAM-Gly-Lys.
A) An in vivo CEST spectrum of the tumor mass before and after direct injection of the agent into the left mouse leg. B) The linewidth of the CEST effect is calibrated with pH. C) The in vivo pHe map and D) the in vivo temperature map is superimposed onto a pre-injection anatomical image. The temperature is determined from the chemical shift of the CEST effect. Reproduced with permission from reference [96].
1.4.4 Exogenous DIACEST agents

The first documented CEST MRI contrast agent was urea, which decreased the water proton signal in *ex vivo* kidney tissue [75]. As the chemical exchange of an amide proton with water is base-catalyzed, the CEST effect of urea was shown to be dependent on pH [92]. This CEST effect is characterized as diamagnetic CEST (DIACEST) as opposed to PARACEST agent with metal ions.

DIACEST agents typically have exchangeable protons with a chemical shift that is less than 10 ppm from the water resonance. For example, a hydroxyl proton has a chemical shift of ~1 ppm, an amine proton resonates at ~2 – 3 ppm, and an amide proton typically has a MR frequency greater than 2.5 ppm, with the basic and aromatic proton shifted more down field [72]. The first reported DIACEST agent, 5,6-dihydrouracil, has a ratio of CEST effects from the two amides that follows a sigmoidal relationship with pH [75].

Many pH dependent DIACEST agents have been investigated, including poly-\(\text{L-lysine}\) (single amide, 3.75 ppm) and polyamidoamine dendrimer (three amides, 3.4 – 3.6 ppm) [119], poly-\(\text{L-arginine}\) (guanidyl amide and amide, 1.8 and 3.6 ppm respectively) and poly-\(\text{L-threonine}\) (hydroxyl and amide, 0.6 and 3.5 ppm respectively) [109]. The DIACEST agents glycogen, \(\text{L-arginine}\) and poly-\(\text{L-lysine}\) have also been encapsulated in liposomes for lymph node mapping *in vivo*. No pH dependence on the CEST effect was reported for these liposome-encapsulated agents, but these pioneering studies offer new approaches for studying the spatial and temporal dynamics of complex biological systems. As another intriguing approach, the hydroxyl peak of poly-\(\text{L-threonine}\) has been
shown to drop as a function of pH, because the exchange rate becomes too fast compared to the chemical shift difference between water and the hydroxyl group at high pH [109].

Iopamidol (Isovue™, Bracco Imaging S.p.A.) is a FDA approved contrast agent for CT/X-Ray imaging that has been shown to be a DIACEST agent (Figure 1.19) [128,129]. This agent has .5 hydroxyl protons, two amide protons that share the same MR frequency, and an additional amide proton with a different MR frequency. Due to the iodinated aryl ring, the amide protons have MR frequencies that are significantly shifted from the water frequency, which facilitates the detection of the CEST effects from these amide protons. The ratio of the CEST effects from these amide protons are correlated to pH in the range of 5.5 – 7.4 via a third-order polynomial function [81]. In vivo kidney pH mapping was performed with healthy mice that were acidified and alkalinized with ammonium chloride and bicarbonate, and with mice that had acute kidney injury [103]. No pH mapping of a tumor was performed, presumably because the agent could not reach a sufficient concentration in the tumor for statistically significant CEST detection.

In our research studies, we plan to use a similar FDA approved CT/X-Ray contrast agent, Iopromide (Ultravist™, Bayer Healthcare Inc.) (Figure 1.20). This agent has an amide and aryl amide that have chemical shifts at 4.2 and 5.6 ppm. Coupled with the CEST-FISP acquisition method, we aim to accurately measure pHe in the tumor microenvironment.
Figure 1.19. CEST MRI of tumor pHe with iopamidol.
A) A CEST spectrum the agent shows two CEST effects at 4.2 and 5.6 ppm. Both CEST effects are dependent on saturation conditions, which indicates that CEST MRI measurements of pH should be performed with optimized saturation conditions for best results. B) The calculated pH based on a ratio of the CEST effects is correlated with experimental pH measured with an electrode. C) An anatomical image shows the locations of the kidneys. D) The pixel-wise pHe map of the kidneys shows a homogenous distribution of pHe values with an average pHe of 5.8 Reproduced with permission from reference [81, 129].

Figure 1.20. Chemical structures of iopamidol and iopromide.
<table>
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<th>Yb-DOTAM</th>
<th>Eu-DOTAM</th>
<th>Dendrimer</th>
<th>5,6-dihydrouracil</th>
<th>APT with creatine</th>
<th>APT</th>
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<td>2.2 ± 0.3 mm</td>
<td>82</td>
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</table>

Table 3: Summary of CEST methods to detect pH
<table>
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<tr>
<th>pH</th>
<th>Tm-DOTAM-Gly-Lys</th>
<th>6.0 – 7.2</th>
<th>2.48</th>
<th>s</th>
<th>4 min 56 s</th>
<th>4.25 x 10⁻³</th>
<th>12 min/kg</th>
<th>6.2 – 7.2</th>
<th>Lognormal</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.0 ± 0.3</td>
<td>Direct injection</td>
<td>50 µL</td>
<td>200 mM</td>
<td>2.8 µT</td>
<td>CEST-RARE</td>
<td>4 min 18 s</td>
<td>4.39 x 10⁻⁴</td>
<td>4 min 18 s</td>
<td>Lognormal</td>
</tr>
<tr>
<td>7.2 ± 0.2</td>
<td>Leg muscle</td>
<td>26 min 36 s</td>
<td>1.28 x 10⁻³</td>
<td>100 mM</td>
<td>Direct injection</td>
<td>6.0 – 8.0</td>
<td>Lognormal</td>
<td>DOTAM-CEA-GLY-LYS</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER 2

PRESENT STUDY
For my graduate study, I focus on the development and application of acidoCEST MRI for evaluating tumor acidosis in pre-clinical cancer models. My published and publishable works are logically connected and integrated into the dissertation in a coherent manner.

In chronological order, my first publication (Appendix A) describes the optimization of acidoCEST MRI for in vivo pHe measurement [74]. In my second publication (Appendix B), we applied this optimized acidoCEST MRI method to measure pHe in lymphoma tumor model of Raji, Ramos and Granta 519 [130]. My third publication (Appendix C) describes the evolution of pixel-wise analysis in more detail [131]. I have included a step-by-step acidoCEST MRI image and data analysis protocol with Matlab® in Appendix E.

This is my original research. My contribution to my paper was 100% from me, with guidance from my co-authors, especially Marty Pagel. My research contributes to the field of CEST MRI in three ways, 1) we are able to produce a parametric pH map of the tumor, 2) evaluate spatial heterogeneity, and 3) determine the % contrast agent uptake. With these advances, we are able to apply CEST MRI to monitor mouse tumor model longitudinally and made comparisons between differ tumor models.
REFERENCES


[96] McVicar N, Li AX, Suchy M, Hudson RHE, Menon RS, Bartha R. Simultaneous


[111] van Zijl PCM, Jones CK, Ren J, Malloy CR, Sherry AD. MRI detection of glycogen in vivo by using chemical exchange saturation transfer imaging


[117] Sun PZ, Wang E, Cheung JS. Imaging acute ischemic tissue acidosis with pH-sensitive endogenous amide proton transfer (APT) MRI—Correction of tissue relaxation and concomitant RF irradiation effects toward mapping quantitative cerebral tissue pH. NeuroImage 2012;60:1–6.


APPENDIX A

EVALUATIONS OF EXTRACELLULAR pH WITHIN IN VIVO TUMORS

USING ACIDOCEST MRI

PUBLICATIONS


PRESENTATIONS AND MEETING ABSTRACTS

1. Measuring Extracellular pH within in vivo tumors using acidoCEST MRI, OctoberCEST, Annapolis, MD, October 15th 2012. [Oral Presentation]
INTRODUCTION

Tumor acidosis changes extrinsic cellular interactions that lead to tumor invasion and metastasis [1,2]. Extracellular acidity can be toxic to surrounding host tissue and can promote invasion [3-5]. Tumor acidosis also promotes metastasis by selecting cells that exhibit metastatic phenotypes [6-8]. Thus, measuring the extracellular pH (pHe) in solid tumors has potential to characterize tumor aggressiveness.

Many chemotherapies, such as doxorubicin (Adriamycin®, Pfizer Inc., New York, NY), are weak-base drugs that exhibit lower therapeutic efficacy against acidic tumors [9-12]. Other potential chemotherapies, such as esomeprazole (NexiumTM, AstraZeneca plc, London UK), are weak-acid chemotherapies that may exhibit higher therapeutic efficacy against acidic tumors [13,14]. Measuring tumor pHe has potential to predict therapeutic efficacy of a weak-base drug or weak-acid drug before the therapeutic treatment is initiated for each patient, which can lead to personalized medicine.

Treatments that alkalinize the tumor microenvironment, such as sodium bicarbonate, may affect invasion and metastasis, and may also potentiate chemotherapeutic efficacies of weak-base drugs [15]. However, alkalinizing treatments remain controversial because the duration of alkalinization to affect outcome is unknown, and the detriments to normal tissues such as kidney and liver are also unknown [16,17]. Accurately monitoring pHe in tumors and normal tissues may greatly aid in evaluating the utility of alkalinizing treatments for cancer care.
Several noninvasive methods have been developed to monitor tumor pH\textit{e} \textit{in vivo}. Optical imaging can measure tumor pH\textit{e}, but can only evaluate surface-accessible tumors and cannot measure the entire tumor volume [18,19]. Positron emission tomography [20,21], electron paramagnetic resonance spectroscopy [22], and MR spectroscopy [23,24] can measure tumor pH\textit{e}, but these methods are inaccurate, insensitive, or difficult to implement especially for clinical diagnoses. Magnetic resonance imaging (MRI) with relaxivity-based MRI contrast agent can be used to measure pH [25]. However, the concentration of the relaxivity-based MRI contrast agent must be known to accurately measure tumor pH\textit{e}, which is technically challenging [26]. Therefore, an imperative remains for a clinically relevant, noninvasive imaging method that can measure tumor pH\textit{e}.

MRI contrast agents that are detected by means of chemical exchange saturation transfer (CEST) have been used to measure pH [27]. To detect a CEST agent, radio frequency saturation is applied at a specific MR frequency of an exchangeable proton on the agent, which reduces the detectable magnetization from this proton. Rapid chemical exchange of this saturated proton with a proton on a near-by water molecule causes the reduced detectable magnetization to be transferred to the water signal. A standard MRI acquisition sequence can then be used to measure the amplitude of the water signal throughout the image [28]. A CEST spectrum is obtained by iterating the saturation frequency and recording the normalized water signal amplitude [29]. Because the average chemical exchange rates of some labile protons are pH-dependent, and because CEST effects from two labile protons can be selectively detected, a ratio of two CEST effects
from the same agent can be used to measure pH in a manner that is independent of concentration, endogenous $T_1$ relaxation time, and incomplete saturation [30,31]. As an example, iopamidol (Isovue™, Bracco Imaging, S.p.A.) is a clinically approved CT contrast agent that has been used to measure pH within in vivo kidney tissue [32].

In the current work, we investigated the use of in vivo CEST MRI for measuring tumor acidosis, which we term “acidoCEST MRI”. We have previously developed in vivo CEST-FISP MRI acquisition protocols that rapidly detect in vivo CEST effects, which is critical for tracking exogenous contrast agents within in vivo tissues [33]. We have developed Lorentzian line shape fitting methods that improve in vivo CEST measurements [34]. We sought to optimize our past technological developments to create the acidoCEST MRI protocol. We investigated the use of iopromide (Ultravist™, Bayer Healthcare, Inc.), which is similar to iopamidol. We have especially focused on the optimization of delivery of iopromide to tumor tissue, to create a method that can noninvasively measure tumor pH. To investigate the practical utility of acidoCEST MRI, we investigated whether this method could identify acidic tumors relative to pH-neutral tumors, and monitor the effect of bicarbonate alkalinization treatment.

METHODS

Optimizing acidoCEST MRI with Iopromide

Chemical samples were prepared using clinical-grade iopromide at 788 mM concentration (300 mg iodine / mL). The iopromide was diluted with distilled water to concentrations ranging from 12.5 mM to 400 mM, and the pH was adjusted to values
between 6.30 and 7.30 using 5 M hydrochloric acid. The addition of < 0.2 mL of hydrochloric acid to 50 mL of each solution had a negligible effect on the solution’s concentration. The pH values of the final solutions were measured with a calibrated pH electrode (Mettler Toledo, Inc., Columbus, OH). Chemical solutions were placed in 300 µL centrifuge tubes, the tubes were placed in a customized cradle, and the cradle was inserted into the MRI scanner. The samples were maintained at 37.0 ± 0.2°C using a heated air and an automatic temperature feedback system (SA Instruments, Inc., Stony Brook, NY).

All MRI studies were performed with a CEST-FISP MRI protocol implemented on a 7 Tesla (T) Biospec MRI scanner with a 72-mm-diameter quadrature transceiver coil (Bruker Biospin, Inc.) [33]. The FISP acquisition used the following parameters: 3.218 ms TR; 1.609 ms TE; 60º excitation angle; 2 mm slice thickness; 250 x 250 µm in-plane resolution 3.2 x 3.2 cm field of view; linear encoding order; unbalanced “FID” mode; 418.54 ms scan time. The CEST saturation period consisted of a series of Gaussian shaped radio frequency pulses with an inter-pulse delay of 10 µs and no additional spoiling or fat saturation pulses.

To optimize the CEST saturation period, the saturation time was varied from 0.5 to 8.0 s while the saturation power was set to 2 µT, the bandwidth was set to 90 Hz, the flip angle was set to 540º, and a series of 54 MR frequencies were saturated to acquire a CEST spectrum in 0.3 ppm increments from +10 to -2.9 ppm, and in 1 ppm increments from -3 to -10 ppm, and at +100 ppm and -100 ppm to measure MR signal without saturation
effects close to water. The saturation power was varied between 0.5 to 4.6 $\mu$T while the saturation time was set to 5.0 s, the bandwidth was set to 90 Hz, the flip angle was set to $540^\circ$, and the same series of 54 MR frequencies were saturated. Bandwidths of 30 and 60 Hz were also tested while using a saturation time of 5.0 s, a saturation power of 2 $\mu$T, and a flip angle of $540^\circ$. For tests with a 30 Hz bandwidth, frequencies were saturated between -10 and -3 ppm in 1 ppm increments, and between -3 and 9 ppm in 0.1 ppm increments, and at +100 ppm and -100 ppm. For tests with a 60 Hz bandwidth, frequencies were saturated between -10 and -3 ppm in 1 ppm increments, and between -3 and 10 ppm in 0.2 ppm increments, and at +100 ppm and -100 ppm.

The signal amplitudes of each series of CEST-FISP images were measured using ImageJ to create a CEST spectrum [35]. Each CEST spectrum was smoothed with a cubic spline function using Matlab R2012B (Mathworks, Inc., Natick, MA) [36]. A sum of three Lorentzian line shapes was fitted to each smoothed CEST spectrum using customized routines written for Matlab R2012B [28]. A $\log_{10}$ ratio of CEST effects from the two amide protons was compared with the pH of each sample to create a linear calibration of CEST versus pH [30,31,34]. The correlation of CEST versus concentration was fit to a linear function based on a two-pool model that represents the chemical exchange between one amide group and water [37].

*Mouse Model of Mammary Carcinoma for Imaging Studies*

All *in vivo* studies were conducted according to approved procedures of the Institutional Animal Care and Use Committee of the University of Arizona. A model of MDA-MB-
231 mammary carcinoma was prepared by injecting one million tumor cells in 0.5 mL of 50% Matrigel into the right lower flank of a 6-week-old female SCID mouse. Imaging studies were conducted when the subcutaneous tumor reached a size greater than 4 mm in diameter as measured by vernier caliper. When needed, a 27 g catheter was inserted in the tail vein or the abdominal cavity to facilitate the i.v. or i.p. injection of contrast agents. At the conclusion of the imaging scan, the mouse was removed from the scanner and cradle, and allowed to recover. To test the ability of acidoCEST MRI to measure a change in pHe within tumors or bladders, acidoCEST MRI was performed one day before and one day after adding 200 mM bicarbonate to their drinking water.

For the “direct” injection procedure, 50 µL of 972 mM (370 mg iodine / mL) clinical-grade iopromide was directly injected into the center of the subcutaneous tumor using a 32 g needle. For the “subcutaneous” injection procedure, 0.5, 1.0 or 1.5 µL of 788 mM iopromide or 1.5 mL of iopromide adjusted to pH 6.3, 6.9 or 7.2, was injected subcutaneously adjacent to the tumor using a 32 g needle, and the injection fluid was allowed to soak into the tumor tissue. For the “IV” injection procedure, 200 mL of 788 mM iopromide was intravenously injected within 60 s through the tail vein catheter. For the “IV-infusion” injection procedure, 200 µL of 788 mM iopromide was injected i.v. followed by slow infusion of the agent at 150 µL/h during imaging. For the “IP” injection procedure, 1500 µL of 972 mM iopromide was injected intraperitoneally (IP).
**In Vivo Micro-Computer Tomography (micro-CT) Protocol**

Thirteen chemical solutions of iopromide at 0, 0.005, 0.05, 0.5, 2.4, 5, 10, 12, 30, 60, 120, 240, and 300 mM concentrations were initially imaged using Inveon micro-CT (Siemens, Munich, Germany) to establish a linear calibration of signal versus concentration. To prepare for *in vivo* imaging studies, a mouse was anesthetized with 1.5 – 2.5 % isoflurane delivered in 1 L/min oxygen gas ventilation, and secured to a customized cradle in the micro-CT scanner. Chemical solutions with 2.4 and 12 mM concentrations of iopromide were placed near the mouse as a concentration reference.

Micro-CT studies of chemical solutions and the *in vivo* mouse model were performed using a Siemens Inveon micro-CT scanner and Inveon Acquisition Workplace software (Siemens Medical Solutions USA, Inc., Knoxville, TN). All scans were acquired with the following parameters: 80 kVp, 395 ms exposure time, 500 µA current, 220 rotation steps with 601 projections, ~ 8.5 min scan time, 0.5 mm aluminum filter, four-fold bin factor, and low magnification yielding an overall reconstructed isotropic voxel size of 105 microns. Scans were reconstructed with filtered back-projection and no down-sampling using integrated high-speed COBRA reconstruction software (Exxim Computing Corporation, Pleasanton, CA). A scalar linear attenuation coefficient in Hounsfield Units (HU) was applied to each dataset to facilitate inter-scan comparisons.

**In Vivo acidoCEST MRI Protocol**

To prepare for imaging studies, a mouse was anesthetized with 1.5-2.5 % isoflurane delivered in 1 L/min oxygen gas ventilation, the mouse was secured to a customized
cradle, probes for monitoring rectal temperature and respiration were connected to the mouse, the core body temperature was regulated at 37.0 ± 0.2°C using an automated feedback loop between the temperature probe and an air heater (SA Instruments, Inc., Stony Brook, NY), and the cradle was inserted into the MRI scanner. At the conclusion of the imaging scan, the mouse was removed from the scanner and cradle, and allowed to recover.

A CEST-FISP MRI protocol used the same FISP acquisition period used for studies with chemical solutions. The saturation period had a 5.0 s saturation time, a 2 µT saturation power, a 90 Hz bandwidth, and a 540° flip angle. A series of 54 MR frequencies were saturated to acquire a CEST spectrum in 0.3 ppm increments from +10 to -2.9 ppm, and in 1 ppm increments from -3 to -10 ppm, and at +100 ppm and -100 ppm to measure MR signal without saturation effects close to water. This procedure was initiated within 30 s after IV injection using the “IV-infusion” protocol or 2 min after subcutaneous injection using the “subcutaneous” protocol. The total time to acquire a single MR CEST spectroscopic image was 4:51 min. MR CEST spectroscopic imaging was repeated for a total of six times, for a total acquisition time of 29:06 min. The MR signals from a region of interest that represented the tumor were used to construct a CEST spectrum, which was then analyzed using the same protocol used to analyze chemical samples described above.
RESULTS

Optimizing acidoCEST MRI with Iopromide

Iopromide, a clinically approved CT contrast agent, has two amide protons that have MR frequencies at 4.2 and 5.6 ppm (Figure 1a). A CEST spectrum showed that the CEST effect from each amide could be selectively detected (Figure 1b). To measure each CEST effect, a sum of three Lorentzian line shapes was fitted to the smoothed CEST spectrum. The low residuals near 4.2 and 5.6 ppm attest that these fittings could accurately measure each CEST effect (Figure 1c). Although the hydroxyl groups of iopromide generated a CEST effect at 0.8 ppm, this effect was difficult to distinguish from the direct saturation of water centered at 0 ppm, and therefore, only a single Lorentzian line shape was fitted to the CEST effect of the hydroxyl groups and the direct water saturation, resulting in higher fitting residuals near 0 ppm.

The concentration dependence of each CEST effect measured at pH 6.44 (Figure 2a) fit to a linear algorithm based on a two-pool model that represented on amide group and water (Figure 2b). The excellent fittings to these models, with $R^2$ correlations greater than 0.967, indicated that each CEST effect was independent of the other CEST effect. Furthermore, this result showed the ratio of the two CEST effects was independent of concentration, with an average ratio of $1.24 \pm 3.7\%$. 
Figure 1. CEST of iopromide.
a) The chemical structure of iopromide. b) A CEST spectrum of 200 mM iopromide at pH 6.69 and 37.0°C with saturation applied at 2 µT for 5 s. c) Lorentzian line shapes fitted to the CEST spectrum show CEST effects at 4.2 ppm and 5.6 ppm. Squares represent the residuals of the fitting process.

Figure 2. Evaluation of the concentration dependence of the CEST effects of iopromide.
a) The CEST effects at 5.6 ppm (squares) and 4.2 ppm (circles) measured at pH 6.44 were dependent on concentration, but the ratio (triangles) was independent of concentration. Solid lines represent the CEST-concentration relationship based on the linear analysis shown in panel (b). The dashed line represents the average of the ratios, 1.24. b) CEST at 5.6 ppm (squares) and 4.2 ppm (circles) from samples with different concentrations were fit using a Hanes-Woolf CEST concentration plot that is based on a two-pool model. The excellent linear fitting demonstrated that each CEST effect was independent of the other CEST effect. CEST was measured using a 5-s saturation period with 2 µT saturation power, followed by a CEST-FISP acquisition.
We optimized the saturation time for acidoCEST MRI with iopromide (Figure 3a). The CEST effects from each amide proton did not reach steady state with 8 s of saturation. However, a $\log_{10}$ ratio of the CEST effect attained a constant value after 1.5 s of saturation. This result demonstrated that incomplete saturation of both CEST effects does not affect the pH measurement with acidoCEST MRI. This obviated a concern for $B_1$ inhomogeneity during *in vivo* studies, and allowed for a shorter saturation time. We set the saturation time to 5 s for subsequent studies to improve sensitivity for measuring each CEST effect.

![Figure 3. Evaluation of saturation conditions.](image)

Although each CEST effect of iopromide at pH 6.74 showed an increase with a) increasing saturation time and b) increasing saturation power, a $\log_{10}$ ratio did not change with saturation times $\geq 2$ s and $\geq 1.8$ $\mu$T. A CEST-FISP acquisition protocol was used for this study.

We also optimized the saturation power for acidoCEST MRI with iopromide (Figure 3b). The CEST effects from each amide proton did not reach a maximum with the range of 0.5 to 5 $\mu$T saturation powers. A maximum CEST effect should have been observed within 5 $\mu$T for protons exchanging slower than 1500 Hz, and our previous studies have shown that the amid protons of iopromide have chemical exchange rates of approximately 100 and 1200 Hz [38,39]. This result indicates that the saturation power affecting the sample
was less than the saturation power generated by the MRI scanner, which was attributed to $B_1$ inhomogeneity. However, a log$_{10}$ ratio of the CEST attained a constant value after 1.8 $\mu$T saturation power, once again indicating that $B_1$ inhomogeneity that causes incomplete saturation of both CEST effects does not affect the pH measurement with acidoCEST MRI. We set the saturation power to 2 $\mu$T for subsequent studies to minimize the specific absorption rate during in vivo studies.

We also optimized the bandwidth of the saturation pulse for acidoCEST MRI using samples at 200 mM concentration. Although the amide protons of iopromide have large chemical shifts relative to other diamagnetic CEST agents, these chemical shifts are still close to each other and to the MR frequency of water. The saturation bandwidth should be minimized to improve the specificity of measuring CEST from each amid proton. Yet the sensitivity of detecting CEST improves with increasing bandwidth. Therefore, an optimal bandwidth is needed to achieve balance between sensitivity and specificity. Our tests of combinations of saturation bandwidth and power showed that a 90 Hz bandwidth at 2 $\mu$T power could calibrate a ratio of CEST with greatest range of pH (Table 1). The optimized saturation conditions were used to calibrate a log$_{10}$ ratio of iopromide’s CEST effects with pH within a range from 6.3 to 7.2 units (Figure 4). This calibration showed excellent linearity with a $R^2$ value of 0.95 and a precision of 0.07 pH units.
Table 1. Measurable pH range

<table>
<thead>
<tr>
<th>Saturation power</th>
<th>Bandwidth</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>30 Hz</td>
<td>60 Hz</td>
<td>90 Hz</td>
<td></td>
</tr>
<tr>
<td>1 μT</td>
<td>pH 6.0-7.1</td>
<td>pH 6.0-7.0</td>
<td>pH 6.0-6.8</td>
<td></td>
</tr>
<tr>
<td>2 μT</td>
<td>pH 6.0-7.1</td>
<td>pH 6.0-7.0</td>
<td>pH 6.0-7.2</td>
<td></td>
</tr>
<tr>
<td>4 μT</td>
<td>pH 6.3-7.0</td>
<td>pH 6.3-7.0</td>
<td>pH 6.3-6.8</td>
<td></td>
</tr>
</tbody>
</table>

a The measurable pH range was defined as the range that could generate two CEST effects from the contrast agent that are \(2\sqrt{2}\) times the standard deviation of the noise of the CEST MR image, which have a 95% probability of being real [40].

![Graph](image.png)

Figure 4. The effect of pH on CEST.

a) The CEST effects of iopromide are sensitive to pH. CEST spectra were acquired with 200 mM iopromide at 37°C with saturation applied at 2 μT for 5 s. The CEST spectra of fitted Lorentzian line shapes are vertically offset to aid the viewing. b) A log_{10} ratio of the two CEST effects is linearly correlated with pH from 6.3 to pH 7.2.

**Evaluation of Routes of Injection**

Micro-CT was used to test the delivery of iopromide to the tumor and normal tissues following “direct”, “subcutaneous”, “IV”, “IV-infusion”, and “IP” injection protocols (Figure 5 and Table 2). As expected, direct injection into the tumor provided the greatest delivery of agent, but this method caused minor bleeding at the needle entrance site, so that direct injection failed to meet our criterion of being a noninvasive method. The IP injection showed no detectable uptake of agent in the tumor, and therefore, was inadequate for acidoCEST MRI studies. The subcutaneous, IV, and IV-infusion
injections delivered 5.12, 4.08, and 3.69 mM of agent to the tumor. The minor differences in these concentrations were assumed to be due to biological variability, so that the subcutaneous, IV, and IV-infusion injections were considered to deliver comparable concentration of agent to the tumor. Furthermore, the concentration of agent in the tumor was relatively stable during the micro-CT scan session, which showed a change in concentration of only 6.69% per 4:51 min which was the acquisition time of a single CEST spectrum. Therefore, the IV-infusion protocol and subcutaneous injection protocol were used for subsequent acidoCEST MRI studies because this route of injection was noninvasive and provided a stable accumulation of agent in the tumor. The IV-infusion protocol was used instead of the IV protocol to potentially improve the stability of the concentration of agent in the tumor.

Figure 5. A comparison of routes of administration using micro-CT. 

a) An axial image of the tumor 9 min after direct injection. b) A sagittal image of bladder, kidney and tumor 27 min after IV-infusion injection. c) An axial image of the tumor 9 min after subcutaneous injection. The concentration in the (d) bladder, (e) kidney, and (f) tumor showed that IV-infusion and subcutaneous injections caused renal clearance of the agent and steady uptake in the tumor.
The optimized acidoCEST MRI protocol with iopromide was used to measure in vivo tumor pH of a mouse model of mammary carcinoma. The IV-infusion and subcutaneous injections were tested using the same five mice on successive days (Figure 6a,b). The CEST spectra of iopromide in the tumor tissue following both routes of injection had more noise than CEST spectra of chemical solutions. CEST measurements that are $2\sqrt{2}$ times the standard deviation of the noise of the CEST MR image have a 95% probability of being real [40]. All CEST measurements were greater than this threshold. Also, the fitting residuals were low near the CEST effects of the amide protons of iopromide, which provided confidence that the CEST effects were accurately determined. The CEST spectrum contained features in the negative ppm range that were attributed to magnetization transfer effects from fat, because fat suppression was not included in our protocol. Yet the Lorentzian line shape fitting method analyzes features in the positive ppm range was not affected by these poorly-fit features in the negative ppm range.

Table 2. Maximum uptake of iopromide following injection

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Direct injection (50 µL)</th>
<th>Subcutaneous injection (1.5 mL)</th>
<th>IV injection (200 µL bolus)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor</td>
<td>349 mM</td>
<td>5.12 mM</td>
<td>4.08 mM</td>
</tr>
<tr>
<td>Bladder</td>
<td>95 mM</td>
<td>208 mM</td>
<td>320 mM</td>
</tr>
<tr>
<td>Kidney</td>
<td>2 mM</td>
<td>52 mM</td>
<td>79 mM</td>
</tr>
</tbody>
</table>

a IP injection: limited uptake in bladder and kidney, and no uptake in tumor and muscle.
Figure 6. A comparison of routes of administration using acidoCEST MRI.

a) Lorentzian line shape fitting to a smoothed CEST spectrum after IV-infusion injection.

b) The difference in extracellular tumor pH determined after IV-infusion and subcutaneous injection had a standard deviation of 0.028 pH units. The average standard deviation of the IV-infusion and subcutaneous injection was 0.137 and 0.138 pH units, respectively, indicating that protocols with the IV-injection and subcutaneous injections produced the same pH value.

The IV-infusion and subcutaneous injections produced comparable measurements of tumor pH. Six CEST spectra were acquired after subcutaneous or IV-infusion injection of the agent to a mouse. The average and standard deviation of the six pH measurements from each CEST spectrum was used to determine the tumor pH (Figure 6b). The average standard deviation of the tumor pH measurements after IV-infusion and subcutaneous injection was 0.137 pH units and 0.138 pH units, respectively. For comparison, the average standard deviation of the difference between the pH measurements with IV-infusion versus subcutaneous injection was only 0.028 pH units. Therefore, the “inter-injection” variance was less than the “intra-injection” variances, indication that both routes of injection produced the same results. This result indicated that subsequent test of the precision of acidoCEST MRI measurements using a subcutaneous injection should also be valid for IV-infusion injection.
Subcutaneous injections with 0.5, 1.0, and 1.5 mL volumes of the agent were performed on successive days using the same eight mice (Figure 7a). The standard deviation of the pHe measurements made from six CEST spectra was determined for each acidoCEST MRI scan session of each mouse (Figure 7b). The average standard deviation of tumor pHe measurements decreased from 0.287 to 0.214 to 0.141 pH units with 0.5 mL, 1.0 mL, and 1.5 mL injections, indicating improved precision with greater delivery of agent, as expected. In addition, the average standard deviation of the difference between tumor pHe values determined with each volume was 0.081 pH units. This “inter-volume” standard deviation was less than each “intra-volume” standard deviation, indicating that the volume of injection did not dilute the tissue and subsequently affect the pH determination, which validated that the acidoCEST MRI protocol was noninvasive.

Subcutaneous injections with solutions adjusted to pH 6.3, 6.9 and 7.2 were performed on successive days with the same six mice (Figure 7c). The average standard deviation of the 18 tumor pHe measurements was 0.142 pH units (Figure 7d), while the average standard deviation of the difference between tumor pHe values determined with solutions at each pH was only 0.055 pH units. The “inter-pH” standard deviation was less than the “Intra-pH” standard deviation, which further indicated that the pH of the injection did not influence the tumor pHe measurement, and further validated that the acidoCEST MRI protocol was noninvasive.
Figure 7. Evaluations of injection conditions on acidoCEST MRI results.

a) The volume of the subcutaneous injection had an average inter-injection standard deviation of 0.081 pH units. b) Greater injection volumes produced a lower intra-injection standard deviation, indicating improved precision with greater delivery of agent. c) Subcutaneous injections of solutions with different pH had an average inter-injection standard deviation of 0.055 pH units, d) and an average intra-injection standard deviation of 0.142 pH units, indicating that the pH of the injection did not influence the accuracy or precision of the pH measurement.

*Monitoring the Effects of Bicarbonate Treatment on Tumor pH*

To investigate the practical utility of acidoCEST MRI, we investigated whether this method could identify acidic tumors and monitor the effect of bicarbonate alkalinization treatment. The average pH of the tumor was measured in a mouse model of MDA-MB-231 mammary carcinoma (Figure 8a,c). Tumor pH was measured with a precision of 0.095 pH units. Three tumors were classified as very acidic (pH 6.5), one tumor was
classified as moderately acidic (pH 6.9), and four tumors were classified as pH-neutral (pH > 7.0). Two of the four pH-neutral tumors showed evidence for necrosis (mouse #7 and #8 of Figure 8c). One day after treating the mouse model with bicarbonate *ad libitum*, the pHe of all tumors increased to pH > 7.1. These results demonstrated that acidoCEST MRI can track changes in tumor pHe.

Figure 8. The effect of bicarbonate treatment on tissue pHe.

a) The MR image shows the location of the tumor and bladder. b) The extracellular pH in the bladder increased an average of 0.53 pH units 24 h after providing 200 mM bicarbonate in drinking water. c) The initial tumor pHe ranged from 6.5 to 7.2. The tumor pHe increased to ≥ 7.1 pH units after bicarbonate treatment. Error bars represent the standard deviation of 4-12 measurements within 30 min. **P < 0.01, *P < 0.02.
The average pH of the bladder was also measured in two mice on two successive weeks, for a total of four measurements (Figure 8b). The bladder pH was measured with a precision of 0.119 pH units. Before treatment with bicarbonate, the bladder pH ranged between 6.3 and 6.6 units. Bicarbonate treatment raised the pH of all bladders to > 6.8 pH units. For each mouse, the increase in bladder pH was statistically significant (P < 0.02).

**DISCUSSION**

We have established an optimized protocol for acidoCEST MRI. However, this optimization included an empirical calibration that was specific to a series of Gaussian-shaped pulses at 7T magnetic field strength. Saturation with other conditions and at other magnetic field strengths may require a different empirical calibration. This optimization included consideration for route of injection. Subcutaneous injection could be helpful for evaluating MRI contrast agents because it can be comparable to IV-infusion injection. These experiments also established that an IV-infusion injection delivered a sufficient and stable amount of agent to tumor tissue for acidoCEST MRI studies. This optimization included the use of Lorentzian line shape fitting to measure CEST effects, and a statistical noise threshold to ensure the statistically significant detection of CEST from both amide protons, which can be easily translated to other CEST MRI studies with other saturation periods and magnetic field strengths.

These results confirm that iopromide can be used as a contrast agent for dual-modality micro-CT and acidoCEST MRI studies. In particular, micro-CT studies with iopromide cross-validated the delivery of agent to the tumor using IV-infusion and subcutaneous
injections. Although cross-validation is critical for engineering new imaging methods, dual-modality imaging has more potential impact when each modality can provide complementary information rather than the same information [41]. For example, micro-CT studies with iopromide have been used to identify solid tumors with high resolution. Iopromide contains three iodine atoms, and, therefore, radiolabeling iopromide with $^{123}$I or $^{124}$I may be feasible, which would provide the opportunity to identify solid tumors with high sensitivity SPECT or PET imaging modalities, respectively. Our study establishes that acidoCEST MRI with iopromide provides complementary information by measuring the average tumor pHe, once the tumor has been identified, which may improve assessments of solid tumors with dual modality CT-MRI, SPECT-MRI or PET-MRI.

Based on our statistical criteria, acidoCEST can only reliably measure a maximum pH of 7.2, and, therefore, our measurements may have underestimated the average pHe of tumors that were classified as pH-neutral. This underestimation is expected to be minor, because pHe in tumors and normal tissues is rarely expected to exceed pH 7.4 [6]. Yet improvements to acidoCEST MRI are warranted that produce pixel-wise parametric maps of pHe within the tumor, to identify intratumoral regions that are acidic and pH-neutral. Pixel-wise pH maps may also provide for investigation of the acidoCEST MRI protocol, such as the influence of $B_0$ inhomogeneities on CEST spectra. Furthermore, our acidoCEST MRI protocol images a single two-dimensional slice. Incorporating multi-slice CEST MRI techniques into an acidoCEST MRI protocol may further improve the evaluation of spatial heterogeneity of tumor pH [42]. In addition, we have not validated the accuracy of tumor pHe measurement using acidoCEST MRI because other methods
that attempt to measure tumor pH are difficult to implement, have their own measurement limitations, and their accuracies have also not been validated. Therefore, future studies are warranted to validate the accuracy of acidoCEST MRI measurements of \textit{in vivo} pH, when other “gold standard” methods become available.

Our tumor pH measurements with acidoCEST MRI demonstrated that the MDA-MB-231 subcutaneous tumor model used in this study had a range of extracellular acidities. This variability within the same mouse model indicates that genotype may not predict an acid phenotype. Therefore, measurements of tumor pH may be needed for each mouse or patient to establish the status of tumor acidosis for studies that investigate or depend on acidosis. However, our proof-of-principle study only used one tumor model, and similar tests with other subcutaneous and orthotopic tumor models are warranted to investigate this variability of acidosis in cancer biology.

Our tumor pH measurements also showed that bicarbonate treatment alkalinized the acidic tumors, but did not change tumors that were classified as pH-neutral. For comparison, bicarbonate treatment alkalinized the bladders in all cases. This evidence indicates that bicarbonate should not be used as an anti-cancer treatment without prior knowledge of the acidic state of the tumor. Otherwise, treating patients with pH-neutral tumors may not affect the pH of the tumor, and yet the patient risks alkalosis of normal organs such as the bladder [43]. Clinical translation of acidoCEST MRI may provide an important diagnostic tool for patients who are considering the use of alkalinizing therapies.
AcidoCEST MRI has great potential for clinical translation and may provide an important diagnostic tool for patients who are considering the use of alkalinizing therapies. Iopromide is a clinically approved contrast agent. Our IV-infusion protocol delivers 3.3-4.6 grams of iodine per kilogram of mouse body weight (gI/kg) to a mouse, based on a mouse body weight of 18-25 grams. Because pharmacokinetics scale between species when normalized to body surface area, the dose used in our study equates to Human Equivalent Dose of 0.27-0.37 gI/kg [44,45]. This dose is 4- to 5.5-fold lower than the 1.5 gI/kg maximum recommended total dose of iopromide for humans, which facilitates clinical translation of acidoCEST MRI. In addition, acidoCEST MRI uses a common FISP acquisition protocol that is also clinically approved. AcidoCEST MRI also uses a long, high-power saturation period, which is not currently approved for routine clinical use. However, long saturation periods with higher saturation powers have been successfully used for clinical imaging studies of solid tumors and other organs, including the brain [46,47]. Based on the compelling evidence of our study that tumor pH measurements may benefit treatment decisions for patients, clinical translation of acidoCEST MRI should be pursued.

REFERENCES


APPENDIX B

IN VIVO IMAGING OF LYMPHOMA CANCER MODELS WITH ACIDOCEST MRI

PUBLICATIONS


PRESENTATIONS AND MEETING ABSTRACTS

INTRODUCTION

Despite significant advances in the diagnosis and treatment of Non-Hodgkin’s Lymphoma (NHL) during the last decade, NHL remains a significant cause of morbidity and mortality in the United States with over 70,000 new cases and almost 19,000 deaths occurring in 2012 [1]. One common feature of NHL is high cellular metabolic activity necessary to fulfill energy demands and nucleotide biosynthesis that are required for proliferation [2]. A consequence of high cellular metabolic activity is enhanced anaerobic and aerobic glycolysis, also know as the Warburg effect, which results in lactic acid production [3,4]. Poorly vascularized tumors are often poorly oxygenated, and this hypoxia further increases anaerobic glycolysis and increased lactic acid production [5]. Glutaminolysis and oxidative phosphorylation result in CO$_2$ production that can also be a source of net H$^-$ production.

Extracellular acidosis may contribute to disease progression and chemotherapy resistance [4,5]. For example, upregulation of Bcl-2, an anti-apoptotic protein, has recently been shown to be upregulated in T-cell lymphoma models via the p38 mitogen-activated protein kinase (MAPK) pathway in response to acidosis [6]. Induction of autophagy has been observed in a cell line model in response to acute and chronic acidity, and has been reported to be implemented as survival mechanism for solid tumor cells [7-9]. Hypoxia-induced extracellular acidosis has been associated with increased activity of p-glycoprotein (pGP) and chemoresistance [10].
The activity of carbonic anhydrase IX (CA IX) can link the intracellular production of lactic acid and H⁺ ions with extracellular acidosis [11]. A member of a family of zinc metalloenzymes, the CA IX isoform is a transmembrane protein with an extracellular catalytic domain that catalyzes the reversible hydration of CO₂ into bicarbonate [12]. The hydration of extracellular CO₂ provides bicarbonate ions for influx by adjacent transporters including the chloride/bicarbonate exchanger 1, 2, 3 and the sodium-bicarbonate cotransporter know as NBCe1 that results in intracellular neutralization [13]. The proton by-products of the CO₂ hydration reaction remain in the extracellular space, acidifying the microenvironment. Thus, CA IX plays a fundamental role in maintaining intracellular pH (pHi) that can result in decreased extracellular pH (pHe).

In our current study, we investigated the expression of CA IX in human lymphoma tissues, in three B-cell lymphoma cell lines in vitro, and in three B-cell lymphoma tumor models in vivo. We also used a novel imaging method, acidoCEST MRI, that can accurately measure tumor pHe with high spatial resolution, using instrumentation and a contrast agent that are routinely available in radiology clinics [14]. This non-invasive method was used to monitor longitudinal changes in tumor pHe, and was also used to evaluate the spatial heterogeneity of tumor pHe in each tumor model. To ensure that acidoCEST MRI can measure a range of pHe among different tumors, we also measured the change in pHe with acidoCEST MRI after administering a mitochondrial poison to a xenograft tumor model.
The high expression of CA IX has also been related to hypoxia. CA IX expression is primarily regulated at the transcriptional level through hypoxia induced transcription factor 1 alpha (HIF-1α) [15], and activating transcription factor 4 (ATF-4), which are activated in response to hypoxia and the unfolded protein response [16]. CA IX is an adverse prognostic factor in multiple tumor types including head and neck, breast, and lung cancers that are known to experience hypoxia [17]. However, a correlation between CA IX and hypoxia has not been established in NHL. More generally, hypoxia and acidosis have been suggested to be related characteristics of the extracellular tumor microenvironment [18]. CA IX may act as a molecular mechanism that links hypoxia and extracellular acidosis. Therefore, another primary aim of our study was to investigate the relationship between CA IX and hypoxia in human lymphoma tissues, and in three B-cell lymphoma cell lines in vitro and in vivo.

**METHODS**

**Reagents**

All reagents were obtained from SigmaAldrich, Inc., (St Louis, MO) unless otherwise indicated. The acidoCEST MRI protocol used clinical-grade iopromide at 788 mM (300 mg iodine/mL Ultravist™, Bayer Healthcare, Inc., Leverkusen, Germany).

**Immunohistochemistry**

Human lymphoma tissue microarrays were obtained from Dr. Lisa Rimsza, Department of Pathology, University of Arizona. Hematoxylin and eosin (H&E) stains were performed with human tissue arrays containing Mantle Cell, Diffuse Large B-Cell and
Follicular Lymphoma tissue. H&E stains were also performed with tissues obtained from lymphoma tumor tissues obtained from xenograft mouse tumor models, obtained by cutting 3-micro thick sections from the formalin-fixed, paraffin-embedded tissue blocks. Immunohistochemistry (IHC) was performed using anti-pimonidazole mouse monoclonal antibody MAb1, clone 4.3.11.3, diluted 1:50 (Hypoxyprobe Inc., Raleigh, NC). CA IX staining was performed using anti-CA IX antibody from Novus Biologicals (Littleton, CO) diluted 1:2000. VEGF-A staining was performed using anti-VEGF-A antibody (Santa Cruz Biotech # sc-7269) diluted 1:70. Tissue sections were stained with a Discovery XT Automated Immunostainer (Ventana Medical Systems, Inc., Tucson, AZ). All steps were performed with VMSI validated reagents, including deparaffinization, cell conditioning (antigen retrieval with a borate-EDTA buffer), primary antibody staining, detection and amplification using a biotinylated-streptavidin-HRP and diaminobenzidine system and hematoxylin counterstaining.

Semi-quantitative scoring of CA IX, VEGF-A, and pimonidazole was performed by a Board Certified Pathologist. Immunoreactivity for VEGF-A was categorized as 0 = negative; 1 = weak; 2 = moderate; 3 = strong. The percentage of immunoreactive tumor cells was also evaluated for each intensity level. The overall scores were calculated by multiplying the intensity by the corresponding percentage of immunoreactive cells (scores range between 0 – 300). For pimonidazole staining, only the percentages of immunoreactive tumor cells were evaluated.
**Tissue Culture**

Granta 519, Raji, and Ramos lymphoma cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were routinely grown in DMEM or RPMI supplemented with 10% HyClone™ fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA) and 100 I.U/mL of penicillin/streptomycin (Cellgro®, Mediatech, Inc., Manassas, MA) in a humidified incubator at 37°C and 5% CO₂. Cells were routinely analyzed to verify cell line authenticity and for mycoplasma contamination. For hypoxia studies, cells were grown in 0.5% oxygen, in a humidified, 37°C Ruskinn Invivo 2 Hypoxia Workstation (Ruskinn Technology Ltd., Pencoed, Bridgend, UK). For growth in acidic conditions, RPMI media supplemented with 15% FBS and 10 mM HEPEs was used as the control. For pH 6.7 lactic acid media, a dilute stock of lactic acid at 85-90% by volume was added to media until a pH value of 6.7 was achieved, with an estimated concentration of 20 mM of lactic acid. For the lactate control media, a solution of 60% w/w sodium DL-lactate was diluted to achieve 20 mM lactate and then was titrated to pH 7.3.

**Quantitative Nuclease Protection Assay (qNPA)**

The HTG Molecular Hypoxia & DNA Array (HTG Molecular Diagnostics, Inc., Tucson, AZ) was used to measure CA IX gene expression: monocarboxylate transporter 1 (SLC16A1; MCT-1) and monocarboxylate transporter 4 (SLC16A3; MCT-4). Briefly, cells were collected and pelleted following treatment. Cells were lysed in HTG Lysis Buffer and heated to 95°C for 10 minutes and then frozen for shipment to HTG Molecular Diagnostics for analysis as previously described [19]. The gene intensities were
normalized to the four housekeeping genes peptidylprolyl isomerase A (PPIA), ribosomal protein L19 (RPL19), ribosomal protein, large PO (RPLPO), ribosomal protein L38 (RPL38). To calculate fold change induced by treatment, intensity values of cells under hypoxia or treated with lactate or lactic acid were divided by intensity values of cells grown in standard media for the same amount of time.

*Western Blot Analysis*

The cells were washed with PBS and suspended in lysis buffer (50 mM Tris-HCL, pH 7.4, 1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 5 mM Na₃VO₄, 200 µM NaF, 21 µM leupeptin, 230 nM aprotinin, and 1 mM PMSF) (100 µL/mL) and lysed by sonication. Xenograft tumors were homogenized using the same lysis buffer solution. Cell lysate was centrifuged at 10,000 x g for 10 minutes at 4°C. Protein concentration of the supernate was determined using the BioRad Protein Assay (Hercules, CA). Lysates were separated by 10% Bis-Tris gel electrophoresis (Invitrogen, Carlsbad, CA) and then transferred to 0.2 µm PVDF membranes (BioRad) and then blocked for 1 hour in 5% non-fat dry milk/TBST. Membranes were immunoblotted with primary antibodies at concentrations recommended by the manufacturer. The membranes were then washed in TBST and then probed with a HRP conjugated anti-rabbit antibody or anti-mouse antibody. Membranes were then visualized with HyGlo Chemiluminescent HRP antibody detection reagent (Denville Scientific Inc., Metuchen, NG). Blots were stripped in 0.2 M NaOH with shaking for 10 minutes at room temperature. Protein levels were normalized to actin or tubulin levels and expressed in arbitrary units relative to control levels. Densitometer analysis was performed using ImageJ [20].
CA IX ELISA

Cell lines were grown in normoxia or hypoxia as described above. Conditioned media was collected by centrifuging cells at 2000 x g and removing the supernate. Media was immediately frozen at -80ºC. Prior to analysis, samples were thawed and gently vortexed. A quantitative human CA IX immunoassay (R&D Systems, Inc., Minneapolis, MN) was used according to manufacture recommendations.

Xenograft Tumor Studies

Lymphoma cells were mixed with 1:1 with Matrigel (BD Biosciences, Inc., Franklin Lakes, NJ), and 10⁷ cells were injected s.c. into 4-6 week old SCID mice. Animals were handled according to the Institutional Animal Care and Use Committee guidelines. Volumes were calculated using T₂-weighted MR images. Animals were euthanized when tumors reached ≥ 2000 mm³. For MIBG studies, MIBG (30 mg/kg) and glucose (3 mg/kg) were administered i.p. 4 hours prior to imaging. Immediately before acidoCEST MRI, another dose of glucose (3 mg/kg) was administered i.p. For exogenous hypoxia biomarker studies, mice were injected i.p. with 60 mg/kg pimonidazole and euthanized 1-4 hours after injection. Tumors were excised immediately and tissue placed in 10% neutral buffered formalin for 24 hours, transferred to 70% ethanol for dehydration, and then mounted in paraffin blocks.
Our acidoCEST MRI protocol has been described in a previous report [14]. Briefly, each mouse was anesthetized with 1.5 – 2.5% isoflurane delivered in 1 L/min oxygen gas ventilation, and then secured to a customized cradle. A 27 g catheter was inserted into the tail vein. The cradle was inserted into a 7T Biospec MRI scanner with a 72 mm-diameter quadrature transceiver coil (Bruker Biospin, Inc., Billerica, MA). The breathing rate was monitored and the core body temperature was regulated at 37.0°C ± 0.5°C using an automated feedback loop between the temperature probe and an air heater (SA Instruments, Inc., Stony Brook, NY). A FLASH MRI acquisition sequence was performed to determine the location of the tumor (500 sec TR, 10 msec TE, 625 x 625 µm in-plane resolution, 2.0 mm slice thickness, 64 x 64 pixels, 4.0 x 4.0 cm FOV, one average, 32 sec total acquisition time). A spin-echo MRI acquisition sequence was performed to obtain images with good contrast for determining the tumor volume (2000 msec TR, 35 msec TE, 312.5 x 312.5 µm in-plane resolution, 1.0 mm slice thickness, 128 x 128 pixels, 4.0 x 4.0 cm FOV, one average, 4:20 sec total acquisition time). A bolus of 200 µL of iopromide was injected via the catheter within 30 seconds. The catheter was then connected to an infusion pump and iopromide was pumped at a rate of 150 µL/hr. Our acidoCEST MRI protocol consisted of 54 FISP MR images acquired at different saturation frequencies, using 2.8 µT saturation power and a saturation period of 5 seconds, which required 4:40 minute of acquisition time. This process was repeated 6 times, for a total acquisition time of 28 minutes. At the conclusion of the imaging scan, the mouse was removed from the scanner and cradle, and allowed to recover.
The acidoCEST MR images were processed with Matlab® R2012B (Mathworks, Inc., Natick, MA). All 6 CEST-FISP images were averaged. To improve signal to noise, groups of 3 x 3 adjacent pixels were binned. The CEST spectrum was fitted with a sum of three Lorentzian line shapes to account for the direct saturation of water and to measure the CEST effects at 4.2 and 5.6 ppm [21]. The pH of each binned pixel with two CEST effects greater than $2\sqrt{2}$ times the standard deviation of noise of the CEST MR image was determined using an empirical calibration based on identical imaging of chemical solution. This empirical calibration can determine pH between 6.2 and 7.0 units with a precision of 0.07 pH units. These pixels were represented as colored pixels in the pHe map. Pixels with only a single CEST effect at 4.2 ppm greater than $2\sqrt{2}$ times the standard deviation of noise of the CEST MR image were set to pH 7.0. These pixels were represented as white pixels in the pHe map. Using a $2\sqrt{2}$ times the standard deviation of noise threshold ensure that the CEST contrast was due to the agent with a 95% probability [22]. A histogram showed pixel-wise analysis and the average pH and standard deviation of pixel distribution.

RESULTS

CA IX is expressed in clinical lymphoma tissue

CA IX expression was evaluated by using IHC to evaluate clinical lymphoma tissue arrays contain cases of Diffuse Large B-cell, Mantle cell (MCL), and Follicular lymphomas (Figure 1). Strong CA IX expression was observed in a subset of each lymphoma subtype, as shown by high median scores for CA IX IHC (Table 1). Yet scores of individual tissue samples showed high variability, as exemplified by high
interquartile ranges. As another indication of this variability, some of the tissues stained negative (zero score) for CA IX, while others had maximal staining (score of 300). This variability demonstrated heterogeneous expression of this protein, which may reflect differences in the tumor microenvironment for each patient. Overall, the detection of CA IX in 79% (45/57) of the clinical samples demonstrated that CA IX expression is substantial in clinical lymphoma.

![Representative images of Mantle Cell lymphoma (MCL), Diffuse Large B-cell lymphoma (DLBCL), and Follicular lymphoma (FL) clinical tissue stained for CA IX (200 magnification).](image)

**Figure 1.** Representative images of Mantle Cell lymphoma (MCL), Diffuse Large B-cell lymphoma (DLBCL), and Follicular lymphoma (FL) clinical tissue stained for CA IX (200 magnification).

**Table 1. CA IX IHC**

<table>
<thead>
<tr>
<th>Lymphoma Type</th>
<th>median score</th>
<th>interquartile range</th>
<th>number of tissues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mantle cell</td>
<td>90</td>
<td>190</td>
<td>19</td>
</tr>
<tr>
<td>Diffuse Large B-cell</td>
<td>125</td>
<td>115</td>
<td>21</td>
</tr>
<tr>
<td>Follicular</td>
<td>50</td>
<td>80</td>
<td>17</td>
</tr>
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</table>

**CA IX expression is induced by hypoxia in vitro**

We investigated the induction of CA IX gene expression in response to hypoxia using a qNPA assay to evaluate three B-cell lymphoma cell lines (Figure 2a). We tested Raji and Ramos lines that represent Burkett’s lymphomas, and a Granta 519 cell line that
represents a Mantle cell lymphoma. Minimal induction was observed in the Raji and Ramos cell lines, but the Granta 519 cell line demonstrated a time dependent increase in CA IX gene expression with more than a 2-fold induction by 48 hours. We also investigated the secretion of CA IX into cell culture media under normoxia and hypoxia (Figure 2b). Similar to message expression, the Granta 519 cells showed the highest induction of secreted CA IX after 48 hours of growth under hypoxic conditions. Western blot analysis of whole cell lysates grown under normoxia or hypoxia for 24 hours demonstrated basal CA IX levels were induced in response to hypoxia in all three cell lines (Figure 2c). Similar to the other results, the Granta 519 cells showed the greatest induction in response to hypoxia, relative to CA IX levels under normoxia. Ramos cells showed minimal induction. The Raji cells also showed some induction in response to hypoxia, although this cell line also showed a relatively high basal level of CA IX under normoxia, which may be a consequence of high basal levels of HIF-1α in this cell line.

CA IX expression is induced by extracellular acidosis in vitro

Because other stress signals in addition to hypoxia can induce CA IX, we investigated the impact of low pH on CA IX gene expression in Raji cells (Figure 2d). Cells were grown in media with 20 mM lactate (pH 7.3), or 20 mM lactic acid (pH 6.7). Interestingly, CA IX message levels were induced 1.69 fold after growth for 24 hours in lactic acid (compared to cells grown in normal media). As a control, we also investigated the gene expression changes monocarboxylate transport 1 (SLC16A1: MCT-1) and monocarboxylate transport 4 (SLC16A3; MCT-4) which also showed gene induction following growth in 20 mM lactic acid.
Figure 2. In vitro evaluations of B-cell lymphoma cell lines. a) A qNPA array was used to measure the fold change in CA IX gene expression in response to hypoxia. b) CA IX expression was measured using an ELISA in conditioned cell culture media from cell lines grown under normoxia or hypoxia. c) Western blot analysis of CA IX protein expression in whole cell lysate from lymphoma cells grown under normoxia or hypoxia for 24 hours. Tubulin expression was used as a loading control. d) A qNPA array was used to measure the fold change in CA IX gene expression in response to growth in lactate or lactic acid. Similar analyses of SLC16A1 and SLC16A3 were performed for comparison.

**CA IX is highly expressed in lymphoma xenograft models**

Lymphoma xenograft tumor Models of the same three B-cell lymphoma cell lines were tested for CA IX expression using IHC (Figure 3a). The three tumor models showed expression of CA IX throughout substantial portions of the tumor areas. The Ramos tumor model had the highest % area that stained positive for CA IX expression, and the Granta 519 tumor model had the most heterogeneous percent of area staining pattern (Figure 3b). We also stained tissues for VEGF-A to assess if similar staining patterns were observed as with CA IX. Moderate VEGF-A staining was observed in all tumor
types (Figure 3c). Similar to CA IX staining results, the Ramos tumor model had the highest VEGF-A score, and the Granta 519 tumor model had the most heterogeneous VEGF-A score.

Figure 3. IHC evaluations of B-cell lymphoma xenograft tumor models. a) Representative images of serial xenograft tumor sections stained for CA IX, pimonidazole, and VEGF-A with 100x magnification. b) The assessment of percent of lymphoma xenograft tumor area stained positive for CA IX (n=4). c) Immunoreactivity scores of lymphoma xenografts stained for VEGF-A (n=4). d) The assessment of percent of lymphoma xenograft tumor area stained positive for pimonidazole (n=4).
CA IX is not highly correlated with hypoxia in lymphoma xenograft models

To evaluate tissue regions of deep hypoxia, we used an exogenous hypoxia probe, pimonidazole, which can be reductively-activated to covalently bind to macromolecules. Following in vivo administration, pimonidazole distributes throughout the body where it covalently binds to normal and tumor tissue regions that have ≤ 14 micromolar dissolved oxygen concentration, which corresponds to a pO$_2$ of ≤ 10 mm Hg at 37°C that is typical of severely hypoxic tissues. Pimonidazole adducts can then be detected by IHC.

Each tumor model showed some staining for the hypoxia probe (Figure 3d). The Raji tumor model had the highest percent area stained positive for pimonidazole. Both the Raji and Granta 519 tumor models showed more heterogeneous staining patterns than the Raji tumors. The percent area of tumor stained for pimonidazole was significantly less than that stained for CA IX (p-value < 0.001) for each tumor type. Using qualitative assessment by a Board Certified Pathologist, the staining patterns of pimonidazole were not similar to CA IX staining patterns in any of the tumor models.

Lymphoma xenograft models have extracellular acidosis

We measured the average tumor pH$_e$ in xenograft models of the same three B-cell lymphoma cell lines, using acidoCEST MRI once per week for three weeks. The average tumor volumes for each imaging session showed that the tumor models experienced tumor growth during this time period (Table 2). In particular, the Ramos tumor model was only imaged for two weeks as these tumors exceeded the maximally allowed volume by week 3 and mice were euthanized. For each group, we calculated the average fraction
of tumor that was neutral or acidic, or had no uptake of the contrast agent (Figure 4a). The Granta 519 tumor model had the least agent uptake during the longitudinal study, while the Ramos tumor model had the most agent uptake.

The average tumor pHe across the three models ranged between 6.78 – 6.86 (Figure 4b). These average tumor pHe values were not significantly different between the three tumor models. The pixel-wise pHe values of the Ramos tumor model were less heterogeneous than Raji and Granta 519 tumor models (p-value < 0.002, Figure 4c). No significant trends were observed for spatial heterogeneities of pHe values relative to growth rates for the three xenograft models. Also, no significant temporal changes in tumor pH were observed in Raji or Ramos tumor models. However, the average tumor pH decreased significantly during the study of the Granta 519 tumor model (p-value < 0.02).

Table 2. Average tumor volume

<table>
<thead>
<tr>
<th>xenograft model</th>
<th>Week 0 (mm³)</th>
<th>Week 1 (mm³)</th>
<th>Week 2 (mm³)</th>
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<tbody>
<tr>
<td>Raji</td>
<td>217 ± 143</td>
<td>1005 ± 381</td>
<td>2505 ± 346</td>
</tr>
<tr>
<td>(n=4)</td>
<td>(n=4)</td>
<td>(n=3)</td>
<td></td>
</tr>
<tr>
<td>Ramos</td>
<td>321 ± 141</td>
<td>1812 ± 384</td>
<td>N/A</td>
</tr>
<tr>
<td>(n=5)</td>
<td>(n=5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Granta 519</td>
<td>181 ± 108</td>
<td>556 ± 280</td>
<td>1585 ± 405</td>
</tr>
<tr>
<td>(n=6)</td>
<td>(n=6)</td>
<td>(n=4)</td>
<td></td>
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</tbody>
</table>
Figure 4. AcidoCEST MRI evaluations of B-cell lymphoma tumor models. a) The fraction of the tumor estimated as having no significant uptake of agent, acidic pH $\leq 7.0$, or neutral pH $> 7.0$. b) The average tumor pH for Raji, Ramos, and Granta 519 tumor xenograft models. The error bars represent the standard deviation of the average of all mice on each day of imaging. c) The average standard deviation of pixel-wise pH values for Raji, Ramos, and Granta 519 tumor xenograft models, which represents the heterogeneity of pH in a tumor model. The error bars represent the standard deviation of this heterogeneity measurement of all mice on each day of imaging.

_AcidoCEST MRI can measure a range of lymphoma tumor pH values_

The three xenograft tumor models of B-cell lymphomas showed a narrow range of average tumor pH values measured with acidoCEST MRI (Figure 4b). This raised a concern that acidoCEST MRI may not be able to measure a range of tumor pH values. To validate acidoCEST MRI for assessments of lymphoma xenograft models, we investigated the ability of this imaging method to monitor changes in tumor pH that are
induced by MIBG. This mitochondrial poison causes a cell to shift from intracellular oxidative metabolism to glycolysis metabolism and thereby produce high levels of lactic acid.

To select a lymphoma xenograft model for this study, we first excluded the Ramos cell line from consideration because of the very rapid growth rate of this xenograft model. We then compared the in vitro induction of lactate in response to MIBG in the Raji and Granta 519 cell lines. The Raji cell line demonstrated the most consistent dose-response relationship between MIBG treatment and lactate expression and was chose for further study in vivo (Figure 5a).

Eight mice bearing Raji xenograft tumors were first scanned with acidoCEST MRI to establish a baseline pHe value two to four days before treatment. Considering that our previous in vivo study of the Raji tumor model showed no significant change in tumor pH over 14 days, the tumor pH was assumed to be the same as the baseline scan at the time of treatment. The mice were treated with MIBG and acidoCEST MRI was performed 4 hours later (Figure 5b).

The mitochondrial poison caused a decrease in average tumor pH in 6 of 8 mice (Figure 5c,d). The range of changes in the average value of tumor acidosis indicated that the effects of MIBG were variable among the mice. Most importantly, this range of pH values demonstrated that acidoCEST MRI can measure a range of tumor pH values,
which confirmed that the narrow range of average tumor pH values among the three tumor xenograft models is not an artifact of the acidoCEST MRI method.

![Image](image_url)

**Figure 5.** AcidoCEST MRI evaluations of the mitochondrial poisoning of the Raji tumor model. a) Analysis of secreted lactate acid following 4 hours of MIBG treatment of Raji and Granta 519 lymphoma cell lines *in vitro*. b) The pH map of a mouse bearing a Raji xenograft tumor before and after treatment with MIBG. Colored pixels have acidic pH values ≤ 7.0 that correspond to the color-bar. White pixels represent tumor regions with only a single CEST effect at 4.2 ppm, which were considered to have neutral pH values > 7.0. c) Cumulative pixel distribution before and after MIBG treatment from the mouse shown in Figure 5B. d) A waterfall plot of the change in average tumor pH in Raji tumors (n=8) following treatment with MIBG. Mice with statistically significant changes are marked with an asterisk (p < 0.01).

**DISCUSSION**

This study is one of the first to report high CA IX expression in clinical samples of B-cell lymphoma and in lymphoma xenograft tissue. We detected CA IX expression in 79% (45/57) of the clinical samples, which represented cases of mantle cell, diffuse large B-
cell, and follicular lymphomas. All three xenograft lymphoma tumor models also showed CA IX expression. For comparison, a previous study reported on the expression of CA IX in only 33% (16/48) of high grade lymphomas [23]. To our knowledge, no other studies of CA XI expression in NHL have been reported, and no studies have attempted to correlate CA IX expression and clinical outcomes in lymphoma. Therefore, our studies indicate that CA IX is a useful biomarker for NHL that warrants investigation in future clinical studies.

The three lymphoma xenograft models showed similar average tumor pH values between 6.78 and 6.86 as measured with acidoCEST MRI. This imaging method can measure a larger range of pH values, as shown by our acidoCEST MRI studies following mitochondrial poisoning of the Raji tumor model (Figure 5d). We have also observed a greater range of measured tumor pH values in our other studies [14]. Therefore, this narrow average pH range in the three lymphoma xenograft models is not an artifact of the imaging method, and instead demonstrates that tumor acidosis is a consistent effect within these xenograft tumor models of B-cell lymphoma.

The relationship between tumor acidosis and CA IX expression should be considered when selecting pre-clinical models for studies of small molecule CA IX inhibitors or anti-CA IX antibody therapies [24], including studies of CA IX inhibitor chemotherapies directed against lymphoma [25].
Conversely, our in vivo studies did not find a correlation between CA IX expression and hypoxia. This finding may be due to the different level of oxygen required to activate pimonidazole than the level needed to induce CA IX through HIF-1α [26]. In addition, our in vitro studies showed that Granta 519 cell line had greater CA IX expression (Figure 2b) and greater induction of CA IX expression under hypoxia (Figure 2a,c). Yet our in vivo studies showed the highest CA IX expression in the Ramos tumor model. This difference between the in vitro and in vivo results strongly indicates that the tumor microenvironment such as studies involving extracellular pH and hypoxia should be studied in vivo.

The trend in contrast agent uptake observed in our in vivo studies of the three lymphoma tumor models paralleled the growth rates of each tumor model. This evidence suggests that the uptake of the contrast agent reflected each tumor models’ uptake of similar small molecule nutrients that can promote tumor growth. Furthermore, the trend in increasing tumor acidity in the Granta 519 tumor model paralleled the growth of this tumor model, again suggesting that tumor acidosis reflects the general metabolic rate that promotes tumor growth. Future studies with biomarkers of general tumor metabolism should be performed to further investigate this connection between acidoCEST MRI evaluations and tumor growth. As shown by the mitochondrial poisoning study of the Raji tumor model, acidoCEST MRI may also be useful for monitoring the early response to inhibitors of tumor metabolism.
REFERENCES


APPENDIX C

EVALUATIONS OF TUMOR ACIDOSIS WITHIN IN VIVO TUMOR MODELS USING PARAMETRIC MAPS GENERATED WITH ACIDOCEST MRI

PUBLICATIONS

INTRODUCTION

The extracellular pH (pHe) of a solid tumor is commonly considered to be acidic, ranging between approximately pH 6.5 and 7.2 [1]. This acidity is often attributed to the Warburg effect, when tumor cells have a propensity to undergo aerobic glycolysis that produces excess lactic acid [2]. This tumor acidosis has been suggested to contribute to growth, invasion, and metastasis and may also cause chemoresistance to weak-base drugs [3,4]. In vivo tumor acidosis has been studied with pH microsensors, including clinical studies of malignant melanomatosis tumor [5]. However, the “minimally” invasive procedure of inserting a microsensor into tissue can cause discomfort and damage, and is inappropriate for probing inaccessible tumors in deep tissues. A variety of non-invasive biomedical imaging methods have been developed to measure in vivo tumor acidosis, but each of these methods has limitations. For example, fluorescence imaging of pHe is limited to surface tissues [6,7]; instrumentation for measuring pHe with in vivo EPR ([8] and hyperpolarized MR spectroscopy [9] is not readily available; PET imaging [10,11] and MR spectroscopic imaging [12] lack spatial resolution for measuring pHe; relaxation-based MRI cannot measure pHe without also accounting for the concentration of an exogenous contrast agent [13].

Chemical Exchange Saturation Transfer (CEST) is a relatively new MRI contrast mechanism [14]. The CEST MRI method applies a radio frequency pulse that selectively saturates the magnetization of a proton, which eliminates the coherent MR signal of this proton (Figure 1a, step 1). Subsequent chemical exchange of this proton with a proton on a near-by water molecule causes a loss of coherent MR signal from the water (Figure 1a,
step 2). CEST spectra, also known as Z-spectra, are obtained by iterating the selective saturation radio frequency and recording the % water signal that remains after the saturation period (Figure 1b). The amplitudes of multiple CEST effects in a single CEST spectrum can be measured by fitting Lorentzian line shapes to the experimental CEST spectrum (Figure 1c) [15].

Figure 1. In situ CEST MRI
(a) Step 1: Selective saturation of one amide proton of iopromide causes a loss of coherent net magnetization from the proton (shown as a conversion from white to black). Step 2: Base-catalyzed chemical exchange of the proton from iopromide to water transfers the saturation to the water. Only protons of the two amide groups and water are shown. (b) CEST spectra show the water signal as a function of selective saturation. The CEST amplitudes at 4.2 and 5.6 ppm are dependent on pH. (c) The CEST amplitudes can be measured with Lorentzian line shape fitting. Residuals of the fitting process are shown as squares. (d) A ratio of the CEST effects is linearly correlated with pH.

The amplitude of the CEST effect involving an amide is pH-dependent because the chemical exchange of an amide proton is base-catalyzed (Figure 1a, step 2) [16]. We and others have shown that the ratio of CEST effects from two protons on the same CESST agent can be used to measure pH in a concentration-independent manner (Figure 1d) [17-20]. Careful attention to consistent image acquisition and processing established a
measurement precision of 0.07 pH units for our method [21]. We have also previously demonstrated that this method, termed “acidoCEST MRI” can monitor relative changes in average tumor pH. Our current report builds on our previous studies by demonstrating that acidoCEST MRI can measure accurate tumor pH values, can be used to produce parametric spatial maps of tumor pH, and can evaluate the differences in tumor acidosis between pre-clinical models of mammary carcinoma.

METHODS

Reagents

All reagents were obtained from Sigma-Aldrich, Inc., (St Louis, MO) unless otherwise indicated. Clinical-grade iopromide at 788 mM was used (300 mg iodine/mL Ultravist™, Bayer Health Care, Inc., Leverkusen, Germany).

Xenograft tumor model

All in vivo studies were conducted according to approved procedures of the Institutional Animal Care and Use Committee of the University of Arizona. A model of MDA-MB-231 mammary carcinoma was prepared by injecting one million tumor cells in 0.5 mL of 50% Matrigel™ into the upper right flank of a 6-week-old female SCID mouse. For the model of MCF7, one million tumor cells in 0.5 mL of 50% Matrigel™ were injected into the upper right flank of a 6-week old nude mouse with an 8-week slow-releasing estrogen pellet implanted in the back of the neck. Imaging studies were conducted when the subcutaneous tumor reached a size greater than 2 mm in diameter, and once every two
weeks thereafter. Each mouse was measured 4 times with acidoCEST MRI, unless the tumor reached $\geq 2000 \text{ mm}^3$ or became necrotic, at which time the mouse was euthanized.

**acidoCEST MRI**

Our acidoCEST MRI protocol has been described in a previous report [21]. Briefly, each mouse was anesthetized with 1.5 – 2.5 % isoflurane delivered in 1 L/min oxygen gas ventilation, and then secured to a customized cradle. A 27 g catheter was inserted into the tail vein. The cradle was inserted into a 7T Biospect MRI scanner with a 72 mm-diameter quadrature transceiver coil temperature was regulated at 37.0 ± 0.5ºC using an automated feedback loop between the temperature probe and an air heater (SA Instruments, Inc., Stony Brook, NY). A FLASH MRI acquisition sequence was performed to determine the location of the tumor (500 msec TR, 10 msec TE, 625 x 625 $\mu$m in-plane resolution, 2 mm slice thickness, 64 x 64 pixels, 4.0 x 4.0 cm FOV, 32 sec total acquisition time). A spin-echo MRI acquisition sequence was performed for the tumor volume measurements (2000 msec TR, 35 msec TE, 312.5 x 312.5 $\mu$m in-plane resolution, 1 mm slice thickness, 128 x 128 pixels, 4.0 x 4.0 cm FOV, 4:20 min total acquisition time). A bolus of 200 $\mu$L iopromide was injected via the catheter within 30 seconds. The catheter was then connected to an infusion pump and iopromide was pumped at a rate of 150 $\mu$L/hr. Our acidoCEST MRI protocol consisted of 54 CEST-FISP MR images acquired at different saturation frequencies using 2.8 $\mu$T saturation power and a saturation period of 5 seconds, which required 4:40 acquisition time. The CEST saturation period consisted of a series of Gaussian shaped radio frequency pulses with an interpulse delay of 10 $\mu$s and no additional spoiling of fat saturation pulses. The FISP acquisition used the following
parameters: 3.218 msec TR; 1.609 msec TE; 60° excitation angle; 2 mm slice thickness; 250 x 250 \( \mu \text{m} \) in-plane resolution; 3.2 x 3.2 cm FOV; linear encoding order; unbalanced “FID” mode; 418.54 msec scan time. This process was repeated 6 times, for a total acquisition time of 28 minutes. At the conclusion of the imaging scan, the mouse was removed from the scanner and cradle, and allowed to recover.

**Image processing**

The acidoCEST MR images were processed using MatLab® R2012B (Mathworks, Inc., Natick, MA). All 6 CST-FISP images were average. To improve the signal-to-noise for individual pixels, a pixel averaging technique was employed that binned 3 x 3 adjacent pixels, which decreased the original spatial resolution. A second pixel averaging technique was used that applied a Gaussian low pass filter with the size of 7 x 7 adjacent pixels with a standard deviation of one pixel, which retained the original number of pixel per image. The CEST spectrum from each of the averaged pixels was smoothed with cubic spline smoothing [22]. Thereafter, the smoothed CEST spectrum was fitted with a sum of three Lorentzian line shapes, to account for the direct saturation of water and to measure the CEST effects at 4.2 and 5.6 ppm [15]. The pHe of each averaged pixel with two CEST effects greater than \( 2\sqrt{2} \times \text{noise} \) was determined using an empirical calibration based on chemical solutions of iopromide (Figure 1d). This empirical calibration can determine pHe between 6.2 and 7.0 units with a precision of 0.07 pH units. These pixels were represented as colored pixels in the pHe map. Pixels with only a single CEST effect at 4.2 ppm greater than \( 2\sqrt{2} \times \text{noise} \) were set to be pH 7.0. These pixels were represented as white pixels in the pHe map. Using a \( 2\sqrt{2} \times \text{noise} \) threshold ensured that the CEST
contrast was due to the agent with 95% probability [23]. The extent of contrast agent uptake was the summation of colored pixels and white pixels relative to the total number of pixels that represented the tumor.

_Tumor microsensor pHe measurements_

Microsensor pHe measurements were performed with mice immediately after acidoCEST MRI imaging sessions, while the mice were still under anesthesia. The anesthesia was supplied throughout the microsensor pHe measurement. The anesthetized mouse was placed on a heating pad to maintain the body temperature at 37°C. There was only a subset of mice on a subset of days were measured with the microsensor, because the fragile microsensors were prone to breaking, and replacement sensors were not always immediately available.

A five-point calibration between pH 4.0 and 9.0 was performed with the needle-type pH microsensor (PreSens Precision Sensing GmbH, Regensburg, Germany) each day prior to _in vivo_ measurements. Five pH solutions were prepared by mixing 40 mM NaH$_2$PO$_4$ and 40 mM Na$_2$HPO$_4$. The pH values of these buffer solutions were determined with a calibrated pH electrode (Mettler Toledo, Columbus, OH USA). The pH microsensor was securely fastened to a micromanipulator and inserted into the subcutaneous tumor tissue. Once inserted, the pH microsensors were extended outside the needle housing by 1 mm to minimize damage to the fiber optic tip. Four pH measurements were recorded in 10-second intervals after the pH recording was stabilized, which typically occurred one minute after extending the microsensor out of the needle housing. After the pH
measurement, the microsensor was retracted back into the needle housing, and the microsensor and housing were withdrawn from the tumor. This procedure was repeated for 2 additional locations in the tumor to assess the heterogeneity of tumor pHc.

Statistical analysis

The Student’s t-test was used for all statistically significant comparisons.

RESULTS

In vivo acidoCEST MRI

We performed in vivo acidoCEST MRI studies with xenograft tumor mouse models of MCF-7 and MDA-MB-231 mammary carcinoma. After preparing each mouse for imaging session, a diamagnetic CEST agent, iopromide (Ultravist™, Bayer Healthcare, Inc., Figure 1a), was injected i.v. and continuously infused during the MR imaging session. In vivo CEST spectra were obtained by iterating a saturation frequency from -10 to +10 ppm, and recording the normalized water signal amplitude using a FISP MRI acquisition protocol (Figure 2a). Pixel averaging methods and a cubic spline smoothing procedure [22] facilitated the selective detection of two CEST effects from iopromide within the pixels of the MR images. The CEST spectrum from each pixel was fitted with Lorentzian line shapes [15] to quantitatively measure each CEST effect (Figure 2b). The low residuals near 4.2 and 5.6 ppm attest that these fittings could accurately measure each CEST effect. CEST amplitudes above a $2\sqrt{2} \times$ noise threshold were retained, because this threshold represented a CEST effect that could be assigned to the agent with a 95% probability.
Figure 2. *In vivo* acidoCEST MRI.
(a) An *in vivo* CEST spectrum of iopromide in a pixel within a tumor shows two CEST effects at 4.2 and 5.6 ppm. (b) The CEST amplitudes are measured with Lorentzian line shape fitting to the CEST spectrum.

Our previous acidoCEST MRI analysis methods only measured the average tumor pH. Our current methods improved on our previous methods by creating parametric maps of pixelwise pH values (Figure 3b,c). Colored pixels indicated regions with two CEST effects greater than $2\sqrt{2} \times$ noise, which were used to measure the pH of the pixel. White pixels indicated pixels with a single CEST effect at 4.2 ppm greater than $2\sqrt{2} \times$ noise, which confirmed that sufficient agent had been delivered to the pixel region, but the pH was too high to generate a measurable CEST effect at 5.6 ppm. These pixels were considered to have a pH of 7.0. Although the pH of these pixels may be higher, our methodology is intended to measure tumor acidity, and pixels above pH 7.0 were considered to be pH-neutral.
Figure 3. AcidoCEST MRI measured tumor pHe in a subcutaneous MCF-7 mouse model with Gaussian low-pass filtering.

(a) A FISP image shows the tumor location. (b,c) The pHe maps show colorful pixels where two significant CEST effects were measured and converted to pHe values, while white pixels show where only one significant CEST effect was measured, which represented pH > 7.0. Gaussian filtering generated a pHe map with better spatial resolution than 3 x 3 binning. (d) A range of water chemical shifts throughout the tumor and torso showed the effects of $B_0$ inhomogeneity, but the CEST effects at (e) 4.2 ppm and (f) 5.6 ppm were measured with homogenous chemical shifts, demonstrating that $B_0$ inhomogeneity did not affect the pH measurements. (g) Similarly, a range of relatively broad water peak widths were measured in the tumor and torso, but more homogenous and narrow peak widths were measured for the CEST effects at (h) 4.2 ppm and (i) 5.6 ppm.

Our first attempts to analyze CEST spectra from individual pixels were unsatisfactory due to low contrast-to-noise (CNR) (Figure 4). To improve the CNR, we average the 6
CEST-FISP images acquired during a single acidoCEST MR imaging session. We then performed pixel averaging by binning a 3 x 3 cluster of pixels into a single pixel, and then smoothed each CEST spectrum from the binned pixel with a cubic spline function. Although this processing procedure provided acceptable CNR for fitting CEST spectra with Lorentzian line shapes, the binning process reduced the spatial resolution to 750 µm. Therefore we performed pixel averaging using a Gaussian low-pass filter, and then performed the cubic spline smoothing and Lorentzian line shape fitting. This procedure retained the original number of pixel per MR image (128 x 128 pixels, FOV 3.2 x 3.2 cm), and was used for all subsequent analyses. The procedures with 3 x 3 binning and Gaussian low-pass filtering produced the same pHe values, spatial heterogeneity, and % uptake values (Table 1), which indicated that the Gaussian filtering did not affect the quantitative measurements.

Figure 4. The effect of binning adjacent pixels on spatial resolution. AcidoCEST MRI measured tumor pHe in a subcutaneous MCF-7 mouse model. (a) A FISP image shows the tumor location. (b-f) The pHe maps with no binning or with binning showed colorful pixels where two significant CEST effects were measured and
converted to pHe values while, white pixels showed regions where only one significant CEST effect was measured which represented pH > 7.0.

Table 1. AcidoCEST comparison for Gaussian low-pass filtering and 3 x 3 binning.

<table>
<thead>
<tr>
<th></th>
<th>MCF-7</th>
<th>MDA-MB-231</th>
<th>Measurement</th>
<th>MCF-7</th>
<th>MDA-MB-231</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average pHe</td>
<td>0.998</td>
<td>0.781</td>
<td>6.70 ± 0.08</td>
<td>6.80 ± 0.05</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>Average standard deviation of pHe distribution</td>
<td>0.786</td>
<td>0.269</td>
<td>0.155 ± 0.0047</td>
<td>0.097 ± 0.032</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>Average % uptake</td>
<td>0.361</td>
<td>0.535</td>
<td>51.0% ± 25.6</td>
<td>61.9% ± 22.8</td>
<td>0.122</td>
<td></td>
</tr>
<tr>
<td>Average tumor volume (mm³)</td>
<td></td>
<td></td>
<td>517.4 ± 555.8</td>
<td>188.8 ± 272.6</td>
<td>0.037</td>
<td></td>
</tr>
</tbody>
</table>

$B_0$ magnetic field inhomogeneity was evident across the image of each mouse including the tumor region, as indicated by the deviation of water chemical shift from 0.0 ppm (Figure 3d; similar results with 3 x 3 binning are shown in Figure 5). However, measurements of the chemical shifts of the CEST effects showed little deviation from the expected values of 4.2 and 5.6 ppm, which showed that our Lorentzian line shape fitting method correctly identified CEST peaks despite the presence of $B_0$ inhomogeneity (Figure 3e,f). Similarly, $B_0$ inhomogeneity and potential variability in endogenous $T_2$ relaxation times were evident in the image as shown by the variability of the width of the direct saturation of water (Figure 3g). This $B_0$ inhomogeneity and $T_2$ variability was more evident in the tumor tissue and other tissue regions relative to the regions of the bladder and phantom of iopromide, because these latter regions were more homogenous and more fluid, leading to lower $B_0$ inhomogeneity and longer $T_2$ relaxation times. Yet measurements of the line widths of the CEST effects at 4.2 and 5.6 ppm showed little
deviation, which showed that the CEST effects were insensitive to $B_0$ inhomogeneity and $T_2$ relaxation in these tumor models (Figure 3h,i).

![Figure 5. Characteristics of the CEST spectrum evaluated with 3 x 3 binning.](image)

(a) A range of water chemical shifts throughout the tumor and torso showed the effects of $B_0$ inhomogeneity, but CEST effects at (b) 4.2 ppm and (c) 5.6 ppm were measured with homogenous chemical shifts, demonstrating that $B_0$ inhomogeneity did not affect the pH measurements. (d) Similarly, a range of relatively broad water peak widths were measured in the tumor and torso, but more homogenous and narrow peak widths were measured for the CEST effects at (e) 4.2 ppm and (f) 5.6 ppm. The 3 x 3 binning created parametric maps with lower resolution compared to the Gaussian low-pass filtering used to create the parametric maps in Figure 2d – i.

*Comparison of tumor pH* measurements with acidoCEST MRI and a microsensor

We compared our tumor pH measurements using acidoCEST MRI with tumor pH measurements made with a pH microsensor (PreSens Precision Sensing GmbH, Regensburg, Germany) (Figure 6c). Assuming that the pH microsensor is the “gold standard”, the accuracy of the acidoCEST MRI method was determined to be 0.034 pH units. The pH values of each tumor were not significantly different ($p = 0.14$). The mean and median standard deviations of the acidoCEST MR measurements of each tumor were
both 0.13 pH units, respectively, and the mean and median standard deviations of the microsensor measurements were 0.12 and 0.10 pH units, which were not statistically different (p = 0.72). Therefore, the precisions of both methods were also similar.

The comparison of pH values measured with acidoCEST MRI and the microsensor required two considerations. Only a subset of mice on a subset of days were measured with a microsensor, because the microsensor was mechanically fragile and frequently broke during use, despite being protected by a stainless-steel needle housing. Also, the fluorescent coating was scrubbed away after repeated measurement, causing the microsensor’s measurements to become increasingly unreliable with each insertion into the tumor tissue. Tumor pH values measured with the microsensor were discarded if the value fell outside the physiological range of pH 6.4 to 7.2 (Figure 6a,b). The insertion of the microsensor also caused necrosis, which demonstrated that the microsensor is invasive. The difficulty in measuring tumor pH with a microsensor emphasizes the need for a non-invasive acidoCEST MRI method.

As a second consideration, the microsensor measure tumor pH values with “biopsy-style” sparse coverage. To account for this sparseness, we assumed that tumor with > 50% of the tumor area that had pH values < 7.0 would be sparsely sampled by the microsensor only in these acidic regions. Therefore, we calculated the average pH of the tumor using all pixelwise pH values below 7.0 when more than 50% of the measured tumor area had pH values below 7.0. We assumed that the other tumors with a larger distribution of regions with pH above and below pH 7.0 would sampled by the
microsensor in any tumor region, so we calculated the average pH of these tumors using all pixelwise pH values above and below 7.0. With this consideration for “biopsy-style” sparse coverage of the microsensor, the pH measurements with both methods were highly correlated ($R^2 = 0.74$; $R^2 = 0.56$ without this consideration; Figure 6).

![Figure 6](image)

**Figure 6.** Comparison of pH measurements with a microsensor and acidoCEST MRI. (a) A comparison of all measurements, including measurements made with deteriorating microsensor ($\Delta$). (b) A comparison of measurement with only non-deteriorating microsensor (○). (c) A comparison of pH measurements with a microsensor and acidoCEST MRI shows excellent agreement. The average pH of tumors with > 50% uptake was calculated using pixels with pH < 7.0 (●), and the average pH of tumors with < 50% uptake was calculated using all pixels of the pH map (○).

**Comparison of mouse models with acidoCEST MRI**

We used MRI to measure tumor volume, which showed that the MCF-7 tumor model grew faster than the MDA-MB-231 tumor model (p-value = 0.006) (Figure 7a). This faster growth was due to implanting the estrogen pellet in MCF-7 model. The MCF-7 model was more acidic than the MDA-MB-231 model (p-value < 0.001) (Table 1 and Figure 7b). This result was similar to a previous observation that measured the average pH of MCF-7 and MDA-MB-231 xenograft tumor models with $^1$H MRS of IEPA [24]. These results are consistent with the Warburg effect, because the faster growth of the MCF-7 model suggests a more rapid glycolytic metabolism that produces more lactic
acid. We also observed that the spatial distribution of pixel-wise tumor pH\textsubscript{e} values for the MCF-7 model was greater than the MDA-MB-231 model, as measured by the standard deviation of the pixelwise values in each tumor pH\textsubscript{e} map (Figure 7c). These results suggest that the tumor microenvironment was more heterogeneous in the faster-growing tumor model.

Figure 7. Longitudinal changes in tumor characteristics.

No statistically significant longitudinal changes were observed in (a) average tumor pH\textsubscript{e}, (b) the distribution of pixelwise pH\textsubscript{e} values, or (c) % agent uptake in the tumor during the 6-week study, for both tumor models. However, the MCF-7 model had lower pH\textsubscript{e} and higher standard deviation of pH\textsubscript{e} distribution relative to the MDA-MB-231 model. The bars of figure 4C are labeled with the number of mice tested each week for each tumor type. (d) Both tumor models grew in volume as expected.

The MDA-MB-231 model had higher % uptake of the agent in the tumor relative to the MCF-7 model, but this difference was not statistically significant (Figure 7d). Higher %
uptake of the agent during the MRI studies suggests higher vascular permeability that facilitates the uptake of nutrients into the tumor, and may also suggest greater tumor angiogenesis [25]. Therefore, a higher % uptake of the agent in the faster growing tumor model was expected. Also, the uptake of the agent at a level above the detection threshold was less than 100% in all tumors, and therefore the tumor pH(e) could only be evaluated in a subset of the tumor. This bias towards acidoCEST MRI measurements of tumor regions with higher uptake of iopromide may bias the average measurement of tumor pH(e). Future developments of CEST MRI methodologies that improve detection sensitivity may improve the interrogation of the entire tumor with acidoCEST MRI [26].

Similar quantitative correlations between tumor pH(e), % uptake, and tumor size were not observed for individual mice. The MCF-7 tumors and MDA-MB-231 tumors reached their respective level of pH(e) at a small size, and these tumors for individual mice maintained their respective acidity levels as the tumor grew (Figure 8a). Similarly, each tumor model reached a level of acidity regardless of the level of % uptake of the agent in the tumor, and this level of acidity did not change for each individual mouse (Figure 8b). The % uptake tended to be higher for larger tumors for the fast-growing MCF-7 model (Figure 8c). No relationship between % uptake and tumor size was observed for the slower growing MDA-MB-231 model (Figure 8d). This last result may indicate that the larger tumors faster growing model required more vascular permeability to facilitate nutrient delivery that maintained growth.
Figure 8. Tumor pH correlation. Tumor pH was uncorrelated with (a) tumor volume and (b) % uptake for both tumor models. (c) The % uptake tended to be greater for larger MCF-7 tumors, (d) but % uptake was not correlated with tumor volume for the MDA-MB-231 model.

DISCUSSION

Our study shows that acidoCEST MRI can accurately measure tumor pH, and can generate parametric maps of pH values with 750 µm in-plane spatial resolution. AcidoCEST MRI improves on previous imaging methods that measure tumor pH, because our MRI method is no limited to interrogating tumor surfaces; can measure tumor pH with excellent spatial resolution; can measure tumor pH in a concentration-independent manner; and can measure tumor pH with pre-clinical MRI instrumentation that is readily available at many institutions.
As another advantage relative to previous imaging methods, acidoCEST MRI has strong potential to be translated to the clinic. The FISP MRI acquisition protocol is available for most clinical MRI scanners. Clinical CEST MRI protocols have been implemented to measure endogenous CEST effects in other organs [27,28]. Although the RF saturation used for acidoCEST MRI raises safety concerns about the absorbance of high radio frequency energies in tissues, these other clinical CEST MRI protocols have used stronger saturation powers and longer saturation times than the saturation power and time used in our study. As a major advantage, iopromide is already approved for clinical x-ray and CT studies, and is approved for i.v. injection into patients at an amount that is 4 – 5.5-fold higher than the amount used during out studies with mouse models [21].

The acidoCEST MRI method has strong potential to be used for many studies with mouse models of human cancers. This report demonstrated that tumor pHe was not correlated with tumor size of vascular perfusion in xenograft models of MCF-7 and MDA-MB-231 mammary carcinoma. Therefore, pHe is an independent biomarker of the tumor microenvironment. Additional studies are warranted to determine if tumor pHe is correlated with biomarkers associated with acidosis such as carbonic anhydrase IX [29], or if tumor pHe is linked to hypoxia [30]. Additional studies with other xenograft, orthotopic and transgenic models are warranted to determine if tumor location within the mouse model affects tumor pHe. These studies may be useful for ensuring that an appropriate mouse model is selected for each cancer biology study.
AcidoCEST MRI may have particular utility for predicting chemotherapeutic efficacy before a drug treatment is initiated. As shown in this study, the MCF-7 mouse model had a significantly lower average tumor pH relative to the MDA-MB-231 mouse model. Tumor acidosis can cause chemoresistance to weak-base drugs, and may potentiate the efficacy of weak-acid drugs [3,4]. Therefore, pre-clinical studies of pH-dependent drugs should select a mouse model with an appropriate tumor pH for best results. When acidoCEST MRI is translated to the clinical, clinical trials can stratify patient populations based on tumor pH to properly assess pH-dependent drugs. Furthermore, an individual patient’s tumor pH may provide decisive evidence for prescribing a weak-base or weak-acid drug, which can provide the paradigm of personalized medicine for each individual patient [31]. For these many reasons, acidoCEST MRI may have great potential to impact patient care.

Reference


APPENDIX D

FUTURE DIRECTIONS
SUMMARY

A novel MRI technique, termed acidoCEST MRI, has been developed to measure extracellular pH in the tumor microenvironment. We measured the CEST effects from the two different amides present on a single DIACEST agent, iopromide (Ultravist™, Bayer Healthcare, Inc.). This ratiometric approach does not require complete saturation of the exchanging protons, and is independent of concentration and endogenous T$_{1\text{sat}}$ relaxation times. However, the ratio of CEST effects is dependent on temperature. Hence all our acidoCEST MRI experiments were conducted at 37° C. We have investigated various routes of administration and determined that IV-infusion delivered a sufficient and stable amount of agent to the tumor tissue. AcidoCEST MRI with ROI analysis was used to longitudinally monitor the effect of bicarbonate treatment on the pHe of tumors and bladders in MDA-MB-231.

We have improved our acidoCEST MRI from ROI to pixel-wise pHe analysis. Pixel-wise pHe maps allow us to identify intratumoral regions that are acidic and pH-neutral and access spatial heterogeneity. To improve contrast to noise ratio for single pixel, we binned 9 adjacent pixels (3 x 3 binning). All CEST measurements that are 2√2 times the standard deviation of noise of the CEST MR image have a 95% probability of being real. With the evaluation of neutral pixels, we developed a new imaging biomarker: % contrast agent uptake. Pixel-wise acidoCEST MRI was used to estimate lymphoma xenografts’ pHe, spatial heterogeneity and % contrast agent uptake. AcidoCEST MRI was also used to monitor these biological changes following pharmacological inhibition of oxidative phosphorylation using m-iodobenzylguanidine (MIBG).
Binning is detrimental to spatial resolution. To maintain our initial resolution of 250 µm x 250 µm, we used a weighted averaging technique – Gaussian low-pass filtering. We have shown that our acidoCEST protocol is robust and insensitive to B₀ and B₁ field inhomogeneity. We compared pHₑ, spatial heterogeneity and % contrast uptake of 2 mammary carcinoma models: MCF-7 and MDA-MB-231. In addition, we also validated our acidoCEST MRI with a microsensor (PreSens, Germany).

Our improved acidoCEST MRI protocol consists of: CEST-FISP acquisition [1], Gaussian low-pass filtering, Lorentzian line shapes fitting [2] and empirical calibration of CEST and pH.

**FUTURE DIRECTIONS**
Based on our statistical criteria, acidoCEST MRI can only reliably measure a maximum pH of 7.2 for analysis of a ROI, and pH 7.0 for pixel-wise analysis. At above neutral pH, the exchange rate for the CEST effect at 5.6 ppm becomes too fast and coalesced with water. Therefore, our measurements may have underestimated the average pHₑ of tumors that were classified as pH-neutral. This under estimation is expected to be minor, because pHₑ in tumors rarely expected to exceed pH 7.0 [3]. Nonetheless, to evaluate the efficacy of alkalinizing treatment such as bicarbonate, or to evaluate neutral/basic organ such as liver (7.34 ± 0.03 [3]), a method that can measure above 7.0 for pixel analysis is crucial.
**Engineering aspects**

Another analogue of iopromide, iopamidol, has been shown to measure a range of 5.5 – 7.4 for pixel-wise analysis in lung [4]. Silvio Aime and colleagues have used a fat suppressed single-shot spin echo RARE sequence with centric encoding preceded by saturation. Iopamidol and iopromide have similar chemical structures and are both clinically approved for X-ray/CT imaging. We can mimic their MRI acquisition as opposed to our current FISP with linear encoding.

To reduce the effect from $T_2$ relaxation, we can use a $30^\circ$ excitation angle rather than the current $60^\circ$ excitation angle. This change will not be prominent in a region with a long $T_2$ relaxation time, such as the tumor. However, this change may be effective for imaging in more ‘solid-like’ environment such as lung.

Preliminary studies performed by our group have shown that dual bandwidth Gaussian pulses can make the CEST effect at 4.2 and 5.6 ppm appear sharper in a CEST spectrum. This process does not improve the contrast to noise ratio. However, the shaper CEST peaks will enable easier fitting with Lorentzian line shapes.

**Reducing the number of saturation frequencies**

An effective way to improve temporal resolution is to reduce the number of saturation frequencies. We have demonstrated that the CEST effects at 4.2 and 5.6 ppm deviates little from their designated values, despite the $B_0$ field inhomogeneity that results in huge shifts in water frequency center from 0.0 ppm. Our acidoCEST MRI protocol consists of
52 saturation frequencies distributed between ± 10 ppm, with higher imaging density at the positive region to detect subtle changes in amide CEST effects. The 52 saturation frequencies also increase the insensitivity of our acidoCEST MRI to B₀ inhomogeneity. Water saturation shift referencing (WASSR) adjusts the water frequency center to 0.0 ppm on a voxel-by-voxel basis independently of spatial B₀ field variation [5]. This process removes the problem associated with B₀ field inhomogeneity, and provides us with a chance to reduce the number of saturation frequencies since B₀ field variation is no longer an issue.

*Image processing prior to Lorentzian line shape fitting*

When we first attempted pixel wise analysis, we used the “imresize” function to bin neighboring pixels together to increase contrast to noise ratio. Unfortunately, binning is detrimental to spatial resolution because it combines a cluster of pixels into a single pixel, and thus reduces the overall number of pixels. Our next step was to use the “imfilter” function that incorporated a Gaussian low-pass filter. As “imfilter” returns the same matrix size as input, the resolution of final image is maintained. The parameter inputs for the Gaussian low-pass filter are width of the Gaussian (sigma) and size of the filter (number of pixels). Future studies can be conducted to optimize these parameters to improve the contrast to noise ratio. Besides optimizing the parameters, we can try to first shift each voxel’s spectra so that the water is on resonance (0.0 ppm), and then perform Gaussian low-pass filtering. This will prevent the Gaussian filtering from smearing out the CEST peaks at 4.2 and 5.6 ppm.
Besides Gaussian low-pass filtering, there are other filtering operators in MATLAB that we can try: “average”, “disk”, “laplacian”, “log”, “motion”, “prewitt”, “sobel” and “unsharp”. There are a few promising filtering operators that we can try. For example, “motion” is a filter for horizontal and vertical motions, and may be useful to reduce motion artifact when we image lung or liver; “unsharp” is an operator to sharpen images by first subtracting a blurred (unsharp) version of the image from itself.

Multi-slice acidoCEST MRI

Our acidoCEST MRI protocol images a single two-dimensional slice. A 3D pHe map can be constructed by incorporating multi-slice CEST MRI techniques into an acidoCEST MRI protocol. This can further improve the evaluation of spatial heterogeneity of intratumoral regions and % contrast agent uptake. However, multi-slice CEST-FISP protocol increases acquisition time. A dual slice CEST-FISP protocol is twice as long as a single slice. A minimum of 3 – 4 slices with 2.0 mm slice thickness of CEST-FISP is required for the entire tumor coverage of a mid-size subcutaneous tumor (~1000 mm$^3$). Our current acidoCEST MRI protocol takes the average of 6 CEST-FISP images in ~ 30 minutes to improve contrast to noise ratio. Our group has also tried 4 averages in an ovarian orthotopic SKOV-3 cancer model that produces good contrast to noise ratio. Multi-slice acidoCEST MRI is feasible with improved contrast to noise ratio that reduces the need for repetition.
Validation with an electrode

We have validated the accuracy of acidoCEST MRI with an external electrode, the microsensor (PreSens, Germany). This microsensor was mechanically fragile and not ideally designed for the use on a hard subcutaneous tumor. When in contact with tumor tissue, the thin fiber optics may break and the fluorescent coating may be scrubbed off. Very often more than one microsensor was required for an acidoCEST MRI imaging session. Due to the cost of the microsensors, they couldn’t be replaced frequently. Furthermore, the reliability of the microsensor deteriorates with each insertion into the tumor tissue. After several usages, the microsensor may produce pH values outside of the physiological range (< 6.4 and > 7.2). We have disregarded these out-of-range values. However, this brings us to question about the validity of the pH values measured by a microsensor that we deemed to be within the physiological range. We can try to measure pHe of softer tissue such as liver or brain to reduce the damage to the microsensor. Nevertheless, the microsensor is still difficult to implement, has its own measurement limitations, and their accuracies to measure tumor pHe has not been validated. Our acidoCEST MRI has been very effective in determining an increase or decrease in pHe of the tumor with bicarbonate and MIBG treatments studies. However, to be a paramount pHe imaging technique, a more stringent validation is required to evaluate the accuracy of acidoCEST MRI. Therefore, future studies are warranted to validate the accuracy of acidoCEST MRI measurements of in vivo pHe when other “gold standard” methods become available.
Cross validation with urine pH

Our acidoCEST MRI has been well established to measure *in vivo* bladder’s pH [6]. Hence, instead of waiting for a “gold standard” for tumor pH to surface, we can validate our acidoCEST MRI by measuring the pH of murine urine collected from metabolic cage with a common pH electrode (Mettler Toledo, Columbus, OH). Silvio Aime and colleagues have done similar validations for their pH measurements with their Yb-HPDO3A PARACEST agent [7]. The mice can be divided into three groups: control mice are fed with regular water, basic mice are fed with 0.2 M bicarbonate solution, and acidic mice are fed with 0.25 M ammonium chloride solution, drinking ad libitum. The mice can then undergo acidoCEST MRI and urine will be collected thereafter. A good linear correlation between pH from the pH meter and acidoCEST MRI acquisition is an indication that our method is accurate and robust.

Applying acidoCEST MRI to other organs

Apart from the subcutaneous tumor and bladder, we can extend the application of acidoCEST MRI to other tumor types, organs and tissue that have sufficient iopromide accumulation. Iopromide is a non-specific targeting agent. High accumulation is typically observed in kidney, bladder and liver. We can use CT imaging to measure iopromide accumulation in other organs/tissue, such as spleen, small intestine, large intestine, colon, heart, lung, pancreas, gonad, bone marrow and brain. IV and IP injects should also be investigated to determine which route of administration is optimal for the specific site.
Monitoring pH-sensitive chemotherapy

The Warburg effect predicts carcinoma tumors to be acidic [8]. Weak acid drugs such as cyclophosphamide, 5-flourouracil and chlorambucil have higher cytotoxicity in a low pH environment, while the reverse is true for weak base drugs such as mitoxantrone, doxorubicin and daunorubicin [9]. Measuring tumor pHe has potential to predict therapeutic efficacy of a weak-base or weak-acid drug before the therapeutic treatment is initiated for each patient, which can lead to personalized medicine. We can use acidoCEST MRI to measure the pHe of the tumor, determine whether the tumor is acidic or basic, and then treat the mice accordingly. The mice can be divided into 4 subgroups: 1) acidic tumor treated with weak acid drug; 2) acidic tumor treated with weak base drug; 3) basic tumor treated with weak acid drug; and 4) basic tumor treated with weak base drug.

Reference


APPENDIX E

acidoCEST MRI IMAGE AND DATA ANALYSIS PROTOCOL WITH MATLAB®
**BACKGROUND**

Our acidoCEST MRI protocol consisted of 54 FISP MR images acquired at different saturation frequencies (Table 1), using 2.8 µT saturation power and a saturation time of 5 seconds, which required 4:50 minute of acquisition time. This process was repeated 6 times, for a total acquisition time of 29 minutes. The CEST spectrum of iopromide is the summation of three Lorentzian line shapes (Equation 1a-d): CEST effects for amides at 4.2 and 5.6 ppm, and the direct saturation of water at 0.0 ppm. The list in Figure 1 summarizes step-by-step image and data analysis using Matlab® R2012B (Mathworks, Inc., Natick, MA) for acidoCEST MRI using iopromide as contrast agent.

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<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
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<td>Identify ROI</td>
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<tr>
<td>2</td>
<td>Average image</td>
</tr>
<tr>
<td>3</td>
<td>Group pixels</td>
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<td>4</td>
<td>Test for baseline</td>
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<tr>
<td>5</td>
<td>Extend baseline</td>
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<tr>
<td>6</td>
<td>Cubic spline smoothing</td>
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<tr>
<td>7</td>
<td>Interpolation</td>
</tr>
<tr>
<td>8</td>
<td>Lorentzian line shapes fitting</td>
</tr>
<tr>
<td>9</td>
<td>Calculate CEST effects</td>
</tr>
</tbody>
</table>

Figure 1. Summary of image and data analysis of acidoCEST MRI
EQUATIONS

\( CEST_{4.2 \text{ ppm}} = \frac{A_{4.2\text{ ppm}} w_{4.2\text{ ppm}}}{(x-\omega_{4.2\text{ ppm}})^2 + w_{4.2\text{ ppm}}^2} \) \hspace{1cm} (1.a)

\( CEST_{5.6 \text{ ppm}} = \frac{A_{5.6\text{ ppm}} w_{5.6\text{ ppm}}}{(x-\omega_{5.6\text{ ppm}})^2 + w_{5.6\text{ ppm}}^2} \) \hspace{1cm} (1.b)

\( CEST_{\text{water}} = \frac{A_{\text{water}} w_{\text{water}}}{(x-\omega_{\text{water}})^2 + w_{\text{water}}^2} \) \hspace{1cm} (1.c)

\( CEST_{\text{total}} = CEST_{4.2\text{ ppm}} + CEST_{5.6\text{ ppm}} + CEST_{\text{water}} \) \hspace{1cm} (1.d)

A: area of CEST peak, in units of % CEST • Hz

w: width of CEST peak, Hz

\( \omega \): frequency for CEST peak maxima, Hz

CEST: Chemical Exchange Saturation Transfer, no unit

The CEST effect has a maximum at \( x = \omega \), where equation 1.a-c can be simplified to

\( CEST_{\text{amplitude}} = \frac{A}{w} \) \hspace{1cm} (2)

For baseline testing:

\( \text{negative baseline} = \text{mean}_{(n=2-6)} \) \hspace{1cm} (3.a)

\( \text{positive baseline} = \text{mean}_{(n=49-53)} \) \hspace{1cm} (3.b)

\( \text{baseline test} = \frac{\text{positive baseline}}{\text{negative baseline}} \) \hspace{1cm} (3.c)
Calculate pH (colored pixels):

\[
pH = \left\{ \log_{10} \left( \frac{CEST_{4.2ppm}}{1 - CEST_{4.2ppm}} \right) \right\} - c / m
\]

where \( c \) is the y-intercept and \( m \) is the slope from an empirical calibration curve.

**TABLES**

Table 1. 54 saturation frequencies

<table>
<thead>
<tr>
<th>n</th>
<th>ppm</th>
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<th>ppm</th>
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<td>1.2</td>
<td>41</td>
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<tr>
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<td>1.5</td>
<td>42</td>
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<tr>
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<td>25</td>
<td>1.8</td>
<td>43</td>
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<tr>
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<td>2.1</td>
<td>44</td>
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<tr>
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<td>27</td>
<td>2.4</td>
<td>45</td>
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<td>28</td>
<td>2.7</td>
<td>46</td>
<td>8.1</td>
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<td>3.0</td>
<td>47</td>
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<td>3.3</td>
<td>48</td>
<td>8.7</td>
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<tr>
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<td>-1.8</td>
<td>31</td>
<td>3.6</td>
<td>49</td>
<td>9.0</td>
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<td>51</td>
<td>9.6</td>
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<td>52</td>
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<td>4.8</td>
<td>53</td>
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<tr>
<td>18</td>
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<td>36</td>
<td>5.1</td>
<td>54</td>
<td>100.0</td>
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</tbody>
</table>
Each saturation frequency takes 5.3 seconds (5 seconds saturation + 0.3 seconds of FISP acquisition) of acquisition time. Increasing the number of saturation frequencies will improve the robustness of Lorentzian line shape fitting. However, this will ultimately increase the total acquisition time. The frequencies listed in Table 1 are sufficient for in vivo Lorentzian line shape fitting with the shortest possible total acquisition time required. The saturation frequencies at ±100.0 ppm act as dummy scans. The in vivo CEST spectrum takes the shape of a super-Lorentzian line, and the data from ±100.0 ppm are not used in further image and data processing. The saturation frequencies from -10.0 ppm to -3.0 ppm are 1.0 ppm apart. This negative regime constitutes the baseline. From -3.0 ppm to +10.0 ppm, the increment becomes 0.3 ppm. The amides’ CEST effects are located at 4.2 and 5.6 ppm respectively. The B₀ field inhomogeneity is ±2.0 ppm for our Biospec MRI scanner (Bruker Biospin, Inc.). Thus, a denser saturation frequency distribution is required to account for the bulk water, and two amide CEST effects.

Table 2. Parameters for Lorentzian line shape fitting

<table>
<thead>
<tr>
<th>Parameter*</th>
<th>Description</th>
<th>Initial Estimate</th>
<th>1st non-linear curve fit</th>
<th>2nd non-linear curve fit</th>
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<td>UB</td>
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<td>-2</td>
<td>-1.0 x 10⁻⁵</td>
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<tr>
<td>5</td>
<td>A₅.₆ ppm</td>
<td>-0.1</td>
<td>-2</td>
<td>-1.0 x 10⁻⁵</td>
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<td>3</td>
<td>Awater</td>
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<tr>
<td>2</td>
<td>w₄.₂ ppm</td>
<td>0.01</td>
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<td>w₅.₆ ppm</td>
<td>0.01</td>
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<tr>
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<td>wwater</td>
<td>1</td>
<td>0</td>
<td>5</td>
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<tr>
<td>7</td>
<td>ω₄.2 ppm</td>
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<td>2.2</td>
<td>6.2</td>
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<tr>
<td>9</td>
<td>ω₅.₆ ppm</td>
<td>5.6</td>
<td>3.6</td>
<td>7.6</td>
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<tr>
<td>8</td>
<td>ωwater</td>
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<td>2</td>
</tr>
<tr>
<td>10</td>
<td>baseline</td>
<td>1</td>
<td>-1</td>
<td>10</td>
</tr>
</tbody>
</table>

* parameter number assignment in Matlab code
These sets of parameters in Table 2 were optimized on three occasions, during studies of
1) bicarbonate studies, 2) pH microelectrode, and 3) m-iodobenzylguanidine (MIBG)
treatment. Most of the decisions are based on empirical observations. After a thorough
trial and error analysis, the initial estimates are the most important to have a good
outcome for non-linear data fitting in least square sense (lsqcurvefit). The lower bound
(lb) and upper bound (ub) limits ensure that Matlab will not accidentally misfits CEST
effect at 4.2 ppm as 5.6 ppm, and vice versa, when performing lsqcurvefit. The lb and ub
for the 1\textsuperscript{st} lsqcurvefit was broader, to account for B\textsubscript{0} field inhomogeneity. The 1\textsuperscript{st}
lsqcurvefit fitting method is Newton-Raphson matrix minimization method. After the 1\textsuperscript{st}
lsqcurvefit, the 10 parameters outcome for this particular experiment will be a more
accurate than the initial estimate blanket for all experiments. These new set of 10
parameters are used as input for the estimate for the 2\textsuperscript{nd} lsqcurvefit. The lb and ub for
then 2\textsuperscript{nd} lsqcurvefit fitting are narrower and this fitting method uses a conjugate gradients
method.

Based on empirical observations, the areas and width for the CEST effects at 4.2 and 5.6
ppm are smaller than water. The width of CEST at 5.6 ppm is broader than CEST at 4.2
ppm. The upper limit for amides’ CEST effects is zero. However having a zero area will
lead to zero CEST effects. This will affect the pH calculation (Equation 4). Hence, the
upper limit for area is fixed at a non-zero value to avoid a disproportionate pH
calculation.
PROCEDURE

1. Identify the region of interest
   a. Circle the tumor region
   b. Circle a noise region (black background)
      i. Standard deviation of noise is calculated for noise thresholding

2. Average the acidoCEST MR images
   a. All 6 acidoCEST MR images are averaged for each saturation frequency
   b. acidoCEST MR images at ±100 ppm is dropped. Only MR images from -10 to +10 are kept (n = 2 – 53).
   c. There are now 52 data points

3. Group pixels to improve signal to noise with either
   a. Gaussian low pass filtering, 7 x 7, 1 ∂; or
   b. 3 x 3 binning of 9 adjacent pixels

4. Test for baseline on the positive and negative frequencies, Equation 3.
   The procedure will continue when baseline test is within 0.9 and 1.1. This is an indication that the positive and negative baselines are in the same order of magnitude. If the baseline test exceeds this range, the fitting process will not proceed further and the outcome will be NaN.

5. Extend baseline to account for B₀ inhomogeneity
   a. Baseline = mean (n = 2-6)
   b. Add this average baseline value to -15, -14, -13, -12, -11, 11, 12, 13, 14 and 15 ppm.
   c. There is now 62 data points
d. This step may not be necessary if the there is good $B_0$ homogeneity. For our MRI machine, there is a ±2.0 ppm inhomogeneity. There is a possibility that the lsqcurvefit cannot proceed smoothing because the CEST effect at 5.6 ppm is left hanging in mid-air, i.e. the peak at 5.6 ppm did not converge to the baseline. Hence by adding a few extra points to make the baseline, the fitting can be completed. ±15.0 ppm is an arbitrary value. This is an artificial addition of data points.

6. Cubic spline smoothing with default parameter
   a. There is now 112 data points
   b. Data from the original sites are replaced by their weighted average. The new values are extrapolated.

7. Interpolation
   a. Increase number of data points in the frequency domain with linearly space vectors within the frequency domain
   b. Interpolate % water signal with linear function
   c. Now there is 11200 data points
   d. The interpolation is at 100-fold. Based on empirical observation, anything more than 100-fold will not increasing the robustness of fitting, but will become more computation intensive.

8. Lorentzian line shape fitting (Equation 1.a-d, Table 2)
   a. 1st least-squares data fitting for nonlinear curve using initial estimate from Table 2.
b. 2nd least-squares data fitting for nonlinear curve using calculated value from 1st least square

9. Calculate CEST effects at 4.2 and 5.6 ppm (Equation 1.a,b)
   a. % CEST effect has to be greater than $2\sqrt{2} \times$ standard deviation of noise.
   b. Colored pixels: % CEST effect @ 4.2 and 5.6 ppm $> 2\sqrt{2} \times$ standard deviation of noise. (Equation 4)
   c. White pixels: % CEST effect @ 4.2 ppm $> 2\sqrt{2} \times$ standard deviation of noise. pH = 7.0
APPENDIX F

PERMISSION
Figure 1.1. Schematic of glycolysis and associated metabolic pathways that create biomolecules for tumor growth.
Figure 1.2. Tumor acidosis causes chemo resistance against weak-base drugs.
Figure 1.3. Treatment of a mouse model of β-galactosidase-labeled MDA-MB-231 mammary carcinoma with NaHCO₃.
Figure 1.4. A pH microelectrode for measuring *in vivo* tumor pH.
Figure 1.5a. Fluorescence imaging of tumor pH.
Figure 1.5c. Fluorescence imaging of tumor pH.
Figure 1.6a. PET imaging of tumor pHe.
Figure 1.6b. PET imaging of tumor pHe.
Figure 1.8. $^1$H MR spectroscopy imaging of tumor pH.
Figure 1.10. $^{19}$F MR spectroscopy of tumor pH.
Figure 1.11. Hyperpolarized $^{13}$C MR spectroscopic imaging of tumor pH.
Figure 1.12. Relaxation-based MRI of Gd-DOTA-4Amp and DyDOTP can measure tumor pH.

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Expected completion date May 2014
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Figure 1.15a. Endogenous CEST MRI of tumor pH.

Using the amide proton signals of intracellular proteins and peptides to detect pH effects in MRI

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Expected completion date May 2014
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Figure 1.15b. Endogenous CEST MRI of tumor pH\textsubscript{i}.

DEVELOPMENT AND APPLICATION OF ACIDOCEST MRI FOR EVALUATING TUMOR ACIDOSIS IN PRE-CLINICAL CANCER MODELS

Expected completion date: May 2014

Expected size (number of pages): 159

Total: 0.00 USD
Figure 1.15c. Endogenous CEST MRI of tumor pH.
Figure 1.16. CEST MRI of tumor pH with Yb-HPDO3A.

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Figure 1.18. CEST MRI of leg pHe with Tm-DOTAM-Gly-Lys.
Figure 1.19. CEST MRI of tumor pH with iopamidol.

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Appendix A: Evaluations of extracellular pH within \textit{in vivo} tumors using acidoCEST MRI