PRO-QUEST: A rapid assessment method based on PROgressive saturation for Quantifying Exchange Rates using Saturation Times in Chemical Exchange Saturation Transfer (CEST)

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Abstract

Purpose: To develop a new MRI technique to rapidly measure exchange rates in Chemical Exchange Saturation Transfer (CEST) MRI.

Theory and Methods: A novel pulse sequence for measuring chemical exchange rates through a progressive saturation recovery process, called PRO-QUEST (**PRO**gressive saturation for **Qu**antifying **E**xchange rates using **S**aturation **T**imes), has been developed. Using this method, the water magnetization is sampled under non-steady state conditions and off-resonance saturation is interleaved with the acquisition of images obtained through a Look-Locker type of acquisition. A complete theoretical framework has been set up, and simple equations to obtain the exchange rates have been derived.

Results: A reduction of scan time from 58 to 16min has been obtained using PRO-QUEST vs. the standard QUEST. Maps of both longitudinal relaxation time of water (T_1) and radio frequency pulse amplitude (B_1) can simply be obtained by repetition of the sequence without off-resonance saturation pulses. Simulations and calculated exchange rates from experimental data using amino-acids such as glutamate, glutamine, taurine and alanine were compared and found to be in good agreement. The PRO-QUEST sequence was also applied on healthy and infarcted rats after 24 hours and revealed that imaging specificity to ischemic acidification during stroke was substantially increased relative to standard APT-weighted imaging.

Conclusion: Because of the reduced scan time and insensitivity to non-chemical exchange factors such as direct water saturation, PRO-QUEST can serve as an excellent alternative for researchers and clinicians interested to map pH changes *in vivo*.

Introduction

Chemical Exchange Saturation Transfer (CEST) magnetic resonance imaging (MRI) is an emerging method for detecting signals from low concentrated species *in vivo* and for monitoring changes in environmental parameters such as pH, temperature, and ion concentration [1]. The chemical exchange rate has been used as a quantitative imaging biomarker for pH mapping or to guide selection of the most appropriate CEST MRI parameters. Indeed, the chemical exchange of protons within exchangeable groups such as amides and amines found *in vivo* is base-catalyzed, and will therefore vary with pH [2],[3]. When combined with other techniques such as diffusion-weighted imaging (DWI) or perfusion-weighted imaging (PWI), pH maps have shown great potential for improving our understanding of acute ischemic damage or for monitoring cancer progression and its response to various treatments [4], [5].

Existing methods for quantitative measurements of soluble labile proton exchange rates in water are either spectroscopy-based, or rely on multiple repetitions of the same acquisition following changes in the experimental parameters [5],[6],[7]. With nuclear magnetic resonance (NMR) spectroscopy, the exchange rates of protons in the slow exchange regime can be estimated from their linewidths measured on solute molecules [5]. Water exchange spectroscopy (WEX) has been used to assess the exchange properties of amides in endogenous mobile proteins and peptides in the brain [6]. Through the build-up of the signal from the exchangeable protons as a function of mixing time the exchange rate can be calculated [6],[8],[9]. Although slow exchange rates are perfectly quantifiable, fast exchanging species require very short mixing times which are difficult to attain due to limitations in hardware, especially in clinical MRI systems.

Recently, measurements of exchange rates using imaging techniques have been developed to overcome the limited spatial resolution of MRS measurements and to provide quantitative maps in both healthy and diseased states. Exchange rates can be quantified as a function of saturation time using **Qu**antification of **E**xchange rates using **S**aturation **T**imes (QUEST) or **S**aturation **P**ower (QUESP) [5],[10]. Moreover, in the case of continuous saturation, analytical solutions of the Bloch-McConnell (BM)

equations have been derived to provide fast and accurate estimates of the exchange rates [11]. Other methods of quantification include the omega plot method, working without a priori knowledge of the agent concentration; or the extended QUEST method with ratiometric analysis (QUESTRA), which considers the rate at which the label and reference scans approach the steady state [12].

While different methods have been proposed to enable correct estimates of the exchange rates, they all have in common that they require too much time for their utility to really be explored in the clinic. To eliminate the need for long CEST saturation times and long TR, MRI fingerprinting methods have also been used for simultaneous multi-parametric mapping to quantify exchange rates [13-15]. Advantages of this approach include sensitivity, specificity and speed [14]. However, even in relatively simple phantom studies, quantification of exchange rates or concentrations can go wrong due to relatively poor fingerprinting trajectory efficiency [14]. In addition, quantification of exchange rates *in vivo* is very difficult due to the interplay of multiple molecular processes between e.g. healthy volunteers and patients.

Here, we propose a novel pulse sequence for measuring chemical exchange rates through a progressive saturation recovery process, called PRO-QUEST for **PRO**gressive saturation for **Qu**antifying **E**xchange rates using **S**aturation **T**imes in CEST. This technique aims to provide direct measures of chemical exchange rates, while at the same time dramatically reducing the total scan time required. It works based on the water magnetization being sampled along a Look-Locker sampling scheme, thereby providing maps of T_1 and B_1, both needed for exchange-rate calculations. Measurements of exchange rates are obtained in phantoms of various amino acids and in rat brains. In addition, PRO-QUEST measurements made in phantoms are compared with those made using the traditional QUEST method, and the sensitivity of the technique to various parameters is explored.

Theory

Progressive saturation T_1 mapping

Fast measurement of longitudinal-relaxation times (T_1) can be performed using a single shot method known as the Look-Locker technique [16] . It is typically based on the use of an inversion or a saturation pulse, followed by multiple small flip-angle pulses, which sample the recovery of the longitudinal magnetization (see Figure 1). The signal evolution during a Look-Locker experiment for a number (n) of RF pulses of the same flip-angle (ϑ) has been derived previously [16], and is given by:

$$M_{zd}(n\tau) = \frac{\mathbf{1}-[(\cos\vartheta)^{n-1}\, e^{-(\boldsymbol{n-1})(\tau R_1)}\,]}{\mathbf{1}-[(\cos\vartheta)\, e^{-(\tau R_1)}]}\, M_{zd}(\tau) \;+ M_{eq}\,(1\text{-}e^{-(t_d R_1)})[\,(\cos\vartheta)^{n-1} e^{-(\boldsymbol{n-1})(\tau R_1)}] \quad \textbf{(1)}$$

where $M_{zd}(\tau) = Meq\,(1 - e^{-(\boldsymbol{\tau} R_1)})$ in the case of a saturation-recovery Look-Locker experiment, t_d is the time to first excitation from preparation, n is the number of excitation pulses, τ is the time between ϑ pulses, R_1 =1/T_1 with T_1 being the water longitudinal relaxation time and M_{eq} is the equilibrium magnetization.

Both T_1 and M_{eq} can be estimated using Equation 1. In practice, an appropriate range of flip angles ϑ, no larger than 20°, has to be selected so that the small flip angle approximation holds and the measured T_1 does not deviate from its theoretical value [17] ,[18] . By using an optimal set of sampling parameters (i.e. n, ϑ and τ), T_1 can be measured with good accuracy and in a reasonable imaging time [18].

Exchange rate measurements through progressive saturation recovery

PRO-QUEST was implemented by inserting either an off-resonance saturation module consisting of a single RF pulse or a spin-lock module before each readout module of the Look-Locker method so that the saturation effects progressively accumulate, while the measured signal is recovering under the longitudinal relaxation. To get accurate estimates of exchange rates, PRO-QUEST is applied with and without saturation module as depicted in Figure 1. The saturation pulse parameters are: pulse width (t_{sat}), flip angle (fa) and number of pulses (n). Imaging parameters for the readout are: imaging RF pulse flip angle (ϑ), number of averages m, TR and the delay (τ - t_{sat}) or (τ -

t_{del}) between the readout and the start of the following acquisition ϑ pulse. Generally, the saturation module used in this method is centered at the offset frequency of interest e.g. amine, amide or hydroxyl frequency offsets (1 - 4 ppm from water), but the module can be extended beyond a single saturation frequency or a spin lock pulse can also be used on resonance for very rapidly exchanging groups resonating close to the water line (e.g. hydroxyl protons).

Mathematical equations have been derived to describe the recovery curves in the presence of CEST saturation pulses [11],[19]. In general, one of the main characteristics of the signal evolution during a CEST off-resonance pulse is that the (apparent) relaxation rate evolves with $R_{1\rho}$ (the relaxation rate along the effective field) rather than R_1. Combining this fact with Equation 1, the longitudinal water magnetization after n off-resonance saturation pulses (M_{zsat}) can be described as (see Appendix for full derivation):

$$M_{zsat}(n\tau) = \frac{\mathbf{1}-\left[(\cos\vartheta)^n\, e^{-\boldsymbol{n}\left(\tau R_1 - t_{sat}(R_1-R_{1\rho})\right)}\right]}{\mathbf{1}-\left[(\cos\vartheta)\, e^{-\left(\tau R_1 - t_{sat}(R_1-R_{1\rho})\right)}\right]} M_{zsat}(\tau)$$

$$+ M_{eq}\,(1-e^{-(t_d R_1)})[(\cos\vartheta)^n\; e^{-\boldsymbol{n}\left(\tau R_1 - t_{sat}(R_1-R_{1\rho})\right)}] \qquad \textbf{(2)}$$

where $M_{zsat}(\tau) = M_{ss}\,(1- e^{-(R_{1\rho} t_{sat})})\cos\vartheta\; e^{-((\tau - t_{sat})R_1)} + M_{eq}\,(1-e^{-((\tau-t_{sat})R_1)})$

and $M_{ss} = \frac{R_1 \cos^2\varphi}{R_{1\rho}}$ is the steady state magnetization, t_{sat} is the duration of the CEST off-resonance saturation pulse, $\cos\varphi = \frac{\Omega}{\sqrt{\omega_1^2+\Omega^2}}$, with φ is the angle between the effective field and the z-axis, Ω is the frequency offset with respect to water (i.e ω_{rf}) and $\omega_1 = \gamma B_1$ is the amplitude of the RF field.

For a continuous-wave irradiation, and assuming that the spin relaxation is dominated by a single exponential decay the relaxation constant along the effective field, $R_{1\rho}$ can generally be expressed as [19] :

$$R_{1\rho} = R_1(\cos\varphi)^2 + (R_2 + R_{ex})(\sin\varphi)^2 \qquad \textbf{(3)}$$

$R_{ex} = \frac{\rho_B\ \delta^2 k_{ex}}{((\delta-\Omega)^2 + \omega_1^2+k_{ex}^2)}$ is the exchange-dependent relaxation which induces the CEST effect, and R_1 and R_2 the longitudinal and transverse relaxation of water in the absence of chemical exchange. Here, ρ_B is the fractional concentration of the labile exchangeable protons, k_{ex} is the exchange rate from the exchangeable protons to water, and δ is the Larmor frequency of the exchangeable labile protons.

For on-resonance spin-locking (SL) ($\varphi = 90°$), $R_{1\rho} = R_2 + R_{ex}$ while for off-resonance SL (where $\varphi = \arctan(\frac{\omega_1}{\Omega})$):

$$R_{1\rho} = R_1 \frac{\Omega^2}{\Omega^2+\omega_1^2} + (R_2 + R_{ex}) \frac{\omega_1^2}{\Omega^2+\omega_1^2} = R_1 + (R_2 - R_1) \frac{\omega_1^2}{\Omega^2+\omega_1^2} + R_{ex} \frac{\omega_1^2}{\Omega^2+\omega_1^2} \quad \textbf{(4)}$$

The exchange-dependent relaxation term for an offset Ω can be written as [11]:

$$R'_{ex}(\Omega) = R_{ex}(\sin\varphi)^2 = \frac{\rho_B\ \delta^2\ k_{ex}}{((\delta-\Omega)^2 + \omega_1^2+ k_{ex}^2)} \frac{\omega_1^2}{\Omega^2+\omega_1^2} \quad \textbf{(5)}$$

R'_{ex} reaches a peak at Ω= δ (i.e. on-resonance with the CEST peak) and assuming that $\delta \gg \omega_1$ it can be simplified to:

$$R'_{ex}(\delta) = \rho_B\ k_{ex} \frac{\omega_1^2}{k_{ex}^2+\omega_1^2} \frac{\delta^2}{\delta^2+\omega_1^2} = \rho_B\ k_{ex} \frac{\omega_1^2}{k_{ex}^2+\omega_1^2} \quad \textbf{(6)}$$

Therefore, for $\delta \gg \omega_1$

$$R_{1\rho}(\delta) = R_1 + (R_2 - R_1) \frac{\omega_1^2}{\delta^2+\omega_1^2} + \rho_B\ k_{ex} \frac{\omega_1^2}{k_{ex}^2+\omega_1^2} \quad \textbf{(7)}$$

For off-resonance spin lock or off-resonance continuous wave irradiation, Equation 7 can be applied to deduce the exchange-dependent relaxation term R'_{ex} as a function of $\omega_1 = \gamma B_1$. However, for off-resonance shaped RF pulses, it was shown previously [20] that $\bar{R}_{1\rho}$ yields better estimates of exchange rates and fractional concentrations rather than using $R_{1\rho}(\overline{\omega_1(t)})$. $\bar{R}_{1\rho}$ is defined as [20]:

$$\bar{R}_{1\rho} = \frac{1}{t_{sat}} \int_0^{t_{sat}} R_{1\rho}(t)\ dt = R_1 + \frac{1}{t_{sat}} \int_0^{t_{sat}} (R_2 - R_1) \frac{\omega_1^2(t)}{\delta^2+\omega_1^2(t)}\ dt + \frac{1}{t_{sat}} \int_0^{t_{sat}} \rho_B\ k_{ex} \frac{\omega_1^2(t)}{k_{ex}^2+\omega_1^2(t)} dt$$
$$= \overline{R_{eff}} + \overline{R'_{ex}} \quad \textbf{(8)}$$

where t_{sat} is the length of the off-resonance CEST saturation pulse.

To estimate exchange rates, T_1, B_1 and M_{eq} maps are calculated by fitting Equation 1 to the signal evolution over time, obtained with the pulse sequence displayed in Figure 1(b), similarly with previous work [21]. The obtained T_1, ϑ and M_{eq} are then used as input parameters for estimating the exchange-induced relaxation term $\overline{R'_{ex}}$ or the exchange rates (k_{ex}) by fitting the progressive saturation recovery curves as a function of number of saturation pulses to Equation 2 in which $R_{1\rho}$ is substituted with $\bar{R}_{1\rho}$, as shown in Equation 8, to obtain an estimate of $\overline{R'_{ex}}$. This is then used in conjunction with ρ_B and the integral $\int_0^{t_{sat}} \frac{\omega_1^2(t)}{k_{ex}^2+\omega_1^2(t)} dt$ which is solved numerically to estimate the exchange rates (k_{ex}) (see Equation 8). In this work, ρ_B is provided to the fit or data are acquired at two irradiation amplitudes $\omega_{1,2}$ and the concentration-free $\overline{R'_{ex}}$ is calculated as $\overline{R'_{ex}}(\omega_1)/\overline{R'_{ex}}(\omega_2)$, which can be then used to extract the exchange rate numerically. The minimum set of measurements to allow calculation of an exchange rate k_{ex} is:

- Measure T_1, B_1 and M_{eq} using the pulse sequence displayed in Figure 1(b) at two different flip angles ϑ_1 and ϑ_2
- Measure $\overline{R'_{ex}}$ using the pulse sequence displayed in Figure 1(a) by applying the CEST off-resonance saturation pulses at two irradiation amplitudes ω_1 (e.g. ω_1 and $2\omega_1$)
- Measure T_2 using a Carr-Purcell-Meiboom-Gill (CPMG) sequence at different echo times. Even echoes are fitted using a mono-exponential signal decay to obtain T_2.

Methods

Several phantom experiments using amino acid samples at different pH values were made to test the theory. QUEST/QUESP experiments were performed in the same samples to validate the exchange rates obtained with PRO-QUEST. pH and solute concentrations were varied to investigate the dependence of the measured exchange rates on various parameters.

PRO-QUEST experiments were also performed in 3 healthy and 3 infarcted rats to demonstrate the feasibility of obtaining exchange rate measurements *in vivo* and their potential usefulness in a clinical setting.

T_2 values, necessary for both PRO-QUEST and QUEST/QUESP methods, were acquired in both phantoms and rats using a standard CPMG experiment.

Precision of T_1 measurements from PRO-QUEST was compared to gold-standard methods using an inversion-recovery spin-echo echo-planar-imaging sequence in both phantoms and healthy rats.

Estimation of B_1 from PRO-QUEST was obtained in phantoms and healthy rats at three imaging flip angles (i.e. 8°, 15°,25°).

Phantom solutions

Taurine, alanine, glutamate and glutamine possess amine protons which are all found to resonate close to 3.00ppm (Gln: 2.87ppm; Glu: 3.00ppm; Tau: 2.87ppm; Ala: 2.87ppm) and produce substantial CEST contrast (see Supporting Figure S1). These metabolites were chosen to assess whether the overlapping CEST signals at 3.00ppm could be discriminated based on their different exchange properties.

All samples were prepared using 0.1% phosphate-buffer-saline (PBS) buffer and scanned within the same day to avoid potential pH drifts (i.e. the gradual absorption of carbon dioxide from the atmosphere leads to an increase in $[H^+]$ which decreases the pH). Exchange rate measurements were obtained from 100mM taurine, alanine,

glutamine and glutamate samples prepared at various pH values ranging between 5.8 and 7.4.

Additional experiments were performed by varying the solute concentration from 12.5 mM to 100mM under constant pH=6.2 for assessing the dependence of the PRO-QUEST curves on the concentration of solute. For all the experiments, the pH was measured using a micro pH probe (Mettle-Toledo International, Inc.) and adjusted where necessary with the use of sodium hydroxide (NaOH) and hydrochloric acid (HCl). Syringes of 1ml volume capacity were used as phantom containers, sealed with silicon glue. All phantoms were grouped in sets of five (same concentration, and five different pH values) or group of four (same pH, and different concentrations). The temperature was kept constant at 23°C throughout the experiment.

In vivo experiments

All animal experiments were performed in accordance with the UK Animals (Scientific Procedures) Act 1986, and the Directive 2010/63/EU of the European Parliament and of the Council on the Protection of Animals Used for Scientific Purposes. Six Sprague-Dawley (SD) rats (weighing 200-250g) were used for MRI experiments. Prior to each scan, animals were anaesthetized with isoflurane. A concentration of 3% isoflurane in air was used for induction of anesthesia, and then the animals were transferred to the scanner bed and a facemask was applied to ensure continuous supply of anesthetic. Isoflurane concentration was decreased to 1.5-2.0% and maintained at this level for the rest of the experiment. During the scanning, the body temperature was maintained at 37°C with a heating pad, while respiration rate was constantly monitored using MR compatible devices (Small Animal Instruments, Inc. Stony Brook, NY, USA).

Stroke procedure

Three male SD rats (weighing 200-250g) were anaesthetized with isofluorane (3% for induction and 2% during surgery) via a vaporizer with a mixture of air. A middle cerebral artery occlusion was performed as follows [22]. A midline neck incision was made over

the trachea and the tissues were retracted gently. The common carotid artery (CCA), internal carotid artery (ICA) and external carotid artery (ECA) were isolated and the CCA and ECA ligated. A filament was inserted from the ECA into the ICA to occlude the origin of the middle cerebral artery. After an ischemic time of 45min, the area was cleaned and rehydrated with saline. Finally, the skin was sutured and analgesics such as Rimadyl and Ketamine were given post operatively. The animals were kept in a warm environment during recovery after MCA occlusion. All the animals were scanned at baseline and at 24 hours post stroke.

Pulse sequence

Phantom experiments

The PRO-QUEST sequence was implemented on a 9.4 Tesla Agilent MRI scanner. A detailed diagram of the pulse sequence is displayed in Figure 1. Using the sequence in Figure 1(a), each of n=300 off-resonance saturation pulses (asymmetric sinc-Gauss) was centered at 3.0ppm with parameters: t_{sat}=6.77 ms, fa=90°, 180°, 300°, 400°, 500°, 700°, 900° (corresponding to B_1=0.87μT, 1.74μT, 2.89μT, 3.85μT, 4.82μT ,6.75μT and 8.67μT, with $B_1=fa/\gamma t_{sat}$) and bandwidth = 1000 Hz (2.5 ppm). Multiple flip angles were used to investigate the dependence of $\overline{R'_{ex}}$ on the irradiation amplitude. However, in principle, two B_1 values are enough to remove the concentration dependency in Equation 8 and provide estimates of the exchange rates. To estimate T_1, B_1 and M_{eq}, an additional scan was acquired without saturation pulses (Figure 1(b)). This was also repeated with flip angles ϑ=8°, 15° and 25° to allow estimation of B_1 and the error in T_1 due to variations in ϑ.

A single-slice 2D-GRE sequence with FOV 20x20mm², data matrix 64x64, TR=2.6s, TE=1.3ms, slice thickness=4mm was employed for readout. PRO-QUEST measurements were made in phantoms using a transmit/receive 33mm RF coil (all RF coils: Rapid Biomedical, Germany). The total scan time for a single PRO-QUEST exchange rate measurement was 28min when 7 irradiation powers are used (~2.8min per acquisition) and can be further reduced to 14min for 2 irradiation powers.

In vivo experiments

Three healthy and three infarcted SD rats were scanned using a 72mm volume coil for radio frequency (RF) transmission and a two-element receive array coil. The optimized sequence consisted of two adiabatic half-passage saturation pulses for improved saturation [23] , followed by 150 off-resonance saturation pulses centered at 3.5ppm with RF pulse parameters: t_{sat}=16.93ms, fa=90° and 180° and bandwidth=400Hz (1 ppm). This off-resonance saturation frequency was chosen to assess the changes in amide proton exchange following ischemia as demonstrated earlier [4]. Images were collected with slice thickness=2mm, FOV=35x35mm², matrix size=64x64, TR=3.0s, TE=1.12ms, and ϑ=8°. For exchange rate measurements using PRO-QUEST, T_1, B_1 and M_{eq} were calculated using the multiple flip angle strategy described above. The same PRO-QUEST MRI protocol was employed for the stroke study with exchange-weighted images collected at fa=90° and 180° (0.35μT and 0.69μT B_1 respectively). Two irradiation powers were used for concentration-free $\overline{R'_{ex}}$, which was calculated as $\overline{R'_{ex}}\,(0.35\ \mu\mathrm{T})/\,\overline{R'_{ex}}\,(0.69\ \mu\mathrm{T})$. It can be seen from Equations 5 and 6 that when we divide the $\overline{R'_{ex}}$ obtained at two different irradiation amplitudes the result will be unaffected by any changes in the concentration of exchangeable protons ρ_B . The total scan time was 16min (~3.2min per acquisition).

QUEST/QUESP MRI

QUEST measurements were acquired in the same samples described above using a single-shot spin-echo (SE) echo planar imaging (EPI), (TR=65.3ms, TE=4.07ms, FOV=20x20mm², slice thickness=5mm, matrix size=64x64) with a saturation train prior to the readout consisting of a saturation pulse train of 1, 21, 41, 61, 101 and 151 Gaussian pulses at two different RF amplitudes: 1.14μT and 1.8μT (t_{sat}=50ms, fa=900° and 1400°, 91% duty cycle). B_1 was calculated as the mean B_1 integrated over an RF pulse and is equal to $B_1 = fa/\gamma t_{sat}$. The total scan time needed for a single QUEST exchange rate measurement was 58min (9.7min per acquisition with different irradiation lengths) and additional 14min were needed for obtaining a T_1 map, leading to a total acquisition time of 1h and 12min.

QUESP measurements were also acquired in the same samples using 151 Gaussian pulses at eleven different irradiation amplitudes (i.e. 0.39μT, 0.76μT, 1.17μT, 1.57μT, 1.96μT, 2.35μT, 2.74μT, 3.13μT, 3.52μT, 3.91μT, and 4.31μT) and similar sequence parameters to the ones used for QUEST measurements.

T_1 and T_2 Measurements *in vivo*

To calculate exchange rates using the standard methods and compare them with the PRO-QUEST sequence, T_1 and T_2 values are necessary (Equation 8). An inversion recovery EPI sequence was used to quantify the T_1 values [24]. A global adiabatic inversion pulse (fa=180°, duration=2ms) was applied followed by 10 inversion times exponentially spaced from 8.1ms to 7.5s (see Supporting Table S3). Other parameters were as follows: TR=15s, TE=25.5ms, slice thickness=2mm, FOV=20x20mm², matrix size=64x64.

For the quantification of T_2 values, a CPMG sequence was used [25], consisting of a 90° Sinc-shaped excitation pulse along the x axis (duration=2ms) followed by 15 Sinc-shaped refocusing pulses along the y axis (fa=180°, duration=1.6ms). Other parameters were as follows: TR=3s, τ_{CPMG}=8.33ms, slice thickness=2mm, FOV=20x20mm²,matrix size=64x64.

Post-Processing

Data processing was performed using custom-written scripts in MATLAB (Mathworks Waltham, MA). All PRO-QUEST images were fitted following the steps described in the theory section. QUEST and QUESP data were analyzed following [10] by fitting full Z-spectra obtained at 77 frequency offsets with different irradiation times or amplitudes respectively. T_1 and T_2 maps were obtained assuming mono-exponential decay for longitudinal and transverse relaxation [24],[25].

Results

Exchange rate measurements in samples using PRO-QUEST and QUEST

Simulated data were obtained using a modified two-pool Bloch equations to assess the effect of different parameters such as T_1, exchange rates, magnetic field strength (B_0), RF saturation power (B_1) and finally the small flip angle (ϑ) on the saturation recovery curves in the presence of off-resonance RF irradiation (see Supporting Figures S2-S6).

Figure 2 shows the experimentally measured saturation recovery curves for PBS and 100mM alanine samples at different pH values with and without off-resonance progressive saturation pulses. The plots in Figure 2(a) demonstrate that saturation recovery curves obtained using a Look-Locker scheme in the absence of off-resonance CEST saturation pulses are not very sensitive to changes in pH. However, changes in pH can be mapped for each sample when the same scheme is used with CEST saturation pulses applied on-resonance with the amine group of alanine (at 3.0ppm) (Figure 2(b)).

Quantitative estimates of exchange rates using both PRO-QUEST and QUEST, T_1 and T_2 of PBS in the presence of alanine, glutamine, glutamate and taurine are displayed in Table 1. As can be seen, T_1 values exhibited no dependence on pH while T_2 and exchange rates varied dramatically with pH, thereby demonstrating that chemical exchange in alanine, glutamine, glutamate and taurine are base-catalyzed. Note that the exchange rates of the (protonated) amine groups in amino acids depend on pH, pKa and the type and concentration of the buffer system used. The pKa values of the amino acids used in this study are: alanine 9.7, glutamate 9.5, glutamine 9.1, and taurine 9.0. If aqueous solutions were used, the expected exchange rates should be the fastest for taurine and slower for alanine. Because the samples were titrated in 0.1 % PBS, the exchange rates are affected by additional reaction pathways.

It is worth noting that the PRO-QUEST exchange rates of glutamine and taurine at the highest pH values (7.4 and 7.2) decreased to $5.80\text{x}10^3\text{s}^{-1}$ and $3.13\text{x}10^3\text{s}^{-1}$ respectively. A reduction in the exchange rates of glutamine and taurine at pH 7.4 and pH 7.2 was also obtained when QUEST analysis was performed. These results suggest

that quantification of exchange rates using saturation times at the power used does not works for exchange rates above ~$15\text{x}10^3 s^{-1}$. This trend was not observed with QUESP analysis (see Supporting Table S1-S2 and Supporting Figures S7, S8) where the exchange rates in taurine and glutamine continued to increase with pH up to $20\text{x}10^3 s^{-1}$.

PRO-QUEST sensitivity to changes in the concentration of exchangeable protons was evaluated by increasing solute concentrations from 12.5mM to 100mM (Table 2). T_1 did not change when the concentration of solute increased and was found to be equal to 2.95±0.01s in all the samples. The exchange rates however were found to be equal within error for 12.5, 25 and 50mM in alanine and glutamine, but deviated for 100mM. The exchange rates of glutamate were $0.77\text{x}10^3 s^{-1}$ on average. The measured exchange rates for taurine at high concentrations were found to be increased by one order of magnitude compared to the exchange rates measured at lower concentrations. In addition, the T_2 was found to vary with the solute concentration for all samples. All data were fitted by keeping the concentration ρ_B to its theoretical value, calculated from the number of protons in the solute multiplied by the concentration of the agent divided by the concentration of protons in the solvent (111.2M), see Table 2, while fitting the saturation recovery curves at different irradiation amplitudes for the exchange rates.

Figure 3(a-d) displays the $\overline{R'_{ex}}$ values obtained for each sample at different mean B_1 values. In all experiments, $\overline{R'_{ex}}$ increased with B_1 (Figures 3 (a-d)). Changes in the exchange rates of alanine, glutamine, glutamate and taurine with pH are shown in Figure 4(a). Figure 4(b) displays the measured exchange rates for all the samples when the solute concentration was increased from 12.5mM to 100mM.

T1 measurements and error analysis in healthy rats

T_1 maps from healthy rats were acquired using PRO-QUEST without the off-resonance CEST pulses at three imaging flip angles (8°, 15°, 25°). Additional T_1 maps were also collected using an inversion recovery spin echo EPI sequence for comparison (Figure 5). Figure 5 (a-d) shows the T_1 values obtained with IR-EPI vs Look-Locker acquisition in 3 different regions in a rat brain. T_1 was significantly smaller (p=0.003) for 25° and

was not significantly different (p=0.19) for 8° when Look Locker acquisition was used comparative to IR-EPI in all ROIs.

B_1 effects on the measured T_1 using saturation recovery Look-Locker sequence

A B_1 map can be calculated by acquiring data at two or more flip angles [21],[26]. If we consider a 3D acquisition, a discrepancy in the flip angle will mainly be due to B_1 effects. However, for a 2D acquisition slice profile effects need to be considered as well. For shaped RF pulses such as Gaussian pulses, the effective flip angle depends on the slice profile, which is not an ideal square profile. Figure 6(a) shows a B_1 map obtained in a healthy rat which includes both B_1 variations and slice profile effects. The B_1 map, calculated with ϑ_1=8°, ϑ_2=15° and ϑ_3=25°, is expressed as a fraction of the ideal flip angle and the mean B_1 in this slice was found to be equal to 0.68 of the prescribed one. Figure 6(b) displays the slice profile effects in the case of a Gaussian RF pulse with ϑ=8°, 15° and 25°. Solid lines represent the ideal or nominal slice profile (2mm slice thickness, ϑ=25°, 15° and 8°) and the dashed lines represent the achieved percentage of RF pulse, calculated by comparing the area under the Gaussian pulses to the area of an ideal pulse with a constant flip angle throughout the slice. The simulations show that an applied flip angle of 8° is underestimated to 74% of its ideal value. No T_1 or T_2 decay was considered during the pulse for slice profile simulations using simple numerical integration of the Bloch equations.

Relaxation exchange weighted ($\overline{R'_{ex}}$) maps in healthy and infarcted rats

Figure 7 shows PRO-QUEST curves obtained in a rat brain at 24 h post stroke. The $\overline{R'_{ex}}$ of normal tissue calculated over the three rats was $2.6\text{x}10^{-2} \pm 3\text{x}10^{-4}\ s^{-1}$ whereas, in the stroke lesion seen in the right side of the brain it dropped to $6.3\text{x}10^{-3} \pm 3\text{x}10^{-4}\ s^{-1}$. Figures 8(a) and (b) display the $\overline{R'_{ex}}$ maps obtained in a healthy rat brain before and 24 h post stroke at 0.39μT, respectively. The ratio of the $\overline{R'_{ex}}$ at 0.39μT and 0.65μT is displayed in Figures 8(c) and (d) before and 24h post stroke respectively. The concentration-free $\overline{R'_{ex}}$ map is sensitive to changes in exchange rate and ω_1 but *not* to the concentration of the CEST pool and therefore it can numerically be solved to probe changes in k_{ex} *in vivo*. Note that the concentration-free $\overline{R'_{ex}}$ was reduced both on the

contra- and ipsilateral side of the stroke in the grey matter, indicating an overall reduction in exchange rate following stroke.

Discussion

In recent years, much effort has been focused on the measurement of amide proton transfer (APT)-weighted signal and other CEST signals using several CEST metrics obtained by various fitting approaches [27], [28], [29]. Here, we have implemented a new pulse sequence, called PRO-QUEST, which enables quantification of exchange rates and therefore isolation of the CEST effect within a clinically applicable total scan time. This sequence samples multiple evenly spaced points along a T_1 recovery curve after a global saturation pulse while off-resonance pulses label protons that are constantly exchanging with water. To progressively follow the water signal during off-resonance irradiation and recovery, a standard Look-Locker sequence has been modified. In addition, a complete set of equations has been derived that describe the recovery of the magnetization due to relaxation and chemical exchange following the Look-Locker sampling scheme.

We showed using both simulated and experimental data that the accuracy in estimating exchange rates is strongly affected by changes in T_1 and ϑ. Therefore, these parameters had to be mapped from additional acquisitions without the use of off-resonance saturation pulses and used as input parameters for calculating the exchange rates in Equations 2 and 8. In addition, since the effect of exchange to the water signal is very small, the main contribution remains the direct saturation effect on the water, which is a function of R_1, R_2 and ω_1. Here, we show that the direct effect can be fitted as a separate parameter $\overline{R_{eff}}$ and therefore the calculated exchange-dependent relaxation rate $\overline{R'_{ex}}$ which describes the CEST effect is isolated from confounding factors (see Equation 6).

Based on Equation 8, exchange rates were calculated in samples containing exchangeable protons in different exchange regimes including slow-to-intermediate and fast exchange regimes. These experiments showed that PRO-QUEST is sensitive to pH alterations through changes in exchange rates. Furthermore, as exchange rates in taurine, alanine, glutamate and glutamine are base-catalyzed, their exchange rates increase with pH (see Figure 3(e)). The range of our calculated exchange rates in

amine protons is in line with previously published results [26],[27] and they are consistent with the results of the QUEST analysis. However, one should keep in mind that the original QUEST approach requires the acquisition to reach steady state and the data to be obtained by repeating the same sequence with at least five different saturation times or pulse train lengths. PRO-QUEST alleviates this requirement through the acquisition of 300 images collected at 300 saturation times in 2.8min. This would have resulted in being able to only acquire 31 images for a single frequency offset using the same sequence in a QUEST experiment. Note also that the use of a saturation-recovery was governed by the need to clearly establish the starting state of the system, but it is not a limiting factor. Indeed, the use of inversion pulses could also be envisaged but might need a longer TR to ensure reaching a steady state. A recent work [32] on positive CEST contrast explored the possibility to use a 180° pulse followed by CEST off-resonance saturation pulse without interleaved readout.

The exchange rate or the exchange-dependent relaxation term Rex represents an additional spectrally selective relaxation pathway to the relaxation of the water magnetization in the rotating frame as can be seen from Equations 3, 7 and 8. Considering that the same functional group (amine) has been studied in small molecules such as amino acids and exchange kinetics was different based on the type of amino acid, the exchange rates in proteins (bigger molecules with smaller correlation times) will be substantially slower than what we detected here mainly because the interior backbone protons are shielded from solvent [33]. In addition, because of the inherent limitations of CEST techniques, we are unable to discriminate the origin of the signals at 3.00 ppm *in vivo*. So the measured Rex will be equal to the sum of each contribution weighted by its concentration (Equation 6). For example, faster or more concentrated species will contribute more. Rex is also dependent on the experimental conditions (i.e. irradiation amplitude and duration) and on the method used to calculate it. Finally, *in vivo*, the chemical exchange is restricted by membranes, while prolonged T_2 effects affect the accessibility to the water pool and the presence of phosphates affect the buffering of the system. MT and dipole-dipole interactions are also confounding factor since they occur simultaneously and cannot be distinguished from

chemical exchange in Z-spectra, although they are slower processes and pH insensitive [34].

Our study demonstrates that PRO-QUEST substantially enhances imaging specificity to ischemic acidification during stroke relative to standard APT-weighted imaging and, when combined with perfusion and diffusion weighted imaging, should permit refined prediction of the salvageable tissue within the penumbra. Because the range of exchange rates *in vivo* seems to be dominated by slow base-catalyzed rates corresponding to amide protons, tissue acidification is associated with a reduced amide exchange rate as seen in Figure 8. Note that tissue acidification is associated with cell death and tissue damage [35]. This is mainly due to the fact that pH alterations are associated with imbalance in glucose/oxygen delivery and consumption during stroke, leading to anaerobic metabolism and mitochondrial poisoning [36]. Therefore, exchange relaxation or pH maps can serve as important surrogate metabolic imaging biomarkers even within the first few hours after stroke. Our results demonstrating a lowering of the exchange rate in the infarcted regions are in good agreement with previous findings [29] [30],[37] . However, the additional decrease in $\overline{R'_{ex}}$ observed on the contralateral side has not been reported previously using APT-weighted imaging [37]. This finding, if confirmed, could be important to understand the pathophysiology of stroke. The lack of appearance of this effect on APT-weighted imaging could be due to low signal-to-noise ratio of the latter method. This would demonstrate an improved sensitivity of PRO-QUEST to this effect as compared with standard APT-weighted imaging.

In a recent study, perturbation of longitudinal magnetization rate in the rotating frame was proposed to reduce the effects of non-chemical exchange-related parameters (i.e. direct water saturation) in the transient state when a continuous wave saturation pulse was used [38]. An exchange-dependent relaxation rate (SPACER) was derived from $\Delta R_{1\rho} = (R_{1\rho}^{ref} - R_{1\rho}^{lab})$ where $R_{1\rho}^{ref} = R_{1\rho}(+\Delta\omega)$, $R_{1\rho}^{lab} = R_{1\rho}(-\Delta\omega)$ based on several approximations including a sufficiently small $|\Delta R_{1\rho}|$ and a fast exchange rate approximation. Although that study showed promise for small B_1 amplitudes, the mapping of faster exchange species requires high B_1 [1] and therefore this method is likely to be limited to slow-to-intermediate exchange regimes. In contrast to SPACER,

PRO-QUEST can be used to map exchange rates in different exchange regimes by replacing the off-resonance saturation pulse with a spin-lock module applied on the water resonance for probing fast exchanging species. In addition, PRO-QUEST measurements can be obtained at different frequency offsets and irradiation amplitudes to capture the build-up of the signal due to chemical exchange and other confounding effects, such as magnetization transfer without the need of acquiring full Z-spectra like SPACER.

Another pulse sequence used to separate and quantify both slow and fast exchanging protons without the need to record full Z-spectra is the variable-delay multipulse (VDMP) method, consisting of a series of saturation pulses separated by an interpulse delay time t_{mix} [39] . Similarly to PRO-QUEST, the VDMP sequence requires only one irradiation frequency, by applying an RF pulse of high bandwidth (200-600Hz), and two images with different delay times: the first is used as a reference image and the second provides the exchange-weighted contrast. The difference between VDMP and the PRO-QUEST method is that the saturation recovery PRO-QUEST curves are based on a T_1 mechanism, while VDMP is based on T_2. In VDPM, the authors manipulate the interpulse delay to gain insight into different exchange properties, while here we vary the PRO-QUEST curves through different irradiation amplitudes to target different exchange regimes. Alternatively, PRO-QUEST has the ability (not shown) to be used with interleaved on-resonance spin lock modules to capture fast exchanging species.

It has been shown that the application of a gradient field simultaneously with the saturation pulse allows obtaining highly accelerated multi-sample Z-spectra. Similar with PRO-QUEST, by repetitively interleaving gradient-encoded saturation and an echo planar imaging (EPI) readout, an incremental saturation time is achieved, which enables exchange rate quantification through a QUEST type of analysis [40]. Both PRO-QUEST and ultrafast CEST accelerate the acquisition by modifying standard pulse sequences and thus making both methods easily available to many potential users.

Limitations

There are a few limitations of our study. First, since only one irradiation frequency is used, this method will be susceptible to B_0 shifts. However, in an attempt to tackle this issue we used high bandwidth saturation pulses (1000 Hz for phantom studies and 400 Hz for the *in vivo* study) and carefully set the frequency offset to zero using a PRESS sequence. Another limitation is that, in our study, exchange rate measurements using QUEST or PRO-QUEST are unreliable beyond $15\text{x}10^3 s^{-1}$. This indicates that we would need higher irradiation powers to target fast exchange rates using QUEST or PRO-QUEST from the ones used here or to use on-resonance interleaved spin lock modules for increased sensitivity in this regime. It is worth noting that if the labelling efficiency is low i.e. $\omega_1^2(t) \ll k_{ex}^2$ the integral $\int_0^{t_{sat}} \frac{\omega_1^2(t)}{k_{ex}^2+\omega_1^2(t)} dt \approx \int_0^{t_{sat}} \frac{1}{\frac{k_{ex}^2}{\omega_1^2(t)}+1} \approx 0$ thus $\overline{R'_{ex}} \approx 0$ and the PRO-QUEST curves will recover mainly with R_1 and R_2. An example of weak labelling is shown in Supporting Figure S6 in which the contribution of $\overline{R'_{ex}}$ to the obtained PRO-QUEST curves is minimal for k_{ex}> 3000 s^{-1} when B_1 = 0.87 μT.

Conclusion

In this study, we developed and validated a new pulse sequence called PRO-QUEST by using full Bloch-McConnell simulations of a two pool system and by acquiring data both *in vitro* and *in vivo*. Based on simulations and experimental data, the calculated exchange rates were found to agree with the original QUEST method. The developed PRO-QUEST sequence offers a much faster alternative for calculating exchange rates and gaining insight into exchange processes *in vivo*. In particular, the application of PRO-QUEST in an animal model of stroke is encouraging as it shows its utility in a typical clinical application.

Appendix

Derivation of the water signal evolution as a function of saturation time with and without saturation pulses or spin lock modules

Consider n off-resonance saturation modules based on RF pulses or spin-lock modules of length t_{sat} separated by an interval τ-t_{sat} as shown in Figure 1. The evolution of the water signal as a function of the saturation time during the off-resonance saturation module can be written as:

$$M_z(t_{sat}) = M(t=0)e^{-R_{1\rho}t_{sat}} + M_{ss}(1 - e^{-R_{1\rho}t_{sat}}) \qquad \textbf{(A1)}$$

where $M(t=0)$ is the initial z-magnetization prior to the first saturation module. In the case of a saturation-recovery Look-Locker experiment $M(t=0) = Meq\left(1 - e^{-\frac{td}{T_1}}\right)$ where td is the time to first saturation pulse from the preparation. The normalized steady state signal is $M_{ss} = \frac{R_1 \cos^2\varphi}{R_{1\rho}}$ where $cos\varphi = 1$ for spin-lock and $\cos\varphi = \frac{\Omega}{\sqrt{\omega_1^2+\Omega^2}}$ for CEST. $R_{1\rho}$ is the longitudinal relaxation rate in the rotating frame. In the presence of off-resonance irradiation, it is equal to the sum of the relaxation rates due to chemical exchange and water saturation.

After the off-resonance saturation module and similar to a typical Look-Locker experiment an RF pulse of small flip angle (ϑ) is used to sample multiple time points during the relaxation of the z-magnetization. The RF (ϑ) pulse is considered to cause instantaneous excitations of M_z. Therefore, the z-magnetization after the (ϑ) pulse becomes $M_z(t_{sat})\cos\vartheta$.

Solution without considering effects of exchange during the recovery between off- resonance saturation pulses

Assuming a recovery of the z-magnetization during the τ-t_{sat} interval with the time constant T_1, i.e. without considering effects of exchange during the recovery between off-saturation pulses, the signal before the second saturation module can be expressed as:

$$M_z(\tau - t_{sat}) = M_z(t = t_{sat})e^{-(\tau - t_{sat})/T_1} + Meq(1 - e^{-(\tau - t_{sat})/T_1}) \quad \textbf{(A2)}$$

Combining equations (A1) and (A2), M_z can be written as:

$$M_z(\tau) = [Meq\left(1 - e^{-\frac{td}{T_1}}\right)e^{-R_{1\rho}t_{sat}} + M_{ss}(1 - e^{-R_{1\rho}t_{sat}})]\cos\vartheta e^{-(\tau - t_{sat})/T_1} + Meq(1 - e^{-(\tau - t_{sat})/T_1}) \quad \textbf{(A3)}$$

To calculate the z-magnetization after the second off-resonance saturation module, equation **(A3)** is substituted into equation **(A1)** as the initial magnetization $M_z(t=0) = M_z(t=\tau)$.

The measured M_z after the second off-resonance saturation pulse and (ϑ) excitation pulse is then:

$$M_z(2\tau) = \left[Meq\left(1 - e^{-\frac{td}{T_1}}\right)e^{-R_{1\rho}t_{sat}} + M_{ss}(1 - e^{-R_{1\rho}t_{sat}})\right][1 + \cos\vartheta e^{-(\tau - t_{sat})/T_1}e^{-R_{1\rho}t_{sat}}] + [Meq\left(1 - e^{-\frac{\tau - t_{sat}}{T_1}}\right)e^{-2R_{1\rho}t_{sat}}\ (\cos\vartheta)^2\ \ e^{-2\frac{(\tau - t_{sat})}{T_1}}\ ] \quad \textbf{(A4)}$$

Each iteration of the next off-resonance saturation followed by a Look-Locker read out multiplies the $M_z(n-1)$ by a factor $\cos\vartheta e^{-(\tau - t_{sat})/T_1}e^{-R_{1\rho}t_{sat}}$ and adds $Meq\left(1 - e^{-\frac{td}{T_1}}\right)e^{-R_{1\rho}t_{sat}} + M_{ss}(1 - e^{-R_{1\rho}t_{sat}})$. Finally, the desired expression for M_z following a train of n off-resonance saturation pulses and (ϑ) excitation pulses is given by:

$$M_{zsat}(n\tau) = \frac{\mathbf{1} - \left[\alpha(\cos\theta)^n\, e^{-\boldsymbol{n}\left(\tau R_1 - t_{sat}(R_1 - R_{1\rho})\right)}\right]}{\mathbf{1} - \left[\alpha(\cos\theta)\, e^{-\left(\tau R_1 - t_{sat}(R_1 - R_{1\rho})\right)}\right]} M_{zsat}(\tau)$$

$$+ M_{eq}\,(1 - e^{-(t_d R_1)})[\,(\cos\theta)^n\, e^{-\boldsymbol{n}\left(\tau R_1 - t_{sat}(R_1 - R_{1\rho})\right)}] \quad \textbf{(A5)}$$

where $M_{zsat}(\tau) = M_{ss}\,(1 - e^{-(R_{1\rho}t_{sat})})\cos\theta\ e^{-((\tau - t_{sat})R_1)} + M_{eq}\,(1 - e^{-((\tau - t_{sat})R_1)})$

Solution with considering effects of exchange during the recovery between off-resonance saturation pulses

Let us consider equation **(A1)** again. Assuming recovery of the z-magnetization with R_{1A} and chemical exchange during the interpulse delay $t_d=\tau-t_{sat}$, the signal before the second saturation pulse or spin-lock module can be expressed as [41],[42]:

$$M_z(\tau - t_{sat}) = daa[M_z(t = t_{sat}) - M_{0z}] + dab\left[\Psi M_z(t = t_{sat}) - \rho_B M_{0z}\right] + M_{0z} \qquad \textbf{(A6)}$$

Combining equations **(A1)** and **(A6)** the M_z can be written as:

$$M_z(td) = daa\left[Meq\left(1 - e^{-\frac{td}{T_1}}\right)\cos\vartheta e^{-R_{1\rho}t_{sat}} + M_{ss}\cos\vartheta(1 - e^{-R_{1\rho}t_{sat}}) - M_{0z}\right] +$$

$$dab\left[\Psi Meq\left(1 - e^{-\frac{td}{T_1}}\right)\cos\vartheta e^{-R_{1\rho}t_{sat}} + \Psi M_{ss}\cos\vartheta(1 - e^{-R_{1\rho}t_{sat}}) - \rho_B M_{0z}\right] + M_{0z} =$$

$$daa.\mathrm{u} + dab.v + M_{0z} \qquad \textbf{(A7)}$$

where

$$daa = \frac{1}{R_{1A} - k_{ex} - \rho_B k_{ex} - R_{1B}}\left[(R_{1B} - 2\rho_B k_{ex} - k_{ex} - R_{1A}).e^{-\frac{(\tau - tsat)}{T_1}} + (2R_{1A} + \rho_B k_{ex}).e^{-(\tau - tsat).(\rho_B k_{ex} + k_{ex} + R_{1B})}\right],$$

$$dab = \frac{k_{ex}}{R_{1A} - \rho_B k_{ex} - k_{ex} - R_{1B}}\left(e^{-\frac{(\tau - tsat)}{T_1}} - e^{-(\tau - tsat).(\rho_B k_{ex} + k_{ex} + R_{1B})}\right),\ \Psi = \rho_B - \frac{R_{ex}}{k_{ex}}. \qquad \textbf{(A8)}$$

To calculate the z-magnetization after the second off-resonance saturation pulse or spin-lock module equation **(A7)** is substituted into equation **(A1)** as the initial magnetization $M_z(t=0) = M_z(t=\tau)$.

The measured M_z after the second off-resonance saturation pulse and (ϑ) excitation pulse is then:

$$M_z(2\tau) = (daa + \Psi.dab)(daa.\mathrm{u} + dab.v).\cos\vartheta e^{-R_{1\rho}t_{sat}} + daa.x_1 + dab.x_2 + M_{0z} \qquad \textbf{(A9)}$$

where $x_1 = M_{0z}\cos\vartheta e^{-R_{1\rho}t_{sat}} + M_{ss}\cos\vartheta(1 - e^{-R_{1\rho}t_{sat}}) - M_{0z}$ and

$x_2 = \Psi M_{0z}\cos\vartheta e^{-R_{1\rho}t_{sat}} + \Psi M_{ss}\cos\vartheta(1 - e^{-R_{1\rho}t_{sat}}) - \rho_B M_{0z}$

After the third module of CEST saturation pulse- ϑ pulse –delay the z-magnetization can be written as:

$$M_z(3\tau) = (daa + \Psi.dab)^2(daa.\mathrm{u} + dab.v).\cos^2\vartheta e^{-2R_{1\rho}t_{sat}} + [(daa + \Psi.dab).\cos\vartheta e^{-R_{1\rho}t_{sat}} + 1].(daa.x_1 + dab.x_2) + M_{0z} \quad \textbf{(A10)}$$

If we set $\alpha = (daa + \Psi.dab).\cos\vartheta e^{-R_{1\rho}t_{sat}} + 1$ and expand using geometric series the desired expression for M_z following a train of n off-resonance saturation pulses and (ϑ) excitation pulses is given by:

$$M_{zsat}(n\tau) = \alpha^{n-1}\left[(daa + \Psi.dab).\mathrm{u} + dab(\Psi - \rho_B)M_{0z}\right] + \left[(daa + \Psi.dab).\mathrm{u} + (daa + \Psi.dab)\left(M_{0z}e^{-\frac{td}{T_1}}.e^{-R_{1\rho}t_{sat}}\right) + dab(\Psi - \rho_B)M_{0z}\right].\frac{1+\alpha-\alpha^{n-1}-\alpha^n}{1-\alpha^2} + M_{0z} \quad \textbf{(A11)}$$

where

$daa + \Psi.dab = (1 - \frac{R_{ex}}{k_{ex}})\, e^{-\frac{(\tau - tsat)}{T_1}}$, and

$$dab = \frac{k_{ex}}{R_{1A} - \rho_B k_{ex} - k_{ex} - R_{1B}}\,(e^{-\frac{(\tau - tsat)}{T_1}} - e^{-(\tau - tsat).(\rho_B k_{ex} + k_{ex} + R_{1B})}) \quad \textbf{(A12)}$$

For data analysis, we used **(A5)** as described in the theory section. However, for completion of our theory we provide the solution **(A11)** when considering the effects of exchange during the recovery between off-resonance saturation pulses which will be crucial for slow exchange rates or for clinical systems where longer inter-pulse delays are used. In our study, the duty cycle was 88 % and the samples used to validate PRO-QUEST possess exchangeable protons that resonate in the intermediate to fast exchange regime and therefore we assume that equation **(A5)** would be sufficient to

describe our data (i.e. $daa + \Psi . dab \approx e^{-\frac{(\tau - tsat)}{T_1}}$ and $dab\left(\Psi - \rho_B\right) M_{0z} \ll 1$) in line with published studies [20],[39].

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Tables

Table 1: Exchange rates and relaxation times (T_1, T_2) in samples containing 100mM alanine, glutamine, glutamate and taurine at various pH values and at 23 °C

Substance			**pH**		
Alanine	**6.02**	**6.33**	**6.74**	**7.06**	**7.42**
T_1 (s)	2.97±0.01	2.95±0.01	2.91±0.01	2.94±0.01	2.97±0.01
T_2 (s)	0.473±0.07	0.295±0.07	0.152±0.07	0.096±0.07	0.105±0.07
$k_{ex}(10^3 s^{-1})$					
PR0-QUEST	0.56±0.01	0.91±0.06	2.27±0.15	6.47±1.06	12.3±0.21
k_{ex} $(10^3 s^{-1})$					
QUEST	0.58±0.13	0.99±0.05	2.42±0.09	7.67±3.51	11.1±3.81
			pH		
Glutamate	**6.1**	**6.4**	**6.6**	**7.1**	**7.4**
T_1 (s)	2.71±0.01	2.76±0.01	2.77±0.01	2.77±0.01	2.81±0.01
T_2 (s)	0.429±0.04	0.341±0.04	0.359±0.04	0.282±0.04	0.222±0.04
$k_{ex}(10^3 s^{-1})$					
PR0-QUEST	0.71±0.03	0.78±0.03	0.84±0.03	1.27±0.20	2.29±0.15
k_{ex} $(10^3 s^{-1})$					
QUEST	0.72±0.22	0.86±0.23	1.04±0.19	1.66±0.28	2.54±0.34
			pH		
Glutamine	**6.1**	**6.4**	**6.6**	**7.1**	**7.4**
T_1 (s)	2.6±0.01	2.6±0.01	2.6±0.01	2.6±0.01	2.6±0.01
T_2 (s)	0.323±0.04	0.180±0.04	0.143±0.04	0.112±0.04	0.141±0.04
$k_{ex}(10^3 s^{-1})$					
PR0-QUEST	0.96±0.03	1.85±0.03	2.54±0.03	12.6±0.20	5.80±0.15
k_{ex} $(10^3 s^{-1})$					
QUEST	0.98±0.15	1.90±0.09	2.58±0.27	12.6±0.26	5.88±0.74
			pH		
Taurine	**5.85**	**6.08**	**6.31**	**6.86**	**7.2**
T_1 (s)	3.04±0.01	3.05±0.01	3.02±0.01	3.08±0.01	3.04±0.01
T_2 (s)	0.148±0.04	0.097±0.04	0.093±0.04	0.131±0.04	0.330±0.04
$k_{ex}(10^3 s^{-1})$					
PR0-QUEST	2.57±0.05	3.27±0.87	5.11±0.19	16.0±0.16	3.13±0.08
k_{ex} $(10^3 s^{-1})$					
QUEST	2.59±0.07	5.27±0.33	6.43±0.51	14.5±0.51	2.91±0.31

Table 2: Exchange rates and relaxation times in samples containing alanine, glutamate, glutamine and taurine at various concentrations. The pH was constant at 6.2 and the temperature was 23 °C.

Substance	**Concentration**			
Alanine	**12.5mM**	**25mM**	**50mM**	**100mM**
T_1 (s)	2.97±0.01	2.95±0.01	2.91±0.01	2.94±0.01
T_2 (s)	0.90±0.14	0.88±0.14	0.62±0.14	0.25±0.14
ρ_B	7.8×10^{-4}	5.1×10^{-4}	1.2×10^{-3}	2.5×10^{-3}
$k_{ex}(10^3 s^{-1})$				
PR0-QUEST	0.70±0.46	0.67±0.26	0.74±0.10	1.24±0.40
	Concentration			
Glutamate	**12.5mM**	**25mM**	**50mM**	**100mM**
T_1 (s)	2.94±0.01	2.93±0.01	2.92±0.01	2.88±0.01
T_2 (s)	1.19±0.16	8.90±0.16	6.31±0.16	3.36±0.16
ρ_B	7.8×10^{-4}	5.1×10^{-4}	1.2×10^{-3}	2.5×10^{-3}
$k_{ex}(10^3 s^{-1})$				
PR0-QUEST	0.71±0.31	0.69±0.44	0.82±0.16	0.87±0.13
	Concentration			
Glutamine	**12.5mM**	**25mM**	**50mM**	**100mM**
T_1 (s)	2.96±0.01	2.95±0.01	2.94±0.01	2.88±0.01
T_2 (s)	1.10±0.18	0.82±0.18	0.49±0.18	0.19±0.18
ρ_B	7.8×10^{-4}	5.1×10^{-4}	1.2×10^{-3}	2.5×10^{-3}
$k_{ex}(10^3 s^{-1})$				
PR0-QUEST	0.64±0.58	0.91±0.41	1.10±0.16	1.82±0.16
	Concentration			
Taurine	**12.5mM**	**25mM**	**50mM**	**100mM**
T_1 (s)	3.04±0.01	3.05±0.01	3.00±0.01	2.96±0.01
T_2 (s)	0.92±0.16	0.53±0.16	0.21±0.16	0.92±0.16
ρ_B	7.8×10^{-4}	5.1×10^{-4}	1.2×10^{-3}	2.5×10^{-3}
$k_{ex}(10^3 s^{-1})$				
PR0-QUEST	0.99±0.58	1.88±0.35	3.29±0.65	5.93±0.97

where $\rho_B = \frac{n\,[Con]}{111.2}$, n: number of protons, [Con]: concentration of the CEST agent

Figure legends:

Figure 1 PRO-QUEST Pulse sequence diagram. An initial 90 °saturation pulse(s) was followed by off-resonance saturation pulses or spin-lock modules (a) or by delays (b) interleaved with the acquisition of segmented exchange-weighted images. Following the application of a 90° pulse, Ny lines in k-space are acquired by the application of the same number of slice-selective ϑ pulses. After full relaxation, a new 90° pulse is applied and the following Ny lines in k-space are acquired in an identical manner. This is repeated until the whole of k-space, at multiple time-points, has been acquired. The sequence in 1b) is used for T_1 measurement and the resulting parameters (T_1, and B_1) are then used as input parameters for fitting the results from the PRO-QUEST sequence in 1(a) to obtain the exchange-related relaxation rate.

Figure 2 Experimental saturation recovery curves without **(a)** and with **(b)** the application of saturation pulses in samples consisting of 100mM of Alanine at pH=6.02, 6.33, 6.74, 7.06 and 7.42. **(c)** Fitted and experimental data for Alanine at pH=7.0 are shown. The fitted curve was obtained by fitting the experimental data to equation 2. **(d)** Theoretical curves of Alanine at pH=6.02 without and with off-resonance saturation pulses obtained using Equations 1 and 2 using the values of the measured parameters reported in Table 1.

Figure 3 (a-d) Measured $\overline{R'_{ex}}$ values using equations (2) and (8) in alanine (Ala), glutamine (Gln), glutamate (Glu) and taurine (Tau) samples respectively for B_1 values ranging from 0.87μT to 8.67μT. The calculated exchange rates for each sample are shown in Table 1.

Figure 4 (a) and **(b)** display alterations in exchange rates of alanine, glutamine, glutamate and taurine due to changes in pH and concentration respectively.

Figure 5 (a-d) T_1 values from three representative regions in a healthy rat obtained using a saturation-recovery Look-Locker sequence at ϑ =8°,15°,25° and IR-EPI sequence. **(e)** T_1 map obtained in a healthy rat brain using an Inversion recovery spin-echo EPI sequence.

Figure 6 (a) B_1 map obtained from a healthy rat with ϑ_1 = 8°, ϑ_2 = 15° and ϑ_3 = 25° **(b)** slice profile effects for Gaussian RF pulses. The solid lines show the ideal slice profile (2 mm slice thickness, ϑ = 8°, 15° and 25°) and the dashed lines show the achieved flip angles calculated as the area under the Gaussian-shaped profiles as a percentage of the area of an ideal pulse with constant flip angle throughout the slice.

Figure 7(a) A single pixel chosen in the stroke lesion (red) and contralateral area (blue) overlaid on a Diffusion Weighted Image (DWI), **(b)** experimental data and fitted PRO-QUEST curves from a pixel in the stroke (red) and contralateral region (blue).

Figure 8 $\overline{R'_{ex}}$ maps in a healthy rat brain **(a)** and at 24 h post stroke **(b)** at irradiation amplitude of 0.39μT. The concentration-free $\overline{R'_{ex}}$ = $\overline{R'_{ex}}$ (0.39μT) / $\overline{R'_{ex}}$ (0.65μT) is shown in 8 **(c)** and 8 **(d)** for healthy and infarcted rat respectively.

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Supplemetary data:

Supporting Figure S1: Z-spectra obtained at 0.78 μT from 100 mM of glutamine, taurine, glutamate and alanine.

Supporting Figure S2: Simulated 2-pool PRO-QUEST curves at different T_1. The simulation indicates the sensitivity of the PRO-QUEST measurements to changes in T_1. For all the simulations B_0 = 9.4T, ϑ = 8°, k_{sw} = 1000Hz, B_1 = 0.87μT.

Supporting Figure S3: displays PRO-QUEST curves obtained by simulating a two-pool system at different B_0. For all the simulations T_1 = 2.9s, ϑ = 8°, k_{sw} = 1000Hz, B_1 = 0.87μT.

Supporting Figure S4 displays simulated data obtained at various tip angles ϑ. For all the simulations T_1 = 2.9s, B_0 = 9.4T, B_1 = 0.87μT, k_{sw} = 1000Hz.

Supporting Figure S5 shows simulated data obtained at different irradiation powers B_1. For all the simulations T_1 =2.9 sec, ϑ = 8°, k_{sw} = 1000Hz, B_0 = 9.4T.

Supporting Figure S6: Simulated two pool saturation recovery curves obtained at various exchange rates. For all the simulations T_1 =2.9 sec, ϑ = 8°, B_0 = 9.4T, B_1 = 0.87μT.

Supporting Figure S7: Z-spectra obtained from taurine at (a) pH=5.85, (b) pH=6.08 and (c) pH= 6.31.

Supporting Figure S8: Z-spectra obtained from taurine at (a) pH=6.86, (b) pH=7.2 and (c) PBS.

Supporting Table S1: Exchange rates in alanine, glutamine, glutamate and taurine at various pH values

SupportingTable S2: Exchange rates in alanine, glutamine, glutamate and taurine at various concentrations

Supporting Table S3: inversion times TI for calculation of T_1 used in phantoms and *in vivo*