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In Vivo Imaging of ParaCEST Agents Using Frequency Labeled Exchange Transfer (FLEX) MRI

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Abstract

Purpose—A main obstacle to in vivo applications of paramagnetic chemical exchange saturation transfer (paraCEST) is interference from endogenous tissue magnetization transfer contrast (MTC). The suitability of excitation-based frequency labeled exchange transfer (FLEX) to separate out such MTC effects in vivo was tested.

Methods—The FLEX sequence measures modulation of the water signal based on the chemical shift evolution of solute proton magnetization as a function of evolution time. Time-domain analysis of this water signal allows identification of different solute components and provides a mechanism to separate out the rapidly decaying MTC components with short T_2^* values.

Results—FLEX imaging of paraCEST agents was possible in vitro in phantoms and in vivo in mouse kidneys and bladder. The results demonstrated that FLEX is capable of separating out the MTC signal from tissues in vivo while providing a quantitative exchange rate for the rapidly exchanging paraCEST water protons by fitting the FLEX time-domain signal to FLEX theory.

Conclusions—The first in vivo FLEX images of a paraCEST agent were acquired, which allowed separation out the tissue MTC components. These results show that FLEX imaging has potential for imaging the distribution of functional paraCEST agents in biological tissues.

Keywords

chemical exchange saturation transfer; magnetization transfer; paramagnetic contrast agent; exchange rate

Introduction

Paramagnetic chemical exchange saturation transfer (paraCEST) has become popular because agents can be designed to respond to several biologically important indices,

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including temperature, glucose concentration, pH, nitric oxide, and enzyme activity (1–9). The paraCEST approach offers several advantages over its diamagnetic counterpart. First, a large chemical shift difference ($\Delta\omega > 10$ ppm) can be achieved between the resonances of the exchanging solute and bulk water proton pools. Consequently, direct saturation of bulk water does not interfere with solute detection. Second, complications that often arise by doing an asymmetry analysis are reduced because interference from several upfield resonances in the chemical exchange saturation transfer (CEST) spectrum, i.e. those due to nuclear Overhauser effects in the 0 to –5 ppm range from mobile macromolecules (1,10–14), can be avoided. Third, faster exchanging compounds can be used while still adhering to the slow to intermediate exchange limit ($k_{\text{ex}} < \Delta\omega$) necessary for CEST detection. This allows more saturated spins to be transferred within a given time frame, resulting in an increase of transfer efficiency as expressed by the proton transfer ratio (PTR). However, detection of paraCEST compounds with such rapid exchange rates ($k_{\text{ex}} \sim 10^4 \text{ s}^{-1}$) requires a high B_1 level to achieve at least partial radio frequency (RF) saturation before exchange occurs. Unfortunately, when performed *in vivo*, this comes with an increase in conventional magnetization transfer (MTC) effects in biological tissues, which, despite the large $\Delta\omega$, can still complicate the analysis in paraCEST *in vivo* studies.

Frequency labeled exchange transfer (FLEX) MRI (10,15–16) is a new approach employing frequency transfer for detecting exchanging protons. In comparison to saturation-based exchange methods, FLEX employs a series of excitation pulses that enable full labeling in a time frame on the order of the length of an RF pulse (microseconds on preclinical systems). Consequently, FLEX is beneficial for detecting rapidly exchanging protons. While CEST is based on a reduction in the water signal intensity due to saturation, FLEX detects the modulation of the water signal based on the chemical shift evolution of solute proton magnetization as a function of an evolution time. This modulation is determined by the frequency difference between the offset (ω_1) of the FLEX excitation pulses and the solute resonance, while the signal decay rate is associated with $k_{\text{ex},s} + 1/T_{2s}^*$, the sum of the solute proton exchange and transverse relaxation rates. Time-domain analysis of this water signal provides the opportunity to identify different solute components or to remove rapidly decaying components (short T_2^*), such as those from MTC effects. Using spectroscopy, such FLEX time-domain analysis previously allowed detection of rapidly exchanging water molecules $k_{\text{ex}} > 10,000 \text{ s}^{-1}$ on paraCEST agents dissolved in an agarose gel without interference of agarose–gel based MTC effects (16). Here, we evaluated the potential of paraFLEX imaging for measuring the distribution of paraCEST agents in biological tissues *in vivo* in an animal model.

Methods

The FLEX pulse sequence (Fig. 1a) contains a preparation time (t_{prep}) consisting of a series of n label-transfer modules (LTMs), followed by detection using a MRI readout, here a fast spin-echo (FSE) sequence (10,15–16). Within each LTM, a pair of selective $90_x/90_{-x}$ RF pulses is applied. The ω_1 of the labeling RF pulses is selected based on the combined requirement of minimal water excitation and keeping the selective pulses sufficiently short (on the order of microseconds) to avoid major signal losses due to the rapid exchange of the coordinating water molecules during the RF pulses. The time period in between these two pulses, t_{evol} , is used to frequency label the exchangeable protons using chemical shift evolution. This frequency information is subsequently preserved in the form of longitudinal magnetization by applying the 90_{-x} pulse and exchange transferred to the solvent during a waiting period (t_{exch}). One powerful aspect of FLEX imaging is that the sequence can be tuned to protons with a particular exchange rate range (exchange rate filtering) by selecting an appropriate FLEX labeling pulse duration and t_{exch} period. For example, if the FLEX labeling pulses are much longer than the average bound water lifetime of the exchangeable

solute protons, the solute protons already exchange before they can be frequency encoded and the FLEX contrast will be weighted to slowly exchanging protons (17). Alternatively, to tune the sequence to rapidly exchanging protons, short FLEX labeling pulses can be used in conjunction with a short t_{exch} period. The short t_{exch} period will allow rapidly exchanging protons to exchange into the water pool while it does not allow sufficient time for slowly exchanging protons (18). In this study of water protons on a paraCEST agent, we therefore used labeling pulses on the order of 27–29 μs and a t_{exch} of 600 μs .

FLEX signal amplification occurs because fresh z -magnetization is present for solute protons at the start of each LTM, as provided through exchange with unlabeled protons from the large water pool. In contrast to CEST, which detects the decrease of water signal, FLEX measures modulation in the water signal as a function of t_{evol} in a manner depending on the frequency difference ($\Delta\omega_{\text{sol}}$) between the ω_1 of the 90_x excitation pulse and the solute resonance.

In general, if there are multiple types of exchanging solute protons, the total effect on the water signal can be described by a convolved time-domain signal:

$$I_w = \sum_s \text{PTR}_s \cdot e^{-(k_{\text{ex},s} + 1/T_{2s}^*) \cdot t_{\text{evol}}} \cdot \cos(\Delta\omega_{\text{sol}} \cdot t_{\text{evol}}). \quad [1]$$

Time-domain analysis of this exponential signal decay term provides the opportunity to separate out multiple components such as the water component as well as any rapidly decaying components, such as the MTC contribution caused by semi-solids if $1/T_{2s}^* > k_{\text{ex},s} + 1/T_{2s}^*$ for the semi-solid components. In addition, the exchange rate of solute protons can be obtained by fitting the signal decay when assuming a negligible contribution of T_{2s}^* . The magnitude of FLEX contrast for exchangeable protons is described by the PTR (Eq. 2) which can be derived under the assumption that RF excitation of the solute pool does not perturb the water pool (15).

$$\text{PTR}_s = x_s \cdot \lambda_s \cdot (1 - e^{-k_{\text{ex},s} \cdot t_{\text{exch}}}) \cdot \sum_{i=1}^n e^{\{-1 + (i-1)/n\} t_{\text{prep}}/T_{1w}} \quad [2]$$

where x_s is the fractional concentration of the solute (s) protons and λ_s is the labeling efficiency for the applied RF pulse pair, which can be obtained by squaring the fractional amount of transverse magnetization created by single excitation pulse (10,15). The exponent in the summation term reflects that magnetization transferred to water (w) in the first LTM will experience T_{1w} decay over the full t_{prep} , while that transferred in the n_{th} module will hardly relax. The above equations also show a limitation of the FLEX sequence. While the duration of t_{exch} is not particularly limited *in vivo*, the most appropriate duration is influenced by the T_1 of the bulk water pool (see Eq. 2), because a longer t_{exch} will reduce the number of useful LTMs in the preparation period.

MRI data acquisition

All MRI data were acquired at 37°C on an Agilent 9.4T MRI small animal system using 38 mm quadrature volume coil. Animal studies were approved by the institutional Animal Care and Use committee at the University of Texas Southwestern Medical Center.

In Vitro FLEX-MRI

Three phantoms in tris buffer (pH=7) were prepared for in vitro FLEX-MRI acquisition. One contained a 10 mM solution of EuDOTA-(gly)[−]₄, the second the same paraCEST

solution but mixed with 4% agarose in order to generate an artificial MTC signal, and the third a 4% agarose solution as control. During the scans, the ambient temperature inside the sample chamber was monitored with a thermocouple and maintained at physiological temperature of 37°C with heated air using an animal monitoring system from Small Animal Instruments (Stony Brook, NY). The FLEX sequence used a pair of $90_x/90_{-x}$ labeling pulses (duration: 27.5 μ s; strength: 211.5 μ T). The frequency offset of the FLEX pulses (ω_1) was set to 88.3 ppm to ensure that the off-resonance effective field generated by FLEX labeling pulses (19) minimized water excitation (0 ppm) while still providing sufficient labeling efficiency ($\lambda = 0.85$) for the paraCEST protons at 50 ppm (Fig. 1b). The condition is satisfied when $\omega_1 = \omega_1 \cdot 15$ (19) and thus depends on the B_1 needed for a certain pulse length. FLEX images were acquired by using multiple LTMs, followed by a FSE acquisition with a TR of 8 s, a TE of 8.65 ms, an echo-train-length (ETL) of 32, a matrix size of 64*64, a FOV of 15*15 mm and a slice thickness of 5 mm. The FLEX time-domain signal was acquired by varying t_{evol} from 1 μ s to 235 μ s using time intervals of 6 μ s. The other parameters for FLEX data acquisition were $t_{exch} = 600$ μ s; number of LTMs: 0, 50, 100, 200, 300, 400, 500, 600, 700, 1000, and 1500. The time taken to acquire FLEX images for each LTM using 40 evolution time points and two acquisitions to fill k-space was 10.7 min.

In Vivo FLEX-MRI

C57BL6 male mice weighing 25 g were used. Anesthesia was performed using 2% isoflurane and mice were placed supine during surgery. A roughly 2 cm ventral midline was made, caudal to the sternum. The abdominal cavity was opened and intestinal contents were shifted gently to the side away from the ureter of interest (e.g. intestine shifted to left to work with the right ureter) and covered with sterile saline-soaked sterile gauze. Gentle blunt dissection with cotton tipped applicators was used to isolate the ureter. Two independent ligatures were tied around the ureter using a nonabsorbable suture. This same procedure was repeated on the other side to doubly ligate the other ureter. The intestinal contents were returned to their prior position in the abdomen. The muscle and skin incisions were closed with a nonabsorbable suture. Mice were given a bolus of approximately 1 ml subcutaneous fluid following the surgical procedure. For MRI, body temperature was monitored with a rectal thermocouple and maintained at 37°C with heated air using animal monitoring system from Small Animal Instruments (Stony Brook, NY). The paraCEST agent (EuDOTA-(gly)⁻⁴) was injected at a dose of 0.25 mmol/kg via a tail vein. The pH and the osmolarity of the paraCEST solution were premeasured at 7.0 and 300 mOsm, respectively. The FLEX time-domain signal was acquired by varying t_{evol} from 1 μ s to 199 μ s using time intervals of 9 μ s for bladder and 6 μ s for kidney. A shorter time interval of t_{evol} was used in the kidney measurement in order to sample the rapidly decaying MTC signal component. Other parameters for FLEX-MRI were TR= 5 s; TE= 8.65 ms; ETL= 32; matrix size= 64*64; FOV= 30*30 mm; slice thickness= 2 mm; duration and strength of FLEX labeling pulses = 28.75 μ s and 204 μ T, respectively; ω_1 = 84.25 ppm; t_{exch} = 600 μ s; number of LTMs were 900 for bladder and 700 for kidney. Note that the ω_1 differs slightly from the in vitro experiments due to the different strength of the FLEX labeling pulses (204 vs. 211.5 μ T), most likely because of a change in coil loading. The FLEX labeling pulse duration and t_{exch} period are most sensitive to rapidly exchanging protons and should filter out most of the contrast from endogenous agents with $k_{ex,s} < 1000$ s⁻¹. Using two TRs to fill k-space, the acquisition time for acquiring FLEX in vivo images for each LTM was 3.8 min (23 evolution time points) for bladder and 5.6 min (34 evolution time points) for kidneys.

MRI data analysis

The FLEX signal is acquired in the time domain (by varying t_{evol}) and analyzing the signal before applying a mathematical transform (i.e., Fourier transform) has advantages because the transform may further complicate the analysis (e.g., with respect to phasing the

frequency spectrum). One main benefit is that the signal components contribute differently to the time domain and the frequency domain. For instance, the semi-solid bound water component and free water components strongly overlap in the frequency domain but not in the time domain, where the semi-solid bound water component decays rapidly with its short T_2 ($\sim 10 \mu\text{s}$), while the water in the paraCEST agent and free water components have much longer T_2 s. As a consequence, time domain analysis is easier than frequency domain analysis to separate out different signal components if there is a limited number of them, especially if the frequency of some of them is known. Using this approach, no asymmetry analysis of CEST spectrum with respect to the water resonance frequency is needed. Time domain analysis for MTC separation is quite straightforward and no complications are expected with this analysis unless the solutes also have extremely short T_2 s or exchange rates on this time scale. Time domain analysis also facilitates the quantification of other parameters such as exchange rate according to Eq. 1. The $k_{\text{ex},s}$ values were determined for each pool by normalizing the FLEX time-domain signal to the average water signal without LTMs and then fitting Eqs [1,2] to the normalized experimental data under the assumption that $k_{\text{ex},s} \gg 1/T_2^*$. A two-pool model (paraCEST bound water and free water) was used for a pure solution of the agent alone and a three-pool model (paraCEST bound water, semi-solid bound water, and free water) was used for the combined phantom and in vivo data. In addition, a low frequency component was added for baseline correction. Prior knowledge of the resonance frequency of the free water and the water in the paraCEST agent was used for the FLEX time domain fitting. We also tried fitting the data without prior knowledge, which was possible for the liquid solution, but not in the gel with the MTC component. All data were processed in Matlab (MathWorks, Natick, MA).

Results

In Vitro data on phantoms

The FLEX time-domain signals and spectra of the three phantom samples are shown in Fig. 2. The rapid initial decay in the FLEX time-domain signal of the agarose phantom (Fig. 2a, left) corresponds to the broad band FLEX spectrum (Fig. 2a, right) of the short T_2^* component of the semi-solid MT effect. The water signal from the paraCEST agent solution (Fig. 2b, left) modulated at a frequency $\Delta\omega_{\text{sol}}$ of 15,300 Hz and generated a FLEX resonance at 50 ppm (Fig. 2b, right), in correspondence with the previously reported CEST spectrum (6). Notice that the 50 ppm resonance in the FLEX spectrum is much larger than the residual free water (0 ppm) due to the limited excitation of bulk water. In the combined phantom (paraCEST agent mixed with agarose), the FLEX time-domain signal (Fig. 2c) indicates a rapid signal drop due to MTC in the first 20–30 μs , followed by a more gradual decay from water signal originating from the paraCEST agent. The FLEX spectrum generated by simple Fourier-transform of the time-domain signal shows that the agent peak at 50 ppm can be detected, but only as part of a multi-component broad resonance. To separate out the paraCEST signal, the FLEX data were processed using time-domain analysis (Eq.1). Using prior knowledge about the offset frequencies of the bound solute water (15,300 Hz) and free water (35,300 Hz) components, the FLEX time-domain signal of the combined phantom could be separated into three signal components (Fig. 2d). The initial rapid signal drop in the FLEX time-domain signal was assigned to the short T_2^* component of the semi-solid MT effect (green line), whereas the moderate decay was assigned to the paraCEST agent (red line). The signal of free water (black line) did not contribute much to the FLEX time-domain signal. The fitted FLEX spectrum (Fig. 2d, right) shows that the paraCEST water resonance (red line) could be isolated from the MTC (green line) and water spectra (black line). Figure 2e show the residuals for time domain signal (Fig. 2e, left) and spectrum (Fig. 2e, right), from which no significant residual was observed, suggesting good quality of fit.

Figure 3a shows a PTR map of the paraCEST agent generated using pixel-by-pixel time-domain fitting. Interestingly, the intensity of FLEX signal differed between the agarose-based and solution phantom, while the same concentration of paraCEST agent (10 mM) was used. The integrated signals over the slice were 3.1% for pure solution and 1.3% for the solution mixed with agarose. When observing the PTR as the function of the number of LTMs in pure solution and combined phantoms, we found that the PTR differed between the two phantoms (Fig. 3b) and increased with the number of LTMs. This was attributed to additional signal losses due to signal dephasing during the evolution time, which is increased in the gel. However, time-domain fitting could still be used to determine the agent exchange rates, which were $16.5 \times 10^3 \text{ s}^{-1}$ and $16.8 \times 10^3 \text{ s}^{-1}$ in pure solution and in the combined phantoms (Fig. 3c), respectively, under the assumption of a negligible contribution of T_2^* s. The 95 percent confidence intervals were $13\text{--}18 \times 10^3 \text{ s}^{-1}$ for pure solution and $12\text{--}19 \times 10^3 \text{ s}^{-1}$ for combined phantoms.

In Vivo data for bladder and kidneys

The excretion route of EuDOTA-(gly) $^{-}_4$ in vivo is mainly through the kidneys and into the bladder. Figure 4 shows in vivo FLEX-MRI acquired of the bladder of a mouse one hour after agent injection via the tail vein. The FLEX time-domain signal within the bladder region of interest (Fig. 4a) does not show a rapid initial decay signal related to semi-solid bound water because of the liquid environment. The gradually decaying modulation of FLEX signal is attributed mainly to the paraCEST agent because the resonance frequency of 50 ppm (Fig. 4b) corresponds well to that of the paraCEST peak in the FLEX spectrum of the agent in solution (Fig. 2b). This is confirmed by performing time-domain analysis (Fig. 4c and 4d). An in vivo PTR map of the paraCEST agent obtained using pixel-by-pixel time-domain fitting is shown in Fig. 4e. A high PTR intensity (8.1%) confirms accumulation of the paraCEST agent in the bladder.

In vivo FLEX-MRI measured in the kidneys is shown in Fig. 5. The FLEX time-domain signal within the left kidney region shows a strong signal in the initial 50 μs (Fig. 5a) attributed to combined MTC and paraCEST signals, which is visually confirmed in the FLEX spectrum (Fig. 5b) which displays a paraCEST peak at 50 ppm overlapping with a broad MTC profile similar to the agarose-paraCEST phantom (Fig. 2c). By performing a time-domain analysis (Fig. 5c and 5d), the in vivo FLEX time-domain signal can be separated into three signal components, the in vivo MTC signal (green line), the paraCEST agent (red line), and free water (black line). An in vivo PTR map of the paraCEST agent in the kidney is shown in Fig. 5e, illustrating high PTR signal (2.6% and 2.7% for both left and right kidney, respectively, especially in the region of renal pelvis. The exchange rates provided by time-domain fitting were $14.3 \times 10^3 \text{ s}^{-1}$ and $15.5 \times 10^3 \text{ s}^{-1}$ for bladder and kidneys, respectively. The 95 percent confidence intervals were $11\text{--}17 \times 10^3 \text{ s}^{-1}$ for bladder and $8\text{--}23 \times 10^3 \text{ s}^{-1}$ for the kidneys.

Discussion

Development of a low power in vivo imaging method for detection of paraCEST agents is important because of the increasing potential for designing responsive molecular imaging agents (3–9,20–23). A main obstacle to in vivo paraCEST applications is interference from strong asymmetric MTC contributions that arise when a high B_1 field is applied for saturating rapidly exchanging paraCEST protons. Currently, there are several efforts ongoing to separate out this contribution, including the use of multi-pool Bloch equation fitting, Lorentzian-line-fit analysis of the CEST spectrum (24), and two-frequency RF irradiation (25). In this study, we implemented the recently developed excitation-based FLEX sequence (10,15–16) on a preclinical MRI scanner to investigate whether FLEX-MRI

provides an alternative way to image paraCEST agents in vivo. The results show that by exploiting the combined advantages of fast labeling by selective pulsed excitation and separating out different exchanging components such as paraCEST agents and semi-solid bound water protons by time-domain analysis, it was not needed to perform asymmetry analysis of CEST spectrum with respect to the water resonance frequency.

Using FLEX-MRI, the paraCEST agent EuDOTA-(gly)⁻₄ could be imaged in vitro (Figs. 2 and 3) as well as in vivo (Figs. 4 and 5). Although the resonance of the agent at 50 ppm in the FLEX spectrum was still obscured by the broad MTC component (Figs. 2c and 5b) when using a direct Fourier transform of the signal, the rapidly decaying MTC signal could be identified and separated out using time-domain analysis (Figs. 2d and 5c,d). However, quantification of the FLEX signal was difficult just based on time-domain fitting. In the phantom studies, we found that PTR for two phantoms with 10 mM agent concentration differed (3.1% and 1.3% for 500 LTMs in pure and agarose-based solution). This effect became more evident with an increasing number of LTMs (Fig. 3b). The most likely explanation for this phenomenon lies in the dephasing properties of the transverse component in FLEX experiments. Rapid dephasing of this component during the evolution time before magnetization is returned to the z-axis causes additional signal loss for solute protons that is then transferred to water and appears as a saturation loss. This dephasing is faster in the less mobile gel environment leading to a larger loss. This dephased component accumulates when applying multiple LTMs. This dephasing can be seen as another type of saturation mechanism besides RF saturation and has been suggested as an additional approach to study CEST agents (10)

In addition to efficient detection of the paraCEST agent, the FLEX method also allowed measurement of exchange rates by fitting the time-domain signal for the individual components. The fitted rate was found to be similar for the paraCEST agent in multiple environments: $16.5 \times 10^3 \text{ s}^{-1}$ for pure solution, $16.8 \times 10^3 \text{ s}^{-1}$ for agarose mixture, $14.3 \times 10^3 \text{ s}^{-1}$ for bladder, and $15.3 \times 10^3 \text{ s}^{-1}$ for kidney. This fitted exchange rate is closed to that reported previously in the literature for this agent ($k_{\text{ex}} \sim 19\text{--}21 \times 10^3 \text{ s}^{-1}$) measured at the same temperature of 37°C (16,26). Notice that the water exchange is not pH dependent, so that differences in pH in the solution, agar, bladder, and kidney do not affect the comparison of the exchange rate measurements.

No initial rapidly decaying signal associated with the MTC component was found in the FLEX time-domain signal for the bladder (Fig. 4). We attribute this to the largely aqueous content of the bladder and relatively low endogenous macromolecular proton concentration. To demonstrate the capability of FLEX-MRI for separating out in vivo MTC effects, we applied FLEX imaging in the kidney (Fig. 5). However, clearance of paraCEST agent from kidneys was quite fast, reaching a maximum concentration in the organ within 10 mins followed by rapid wash out (27–28). To prevent the paraCEST agent from being washed out, both ureters were ligated. FLEX images acquired this way showed that the paraCEST agent could be detected in the kidney regions and that the in vivo MTC signals could be separated out using time-domain analysis (Fig. 5). When comparing the bladder and kidney, the bladder PTR image was homogenous, reflecting the equal distribution of paraCEST agent in this liquid storage area. The intensity reduces at the edges due to partial volume effects with other tissues. However, strong heterogeneity in the PTR map of the kidney was observed. We interpret the bright area to be the top of the ureters centered inside the kidney, now filled up with agent, because of the ligation outside the kidney (29). The other areas in this organ contain a lot of tissue in addition to some free liquid and therefore show much reduced paraCEST signal. Notice that the B_1 field is expected to be homogeneous over these small areas and not expected to contribute to the PTR inhomogeneity. The PTR images in bladder and kidney reflect the FLEX signal intensity at 50 ppm, and there is no signal

expected outside these organs. However, several bright spots are visible in the image in regions outside the organs, but these are random and interpreted as noise. The noise is higher inside the body (Fig. 5) indicating some physiological origin for misfitting of the time domain signal, most likely motion.

Despite having many advantages such as the possibilities for exchange rate filtering and separating out the MTC effect by time domain analysis, the FLEX-MRI approach also has some issues. While there are no theoretical limits on the length of t_{exch} , there are preparation time length considerations because the length of T_1 reduces the number of useful LTMs (Eq. [2]). In addition, when choosing the duration of the FLEX labeling pulses for *in vivo* experiments, one important consideration is the amount of MTC induced by the FLEX pulses. Shorter excitation pulses require larger amplitudes, which increase the MTC. Even though FLEX time domain analysis can separate out the components from the CEST agent and MTC, MTC-based dephasing causes a reduction in the bulk water signal, which reduces the attainable CNR. The shortening of the FLEX labeling pulses can also be limited by the scanner hardware and the use of multiple LTMs may produce SAR problems for high power pulses and hamper translation to human clinical studies. However, the SAR for FLEX can easily be reduced by lengthening the labeling pulse duration ($SAR \propto B_1^2 t_{label}$) if exchange loss during excitation is not a big factor. This would be favorable for more slowly exchanging protons such as those in many diaCEST agents based on amide and many amine protons, for which exchange losses would be minimal even with pulse duration on the order of a millisecond. In addition, a more favorable paraCEST agent having a longer life time may be designed. Second, similar to any other MRI technique, spatial B_1 inhomogeneity complicates PTR quantification in the FLEX method. However, it can be reduced substantially by using multichannel transmit hardware (30–31) that is now becoming available. Finally, frequency shifting caused by variation of the B_0 field over the subject could affect modulation of the FLEX signal. However, while this may be a concern when studying diaCEST agents with proton resonances close to water, this is less of a concern for the well-separated paraCEST agents. If needed, similar to the CEST method, errors caused by frequency shift in FLEX method can also be corrected by acquiring a B_0 reference map.

Conclusion

The first *in vivo* images of a paraCEST agent collected using FLEX-MRI techniques have been demonstrated. The FLEX approach allowed separation of the endogenous tissue MTC signal from the CEST exchange signal in the time-domain and also provided a quantitative exchange rate for the agent.

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Figure 1.

a: FLEX pulse sequence. FLEX preparation consist a series of n label-transfer modules (LTMs) in which exchangeable solute protons are frequency labeled during evolution time, t_{evol} and subsequently transferred to water during exchange period, t_{exch} . **b:** Simulated excitation profile for two rectangular FLEX pulses. The offset frequency ($o1 = 88.3$ ppm here) of the labeling RF pulses is selected based on the combined requirement of minimal water excitation (0 ppm) and keeping sufficient labeling efficiency for the paraCEST protons (50 ppm).

Figure 2. FLEX time-domain signals (500 LTMs) and FLEX spectra of three phantoms (top: 4% agarose; lower left: pure solution (10 mM EuDOTA-(gly)⁻₄); lower right: 10 mM EuDOTA-(gly)⁻₄ mixed with 4% agarose) at 37°C

Experimental data and fitted FLEX time-domain signals as a function of evolution time (left) and corresponding FLEX spectra (right) in (a) a 4% agarose phantom, (b) a solution of 10 mM EuDOTA-(gly)⁻₄ in tris buffer, and (c) 10 mM EuDOTA-(gly)⁻₄ mixed with 4% agarose in tris buffer. (d) Details of time-domain analysis (left) and spectral components (right) of the FLEX signal for (c) showing three signal components (paraCEST agent bound water, red line; free water, black line; semi-solid bound water, green line). The black dashed line shows the base-line correction. (e) Residuals from the fits in (d).

Figure 3. Quantitative analysis of the FLEX-MRI data for the three phantoms in Fig. 2
 (a) PTR maps for 500 LTMs generated from pixel-by-pixel time-domain fitting show that paraCEST signal can be detected for the pure agent solution (lower left) (3.1%) and combined phantoms (lower right) (1.3%) but not in the agarose-only sample (top). (b) Graph of PTR as a function of the number of LTMs for the agent in solution (red line) and in agarose (blue line). (c) Exchange rate maps obtained by fitting the FLEX time-domain signal under the assumption of a negligible contribution of T_2^* gives exchange rates of $16.5 \times 10^3 \text{ s}^{-1}$ and $16.8 \times 10^3 \text{ s}^{-1}$, resp.

Figure 4. In vivo FLEX-MRI of a mouse bladder after i.v. injection of paraCEST agent (0.25 mmol/kg) via the tail vein

(a) FLEX signal decay (900 LTMs) measured in the bladder ROI indicated in the anatomical image. (b) FLEX spectrum generated by Fourier transform of (a), showing a paraCEST peak at 50 ppm. (c) Details of time-domain analysis and (d) spectral components of the FLEX signal showing two components (paraCEST agent bound water, red line; free water, black line). The black dashed line shows the base-line correction. (e) Pixel-by-pixel in vivo paraCEST map showing that the paraCEST bound water was observed primarily in the bladder region.

Figure 5. In vivo FLEX-MRI of mouse kidneys after an injection of paraCEST agent (0.25 mmol/Kg) via the tail vein

(a) FLEX signal decay (700 LTMs) within a region of the left kidney. (b) FLEX spectrum generated by Fourier transform of (a) shows the paraCEST peak at 50 ppm convolved with a broad MTC profile. (c) Details of time-domain analysis and (d) spectral components of the FLEX signal showing paraCEST agent bound water (red line), free water (black line) and semi-solid bound water (green line). The black dashed line shows the base-line correction. (e) Pixel-by-pixel in vivo paraCEST map shows that high PTR level of the paraCEST agent was observed in the kidney regions.