# Pulse sequences for measuring exchange rates between proton species: from unlocalised NMR spectroscopy to chemical exchange saturation transfer imaging

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## Abstract

Within the field of NMR spectroscopy, the study of chemical exchange processes through saturation transfer techniques has a long history. In the context of MRI, chemical exchange techniques have been adapted to increase the sensitivity of imaging to small fractions of exchangeable protons, including the labile protons of amines, amides and hydroxyls. The MR contrast is generated by frequency-selective irradiation of the labile protons, which results in a reduction of the water signal associated with transfer of the labile protons' saturated magnetization to the protons of the surrounding free water. The signal intensity depends on the rate of chemical exchange and the concentration of labile protons as well as on the properties of the irradiation field. This methodology is referred to as CEST (chemical exchange saturation transfer) imaging. Applications of CEST include imaging of molecules with short transverse relaxation times and mapping of physiological parameters such as pH, temperature, buffer concentration and chemical composition due to the dependency of this chemical exchange effect on all these parameters. This article aims to describe these effects both theoretically and experimentally. In depth analysis and mathematical modelling are provided for all pulse sequences designed to date to measure the chemical exchange rate. Importantly, it has become clear that the background signal from semisolid protons and the presence of the Nuclear Overhauser Effect (NOE), either through direct dipole-dipole mechanisms or through exchange-relayed signals, complicates the analysis of CEST effects. Therefore, advanced methods to suppress these confounding factors have been developed, and these are also reviewed. Finally, the experimental work conducted both *in vitro* and *in vivo* is discussed and the progress of CEST imaging towards clinical practice is presented.

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## Introduction

In this review, we begin with an overview of early saturation transfer experiments and an explanation of the basic mechanisms underlying chemical exchange saturation transfer (CEST) contrast. Chemical exchange is one pathway through which magnetization is transferred in tissues. However, there are several other confounding effects present in vivo that need to be accounted for in order to quantify chemical exchange phenomena in vivo. These effects, which generate the so-called magnetization transfer (MT) contrast, are discussed in a separate section (section 5). We also present a mathematical description and modelling of the CEST effect , followed by an extensive review of pulse sequences used to measure the chemical exchange rate. We separate these techniques into four categories, namely, sequences and techniques to directly assess exchange rates, measurements of exchange rates based on solutions of the Bloch-McConnell equations, measurements of exchange rates through its interactions with relaxation mechanisms, and finally pulse sequences that improve exchange rate sensitivity by suppressing the other magnetization transfer mechanisms which compete with the CEST effect *in vivo*. For each method, the general concept is presented followed by the pulse sequence and applications. The advantages and disadvantages of each technique are discussed for providing guidance when applying each method, and the section on each method is written in a self-contained fashion, avoiding requirements for further background.

## 1. Early saturation transfer experiments

The sensitivity of the NMR spectrum to chemical exchange was reported in the 1950s though at that time studies were confined to small molecules. In the ensuing years, developments in NMR methodology and in particular the discovery of two-dimensional (2D) NMR enabled significant advancements in the field. In a landmark paper Forsen and Hoffman developed the theory for the method known as saturation-transfer to measure the rate of proton transfer of salicylaldehyde using nuclear magnetic double resonance (2). Some years later Campbell et al. described NMR Fourier transform pulse methods for measuring rates of slow exchange in valium solutions (3). *In vivo* the first analysis by means of saturation transfer was reported by Brown et al in 1977 who measured the exchange rates between inorganic phosphate and

adenosine triphosphate (ATP) catalyzed by the dicyclohexylcarbodi-imide-sensitive ATPase of *Escherichia coli (4)*. Other applications of inversion or saturation transfer techniques used in $^{31}$P NMR spectroscopy included the measurement of the activity of creatine kinase in frog and rabbit skeletal muscle (5) (7). Following this work Balaban and co-workers applied a similar methodology to proton-proton transfer and demonstrated that exchange between labile protons at high concentrations of exchangeable molecules such as urea and ammonia in water offers the possibility of increasing the sensitivity of MRI detection of these molecules (see Figure 1) (8). However, it was more difficult to make observations of this type in tissues. Indeed, this newly-detected effect was found to be very small in vivo in comparison with the conventional MT contrast (MTC) because of the larger number of semisolid structures affected by the off-resonance irradiation (9). For this reason, it took the same group nearly a decade to come up with the first study on imaging of the distribution of urea and other molecules in the kidney (10). Two years later, they published the first paper introducing a brand new type of contrast agent to be used for MRI based on chemical exchange of all exchangeable species possible, and coined the acronym CEST (6).

CEST effects involve chemical exchange between a small pool of nuclei that is being irradiated and a large pool being detected (see Figure 1). If we only consider water-related exchange processes then water represents the large pool and low-concentration mobile solutes are the sources of exchangeable protons. Importantly, the CEST technique offers a sensitivity enhancement mechanism in which continuous irradiation of the small pool causes cumulative saturation of the water; as a result of chemical exchange, the saturated protons of the small pool are continuously replaced by non-saturated water protons which in turn can be saturated and exchanged. This provides an amplification process for the small solute signal, for it gives rise to a substantial decrease in the water signal (11), (12). Finally, CEST mechanisms can be distinguished from conventional MTC based on the size and mobility of the molecules involved and the widths and frequency offsets of their signals, which are typically closer to the water resonance (see sections 4,5,6).

Early *in vivo* CEST experiments included imaging of endogenous mobile proteins and peptides based on the properties of amide protons. Zhou et al were the first to demonstrate in preclinical settings that it was possible to achieve a significant increase in sensitivity for detection of amide protons at mM concentration by detection of a few percent change in the water signal of rat brain following infarction (13). The data collected by Zhou and co-

workers show the presence of amide proton exchange *in vivo* and *post-mortem*. In these initial experiments, a long frequency-selective saturation pulse of 4s centred at 3.5ppm from the water peak was used to reach maximum labelling efficiency of amide protons. In addition, the researchers realised that amide-weighted contrast depends on several MR and tissue related parameters such as exchangeable proton concentration, exchange rate, water content and $T_1$. However, the effect of amide proton transfer exceeds these contributing factors, and a decrease of the amide proton-related CEST signal, dubbed Amide Proton Transfer (APT) signal by 1-3% during ischaemia was attributed to the difference in the exchange rate of the amide protons due to a drop in pH. A further publication reported an increase in APT signal by 3-4% in brain tumours associated with elevated amide proton content (11).

**<<< Insert Figure 1 here >>>**

In addition to the emergence of this new native contrast, several dedicated contrast agents started to be developed conjointly. This started with the accidental discovery of a separate proton resonance in the DOTA-tetra(amide) derivative, 1,4,7,10-tetraazacyclododecane tetrakis (ethyl-acetamidoacetate), in which the $Eu^{3+}$ complex shifted the amide resonance 50 ppm away from the water peak, as assessed by in high-resolution NMR, which gave birth to the new family of paramagnetic CEST contrast agents (14). The so-called paraCEST agents act as negative contrast reagents by reducing the water signal intensity under frequency-selective irradiation of the exchangeable groups in a similar manner to the endogenous pools of exchangeable protons found in proteins or metabolites (later termed diaCEST agents) (15). However, in the case of paraCEST agents the chemical shift difference with water is generally much larger ($\Delta\omega \geq 10$ ppm) and therefore faster exchange rates can be detected using stronger irradiation pulses without affecting the water signal through direct saturation (see section 1.7). In a theoretical study, simulated data of a two site exchange system based on the Bloch-McConnell model, which is currently used to describe chemical exchange processes (see mathematical description of CEST section**Error! Reference source not found.**), show that the CEST effect of these agents is maximised when the amplitude of the off-resonance saturation pulses matches the exchange rate of the labile protons with water (16)(7).

Early classifications of CEST contrast agents have been made in terms of molecular size or type of protons involved e.g. glucoCEST for glucose exchange (17), glycoCEST for glycogen (18) etc. A useful and technically more correct classification is based on the exchange mechanism involved and on the different exchange pathways that produce CEST contrast: atom (proton) exchange, molecular exchange and compartmental exchange (19) as shown in Figure 2. This review paper will use the proton exchange terminology, because it dominates the CEST field.

**<<< Insert Figure 2 here >>>**

## 2. Motional timescales of relaxation-inducing mechanisms

For most MRI applications, the basic contrast originates from the relaxation processes of water molecules, and the theoretical model of Bloembergen, Purcell and Pound (BPP) which was published in 1948 remains the primary reference on relaxation effects relevant to studies of tissues (1). In this section, we briefly discuss the dynamics of a spin system in the absence of off-resonance RF irradiation, which is mainly governed by longitudinal and transverse relaxation. We do this to present the complexity underlying the physical mechanisms responsible for $T_1$ and $T_2$ relaxation in biological tissues and to discuss how these effects can influence a CEST experiment. Following this, in section 1.7 we demonstrate that in the presence of RF pulses, an effective magnetic field $B_{eff}$ is formed which captures dynamic processes with rates close to the precession frequency $\omega_{eff}$ about $B_{eff}$ through different magnetization transfer pathways mediated either via chemical or dipolar interaction. In this case, the relaxation processes are mainly governed by the $T_1\ \rho$ time constant.

To detect an MR signal, the spin system is excited by the application of an RF pulse, which generates transverse magnetization and reduces the longitudinal magnetization Mz by tilting it into a plane perpendicular to $B_0$. The longitudinal component Mz then relaxes exponentially back towards thermal equilibrium with a time constant $T_1$. This relaxation is mediated by random molecular motions that produce local fluctuating magnetic fields. At the most basic level, $T_1$ relaxation is an energy flow between spins and their external environment (i.e. nearby nuclei, atoms and molecules).

Mathematically, a correlation function $g(\tau_c)$ is used to describe the temporal dynamics of a spin system approaching its equilibrium due to random processes. For instance, in the case of isotropic molecular tumbling, $g(\tau_c)$ is assumed to be exponential, and the correlation time $\tau_c$ is defined as the time needed for the molecule to rotate by 1 radian about any axis; this depends on the viscosity of the solvent, the structure and size of the molecule and the temperature T. $\tau_c$ can be approximated as $10^{-12}$ $W_M$ where $W_M$ is the molecular mass in Daltons. The frequency distribution of the motion of a randomly tumbling molecule $J(\omega)$ is described by the spectral density function which models how the energy available from the lattice induces relaxation as a function of molecular tumbling rate, and can be represented mathematically by the Fourier transform of $g(\tau_c)$. It can be shown that fluctuations at the resonance frequency $\omega_0$ provide the most efficient contributions to the relaxation rate $1/T_1$.

After the application of an RF pulse, spins precess at different frequencies because they experience slightly different local magnetic fields. The distribution of frequencies results in a loss of phase coherence between the spins, leading to a random phase distribution and to the disappearance of the detectable signal. The decay of the transverse component $M_{xy}$ of the magnetization is known as $T_2$ (spin-spin) relaxation. $T_2$ originates from spin dephasing caused by the interactions between spins such as intramolecular (i.e. spins residing on the same molecule) and intermolecular (i.e. spins residing on different molecules) dipolar interactions. These interactions primarily involve energy exchange between the spins (secular part) with only limited energy transfer to the surrounding lattice (non-secular part). Other sources of spin interactions include diffusion through local field gradients and proton exchange. In the case of diffusion or exchange, the protons change their location and thereby experience different local fields, leading to dephasing and enhanced relaxation. Spin-spin relaxation causes a broadening of the resonance linewidths. In a solution, motion of the molecules tends to average the interactions between spins, leading to a single narrow line. Thus, as motion increases the linewidths decrease.

In summary, fluctuating magnetic fields are responsible for both spin-spin and spin-lattice relaxation. However, the contributions to the two types of relaxation depend on the time-scales of these fluctuations. In particular, slow movements contribute only to spin-spin relaxation, while components of motion at the Larmor frequency contribute to both types of relaxation.

Biological tissues are complex with internal microstructures containing water and larger molecules distributed nonuniformly and within compartments. As such, during the course of an NMR experiment there are many different physical processes that cause relaxation (see Figure 3).

Chemical exchange in tissues requires more complicated models including multiple molecular environments with different exchange rates between compartments. In addition, chemical exchange mechanisms result in a field-dependent shortening of both $T_1$ and $T_2$, with typically a much larger effect on $T_2$. Importantly, exchange phenomena occurring with time frames much longer than $T_1$ do not significantly affect spectral linewidths or the measured MR signal.

**<<< Insert Figure 3 here >>>**

## 3. Chemical shift dispersion of exchange-mediated phenomena and current applications

The chemical shift enables the discrimination of NMR signals between different molecules and individual atoms within a molecule. It is used in conjunction with peak intensities for providing substantial information about molecular structure. It originates from a magnetic shielding field produced by electronic currents in atoms and molecules, which is additive to the large magnetic field of strength $B_0$ in NMR experiments. Because the secondary shielding fields are weaker than the main magnetic field $B_0$ the frequency spread is also small. The chemical shift is defined as the separation of resonance frequencies, expressed in parts per million from a chosen reference frequency. In $^1$H NMR, peak positions are conventionally measured relative to tetramethylsilane (TMS) and the water resonance at 293K is 4.75 ppm. However, in a CEST imaging experiment the water frequency is usually set at 0 ppm and the frequency offsets of the exchangeable protons groups are referred relative to it. Table 1 shows the three main groups of endogenous exchange contrast and highlights important current applications.

Pulse sequences for measuring exchange rates between proton species

### 3.1. Amide protons

A segment of a peptide chain contains many groups that contain exchangeable protons: –OH (hydroxyl), amines (in which nitrogen is bonded to an aliphatic carbon, e.g. R-$NH_2$), amides (in which nitrogen is bonded to a carbonyl group, e.g. CO-$NH_2$) and many aliphatic protons. However, the functional group that has been studied the most in biomedical applications is the amide proton. Amides are component of the 'backbone' in proteins, side chains and small peptides, and the proton exchange of this group $(\Delta_\omega \approx k_{sw})$ usually falls within the slow-to-intermediate regime.

Recent studies have explored the Amide Proton Transfer (APT) effect in animal models with ischaemia and in human patients with stroke (24),(25). Further applications of APT include investigations of multiple sclerosis (26), Huntington's disease (27) and breast cancer (28). Another important application of APT is in the imaging of tumours, with a recent study from Zhou et al. suggesting that APT could be used for differentiation between glioma and radiation necrosis (29).

### 3.2 Hydroxyl protons

Hydroxyl protons are another abundant group of exchangeable protons. In general, OH protons resonate at a frequency 0.5–3.0 ppm downfield from water and have exchange rates in the range of 700–15,000 Hz (30). The majority of OH protons have fast exchange rates that do not satisfy the slow-to-intermediate exchange condition, particularly at low magnetic field strength, and therefore cannot be detected. Applications of hydroxyl CEST imaging include: glycosaminoglycan (GAG) CEST (31), glycogen CEST (glycoCEST) (18), glucose CEST (glucoCEST) (17)(32) and myo-inositol CEST (MICEST) (33).

The capability to detect –OH protons has clinical utility in disorders affecting glucose metabolism, such as tumours, or in neurodegenerative diseases such as Alzheimer's disease (AD), which show a proliferation of glial cells (45).

### 3.3 Amine protons

Another important source of endogenous CEST contrast is the amine proton group. Amine protons originate from the amino acid side-chains and resonate 2–3 ppm downfield from the water with exchange rates in the range of 150–1000 Hz (34),(35).. Glutamate and creatine are

examples of endogenous metabolites that possess amine protons suitable for CEST imaging (34),(36). Other amine protons can be found in neurotransmitters such as dopamine, epinephrine and GABA.

Amine protons exhibit a concentration- and pH-dependent CEST effect (36). Elevated CEST contrast at the glutamate resonance frequency (detectable at 3.0ppm) was observed in a rat brain following middle cerebral arterial occlusion (MCAO), possibly due to a decrease in pH (35). Moreover, glutamate imaging (known as GluCEST) was used to investigate changes in amine exchange in an animal model of AD (37) and also in spinal cord studies (38). Amines have fast exchange rates, thus requiring high field imaging to be able to detect them properly (see Section 6).

**<<< Insert Table 1 here >>>**

## 4. Basic principles of CEST imaging

*In vivo* CEST MRI is mostly based on detection of changes in the water signal. Indirect imaging of protons of small molecules as described in the previous sections is carried out by saturating these protons and detecting the transfer of their (reduced) magnetization to the water signal. In general, a saturation transfer experiment is divided into two parts: 1) a saturation part using selective RF pulses at the resonance frequency of the exchangeable species of interest; and 2) a read-out part for the acquisition of the water magnetization. To achieve CEST contrast, the spin population must remain in a saturation state for long enough to ensure sufficient magnetization exchange with water. Typically, the duration of the saturation is in the order of seconds and often represents the longest part of a CEST experiment. Due to the variety of exchange rates and range of chemical shifts relative to the water resonance, it is necessary to optimise the irradiation amplitude and duration for maximum saturation efficiency.

Apart from the saturation scheme the read-out also impacts the CEST contrast. In principle, the signal is acquired rapidly after saturation to avoid relaxation of the labelled exchangeable protons during long acquisitions of multiple k-lines acquired after individual preparation modules. There are two ways to speed up the CEST acquisition, according to Liu et al. (19).

The first and most popular way is to use a train of RF pulses followed by a fast read-out such as Echo Planar Imaging (EPI) or fast Gradient Echo (GE). A second approach is to use a 'pulse-acquire' method, which uses saturation pulses prior to each phase encoding step. With this approach, a steady state can be achieved when the centre of K-space is reached; however the acquisition time is generally longer than with the first approach.

CEST effects are visualized as the normalised water signal amplitude as a function of irradiation frequency offsets defining the so-called *Z* or CEST spectrum. Generally, these spectra reflect the loss in longitudinal magnetization of the water protons upon saturation at a frequency offset which corresponds to the resonance of a CEST agent. Characteristic *in vivo* features of *Z*-spectra are the direct water saturation around the water resonance (i.e. 0 ppm), amide proton exchange at 3.5 ppm and NOE-mediated signal at a range between –2.0 and –5.0 ppm (see Figure 4). Isolation of the CEST effect is done by acquiring three images: one (the label image) is acquired with selective irradiation at the offset frequency of interest (i.e. solute frequency), the second is a control image acquired with selective irradiation at the exact opposite frequency (relative to the water signal) and the third image is used for normalisation. This is either an image where no saturation pulses are applied or an image where saturation pulses are applied very far from the water resonance. The CEST effect is calculated as the difference between the normalised label and control images.

**<<< Insert Figure 4 here >>>**

## 5. Magnetization transfer pathways in tissues

Besides proteins in solution, human tissue contains many other hydrogen nuclei-rich molecules forming semi-solid or solid structures. While the NMR proton signal from these molecules is not directly measurable by MRI (hydrogen nuclei belonging to such semi-solid structures usually have $T_2$ relaxation rates of ~10μs), indirect detection can be achieved through the so-called Magnetization Transfer (MT) effect. The basic principles behind MT measurements are very similar to those for measuring CEST effects. The basic pulse sequence is the same, composed of a frequency-selective pulse applied off-resonance from the water peak, and measuring the reduction in signal on the water peak elicited through exchange processes between both proton species. Importantly, due to the very short $T_2$ decay

of these protons, the width of the Z spectrum coming from this semi-solid pool of membrane-bound and other macromolecules is very large, with an overall width determined by $1/T_2$, and thus about 100-200kHz broad. Its shape has been determined *in vivo* to be best represented by a Super-Lorentzian (21), and to be centered around 2.34ppm upfield from the water peak (22).

Various pathways exist through which MT effects take place in tissues, and, unlike CEST effects, these combine dipolar coupling with chemical exchange. For example, the semi-solid proton pool can be saturated in part via intramolecular dipolar transfer, which can be followed by intermolecular transfer via smaller interacting molecules. Thus, magnetization can be transferred to water molecules trapped very closely to biological membranes through intermolecular NOEs, due to the spatial proximity of the water protons to the saturated membrane-bound ones. In addition, intramolecular NOEs enable magnetization to be transferred through exchange-relayed pathways, e.g. fast exchanging protons such as OH or amines, which are therefore efficiently saturated during the MT preparation pulse, and can transfer their magnetization to neighbouring spins via cross-relaxation. Alternatively, intramolecular NOEs may also take place through direct dipolar relaxation between saturated protons from peptides and proteins. The relative contributions of each pathway depend on both the RF irradiation and on the molecular properties of the individual membrane-bound or other macromolecules, such as their molecular mobility and conformation, as well as dipolar transfer efficiency and water accessibility.

## 6. More details on chemical exchange mechanisms

Chemical exchange can affect resonance frequencies, intensities and linewidths in NMR spectra. For example, if two protons in different sites and with different chemical shifts exchange with each other, then as the exchange rate increases, the two signals will first broaden and start to merge and eventually sharpen in the form of one single peak (Figure 5). Experimental methods aimed at direct quantitative measurement of chemical exchange include the quantification of interconversion kinetics or the quantification of structural features of populated conformational states in equilibrium. Applications of such methods in protein NMR include studies of folding, molecular recognition, catalysis and allostery by proteins and nucleic acids (23).

Chemical exchange between labile groups and water can be categorised as slow, intermediate or fast (see Figure 5). For instance, slow water-solute exchange is characteristic for amide protons of peptides and proteins, while fast exchange occurs in all other labile proton groups, including hydroxyl, amine and imino groups. According to the theory, for a substantial CEST contrast to take place, the exchange rate of labile protons with those of the water must be slow enough for the CEST agent and the water to give two separate signals (i.e. $k_{sw} <= \Delta\omega$), but at the same time it must be fast enough so that a significant magnetization flux between them can be measured (i.e. $k_{sw} >= (1/T_1)$). In other words, the system should be in the slow-to-intermediate exchange regime (16). It is worth noting that these conditions depend on the main magnetic field strength, which will mainly determine the frequency separation $\Delta\omega$. Therefore, high field imaging favours fast exchangeable species because of its higher spectral separation between exchangeable proton frequencies. Furthermore, $T_1$ increases with field strength, which enables the build-up of a larger CEST effect with time.

**<<< Insert Figure 5 here >>>**

## 7. Mathematical description of CEST: Bloch- McConnell equations

Figure 6 shows a simplified two spin pool system connected via Chemical Exchange (CE). The small pool represents exchangeable protons (s) in soluble proteins and metabolites and the large pool consists of bulk water (w). Note that here, we consider exchangeable protons with long T2 values, rather than the short T2 species leading to conventional magnetization transfer effects, that are associated with larger, more immobile macromolecules (9). Each of these pools has its own spin–lattice ($R_1$) and spin–spin relaxation rates ($R_2$). $M_{0w}$ and $M_{0s}$ are the equilibrium states of magnetization of pool w and pool s, respectively.

**<<< Insert Figure 6 here >>>**

When an RF pulse ($B_1$) is applied along the *x*-axis in the rotating frame of reference, the Bloch-McConnell (BM) equations for a two-pool exchange model are written as follows:

Pulse sequences for measuring exchange rates between proton species

$$\frac{dM_{xs}}{dt} = -\Delta\omega_s M_{ys} - R_{2s} M_{xs} - k_{sw} M_{xs} + k_{ws} M_{xw} \quad \text{(Equation 1)}$$

$$\frac{dM_{ys}}{dt} = \Delta\omega_s M_{xs} + \omega_1 M_{zs} - R_{2s} M_{ys} - k_{sw} M_{ys} + k_{ws} M_{yw} \quad \text{(Equation 2)}$$

$$\frac{dM_{zs}}{dt} = -\omega_1 M_{ys} - R_{1s}(M_{zs} - M_{0s}) - k_{sw} M_{zs} + k_{ws} M_{zw} \quad \text{(Equation 3)}$$

$$\frac{dM_{xw}}{dt} = \Delta\omega_w M_{yw} - R_{2w} M_{xw} + k_{sw} M_{xs} - k_{ws} M_{xw} \quad \text{(Equation 4)}$$

$$\frac{dM_{yw}}{dt} = \Delta\omega_w M_{xw} + \omega_1 M_{zw} - R_{2w} M_{yw} + k_{sw} M_{ys} - k_{ws} M_{yw} \quad \text{(Equation 5)}$$

$$\frac{dM_{zw}}{dt} = -\omega_1 M_{yw} - R_{1w}(M_{zw} - M_{0w}) + k_{sw} M_{zs} - k_{ws} M_{zw} \quad \text{(Equation 6)}$$

where $\omega_0 = \gamma B_0$ and $\omega_1 = \gamma B_1$ ($\gamma$ is the gyromagnetic ratio, $B_0$ is the main magnetic field strength and $B_1$ is the applied RF field on the *x*-axis), $\Delta\omega_s = \omega_s - \omega_0$ and $\Delta\omega_w = \omega_w - \omega_0$ with $\Delta\omega_s$ and $\Delta\omega_w$ representing the chemical shift differences between the offset frequency of the RF pulse and the solute or water resonance frequency, respectively. Exchange occurs between the two pools at a forward ($k_{sw}$) and backward rate constant ($k_{ws}$) according to the equilibrium condition: $k_{sw} M_{0s} = k_{ws} M_{0w}$. This system of equations can easily be expanded to add more solute pools and magnetization transfer with water.

The proton transfer ratio (PTR) can be defined as the change in water amplitude due to the CEST effect, and is usually measured and expressed as a percentage of reduction in the water signal following application of a selective RF pulse of duration $t_{sat}$.. PTR can be expressed by an analytical approximation of the two-site Bloch–McConnell equations (i.e. water and exchangeable protons), under the assumption that RF irradiation is applied at the resonant frequency of the exchangeable protons while the water resonance is assumed to be unaffected by the RF pulse (41),(42).This approximation is given by:

$$PTR = \left(1 - \frac{M_{zw}(t_{sat})}{M_0}\right) = x_s . \alpha . k_{sw} . T_{1w} \left(1 - e^{-tsat/T_{1w}}\right) \quad \text{(Equation 7)}$$

$$x_s = \frac{[exchangeable\ protons]}{[water\ protons]} = \frac{k_{ws}}{k_{sw}} \quad \text{(Equation 8)}$$

where $x_s$ is the fractional concentration of the exchangeable protons, $\alpha$ is the saturation efficiency, $k_{sw}$ is the forward exchange rate constant in $s^{-1}$ and $T_{1w}$ is the longitudinal relaxation time of water in seconds. The saturation efficiency α is given by:

$$\alpha \approx \frac{(\gamma B_1)^2}{(k_{sw})^2+(\gamma B_1)^2}, \qquad \text{(Equation 9)}$$

where $B_1$ is the applied irradiation field in μT and $\gamma$ is the gyromagnetic ratio in MHz/T. The maximum PTR is obtained when $\alpha$ is equal to one. For amide protons with exchange rate constant ~ 30 $s^{-1}$, a $B_1$ of 1 μT is sufficient to saturate this spin system; however, faster exchangeable protons require higher $B_1$ to achieve full saturation. Under slow exchange conditions (i.e. $k_{sw} \ll \Delta\omega$ and assuming that each resonance can be resolved separately) PTR increases with increasing exchange rate constant $k_{sw}$ until it reaches a plateau. Figure 7 displays the dependence of the saturation efficiency on the exchange rate.

In the case of molecular or compartmental exchange, Equation 7 is adjusted and the proton transfer enhancement (PTE) is given as the number of exchangeable protons ($N_E$) per molecule or contrast agent (CA) (11) as follows:

$$PTE = N_E \,.\, \alpha \,.\, k_{SW} \,.\, T_{1w} \,(1 - e^{-tsat/T1w}) \qquad \text{(Equation 10)}$$

*For proton exchange:* $N_E = N_{CA} \,.\, M_{CA}$, (Equation 11)

where $N_{CA}$ is the number of exchangeable protons per molecule, $M_{CA}$ is the molecular weight of the substance in kDa.

*For molecular exchange:* $N_E = C_m \,.\, N_m . M_{CA}$, (Equation 12)
where $C_m$ is the number of molecules, $N_m$ is the number of protons in each molecule.

*For compartmental exchange:* $N_E = 2 \,.\, N_A \,.\, [\mathrm{H_2O}] . V_{comp}$ (Equation 13)
where $N_A$ is the Avogadro constant, $[\mathrm{H_2O}]$ is the the water concentration (110M), $V_{comp}$ is the compartment volume (litres).

Finally, the PTE is related to the PTR by the following equation:

$$PTR = \frac{[\mathrm{solute}].PTE}{2[\mathrm{H_2O}]} \qquad \text{(Equation 14)}$$

PTR is used to measure the chemical exchange rate of a CEST agent as a function of saturation time or saturation power (see Equations 7 and 9 and section 9). To account for the experimentally observed direct water saturation, PTR is replaced by the asymmetry between the two sides of the Z spectrum, which *in vivo* includes all magnetization transfer effects, and has been therefore named, somewhat confusingly, as the asymmetry in magnetization transfer ratio, or MTRasym.

Note that it can be shown that accurate measurements of the CEST effect require refined equations in the case of weak labelling ($\gamma B_1 < k_{sw},\ \alpha < 0.5$) as well as non-equilibrium initial magnetization conditions. In such cases, PTR can be replaced with Equation 15 (43).

$$\mathrm{PTR} \approx \mathrm{MTRasym} = \frac{M_{sat}(-\Delta\omega)}{M_0} - \frac{M_{sat}(+\Delta\omega)}{M_0} = \frac{x_s\, k_{sw}.\alpha}{R_{1w} + x_s k_{sw}.\alpha} + \left(\frac{M_{init}}{M_0} - 1\right).e^{-R_{1w}t_{sat}} - \left(\frac{M_{init}}{M_0} - \frac{R_{1\alpha}}{R_{1\alpha} + x_s k_{sw}.\alpha}\right).e^{-(R_{1w} + x_s k_{sw}.\alpha).t_{sat}}$$

(Equation 15)

where $M_{init}$ is the initial magnetization.

In the case of 'strong labelling' the saturation amplitude is larger than the exchange rate constant ($\gamma B_1 > k_{sw}$) and the magnetization of the CEST pool is close to zero during irradiation. In that case, the CEST effect is maximised and the labelling efficiency $\alpha$ approaches 1. Note that when 'strong labelling' is achieved and $M_{init} = M_0$ Equation 15 is equivalent to Equation 7.

**<<< Insert Figure 7 here >>>**

## 8. Spin lock (SL) pulse sequences as an alternative to CEST

### 8.1. SL pulse sequence

The spin lock (SL) sequence was first introduced by Jones in 1966 (44) and the first SL experiments were performed later by Sepponen et al. (45) and Santyr et al. (46). This technique provides an efficient way to measure another relaxation term, the spin–lattice relaxation in the rotating frame, termed $T_{1\rho}$, at lower field strengths without compromising the signal-to-noise ratio (SNR). $T_{1\rho}$ is obtained under a locking RF field (i.e. $B_1$) several

orders of magnitude smaller than the main magnetic field $B_0$, but of sufficient magnitude to ensure maintenance of the magnetization along that field. While the magnetization is locked along $B_1$, it relaxes exponentially with decay constant $T_{1\rho}$: $S(t_{sL}) = S_o e^{-(t_{sL}/T_{1\rho})}$ where $t_{sL}$ is the spin lock time and $S_o$ is the maximum signal at the minimum $t_{sL}$ (45). Figure 8 describes the pulse sequence and the geometric representation of the magnetization under a locking field $B_{eff}$.

The standard SL module consists of a 90° excitation pulse followed by a locking pulse placed on-resonance. When the SL period finishes, the magnetization is returned back to the *Z*-axis with a second 90° pulse. For this type of experiment, the locking pulses are of high amplitude, with $B_1$ values such that $T_{1\rho}$ is sensitive to molecular fluctuations with frequencies in the order of kHz. The locking RF frequency can alternatively be applied off-resonance, in which case the magnetization relaxes along the direction of the effective field $B_{eff}$ with a time constant known as $T_{1\rho off}$.

After the SL preparatory module, a rapid imaging acquisition scheme is generally used to sample the $T_{1\rho}$ dependent signals. In a similar manner to $T_1$ and $T_2$ weighted contrast, $T_{1\rho}$ weighted contrast can be established. $T_{1\rho}$ is commonly quantified for a particular SL amplitude using several durations of the locking pulse. It is also feasible to obtain $T_{1\rho}$ dispersion measures by measuring the $T_{1\rho}$ for different SL amplitudes. Figure 8 displays a pulse sequence diagram for on/off-resonance SL experiments.

**<<< Insert Figure 8 here >>>**

### 8.2. SL and chemical exchange

The SL contrast has the potential to be used as an alternative to CEST and to better characterize exchange processes falling in the intermediate to fast exchange regime through the tuning of SL pulse parameters. The theoretical model of Trott and Palmer describes the dependence of $R_{1\rho}$ ($1/T_{1\rho}$) on CE for conditions outside the fast exchange limit in the case of two unequal populations (47). Under these approximations, the relaxation decay is a single exponential and the relaxation rate constant $R_{1\rho}$ can be obtained by finding the largest real

eigenvalue $\lambda_1$ that dominates the evolution of the magnetization according to the Bloch–McConnell equations.

It has been demonstrated that CE dynamics are equally well described for both saturation transfer and off-resonance SL experiments. Solutions of the Bloch–McConnell equations lead to the same eigenvalue, which is the relaxation rate in the rotating frame $R_{1\rho}$ and to the same steady-state values for both experiments (48).

Off-resonance SL only differs in the initial orientation of the magnetization compared with CEST. In the case of a spin-lock experiment, before the magnetization is locked, it is set along the effective field $B_{eff}$ by the first RF pulse, while for CEST no initial flipping of the magnetization is performed and the magnetization stays parallel to the *Z*-axis. In other words, a CEST experiment can be described as an off-resonance SL experiment with insufficient locking at the start. While the spins are 'pseudo-locked' they precess about the effective field, whereas in a SL experiment the magnetization avoids these oscillations and remains parallel to the effective field throughout the locking time (49). Figure 9 displays the initial orientation of the magnetization for both SL and CEST approaches.

Studies comparing the CEST effect with on-resonance and off-resonance SL experiments have been applied for exchange processes in the slow, intermediate and fast regimes (50). On-resonance SL was found to be more sensitive to hydroxyl and amine protons. In contrast, off-resonance SL is identical to CEST using low-power RF irradiation and it is more sensitive to amide proton exchange.

**<<< Insert Figure 9 here >>>**

## 9. Theoretical description of CEST experiments based on SL theory

This section describes the analytical solutions of the BM equations obtained by an eigenspace approach in which the time dependent terms of the BM equations are transformed into a diagonal matrix (48). Analytical solutions of the z-magnetization in a transient state as well as in the case of pulsed experiments are provided for gaining insight into the intrinsic structure of Z-spectra and for optimal design of CEST experiments.

## 9.1. Transient vs. steady-state experiments

Depending on the timing of the irradiation pulse(s) two types of CEST experiments can be distinguished: steady-state vs. transient experiments. Generally, steady-state refers to the state in which the system does not change further and is time independent, whereas in the case of transient experiments the magnetization evolves as a function of time. Experimentally, the simplest way to achieve steady-state is to use irradiation pulses longer than $\sim 5 \cdot T_1$. However, the irradiation does not have to be continuous, nor does the steady-state condition imply complete saturation of the exchangeable proton pool. In many cases however, longer saturation pulses are preferable for the detection of slow exchange processes and for simplifying the mathematical equations used to describe the dynamics of a system undergoing chemical exchange.

Based on the equivalence of CEST experiments with spin lock theory (see previous section), the mathematical equations used to describe the CEST effect in both steady $\frac{M_{wz}^{ss}(\Delta\omega)}{M_0}$ and transient states $\frac{M_{ws}(\Delta\omega,t_{sat})}{M_0}$ can be written as follows (52):

$$\frac{M_{ws}(\Delta\omega,t_{sat})}{M_0} = \left(cos^2\theta - \frac{M_{wz}^{ss}(\Delta\omega)}{M_0}\right) \cdot e^{-R_{1\rho}(\Delta\omega)t_{sat}} + \frac{M_{wz}^{ss}(\Delta\omega)}{M_0} \qquad \text{(Equation 16)}$$

$$\frac{M_{wz}^{ss}(\Delta\omega)}{M_0} = \frac{cos^2\theta\, R_{1w}}{R_{1\rho}(\Delta\omega)} \qquad \text{(Equation 17)}$$

where $R_{1\rho}$ is the relaxation rate in the rotating frame and is defined as:

$$R_{1\rho} = R_{1w} + (R_{2w} - R_{1w}) \cdot \frac{\omega_1^2}{\omega_1^2+\Delta\omega^2} + x_s k_{sw} \cdot \frac{\omega_1^2}{\omega_1^2+ k_{sw}^2} = R_{eff} + R_{ex} \qquad \text{(Equation 18)}$$

and $cos^2\theta = \frac{\omega_1^2}{\omega_1^2+\Delta\omega^2}$

According to Equation 18 chemical exchange adds a spectrally selective relaxation pathway (termed $R_{ex} = x_s k_{sw} \cdot \frac{\omega_1^2}{\omega_1^2+ k_{sw}^2}$) to the intrinsic longitudinal and transverse relaxation of the water magnetization in the rotating frame.

## 9.2. Pulsed experiments

In many cases, due to hardware limitations or SAR restrictions researchers employ a series of pulsed CEST RF saturation pulses rather than a CW irradiation pulse, sometimes interleaved

with delays and crusher gradients (53),(39). The fundamental physics of the experiment does not change; however, the mathematical equations used to describe a pulsed CEST experiment are more complex.

There are two analytical models that can be used to describe the decay of the z-component of the magnetization, depending on whether we assume chemical exchange during the inter-pulse delay or not.

If one considers the case in which no chemical exchange is considered during recovery, that is under the assumption of $R_{1\rho}$ relaxation during the saturation pulse of duration $t_{sat}$ and $R_{1w}$ recovery during the inter-pulse delay $t_d$, the steady-state $M_{wz}^{ss}(\Delta\omega)$ is given by (54):

$$\frac{M_{wz}^{ss}(\Delta\omega)}{M_0} = \frac{R_{1w}}{\overline{R}_{1\rho}(\Delta\omega)DC + R_{1w}(1-DC)}$$, where DC is the duty cycle defined as $\frac{t_{sat}}{t_{sat} + \mathrm{td}}$

(Equation 19)

For Gaussian-shaped RF pulses $R_{1\rho}(\Delta\omega, \omega_1(t))$ is averaged to (49):

$$\overline{R_{1\rho}} = R_{1A} + (R_{2A} - R_{1A}) \cdot c_1\, x_s k_{sw} \cdot \frac{\omega_1^2}{\omega_1^2 + k_{sw}^2} + x_s k_{sw} \cdot c_1 \frac{\omega_1^2}{\omega_1^2 + k_{sw}^2 \cdot c_2^2} = \overline{R_{eff}} + \overline{R_{ex}}$$

(Equation 20)

where $c_1$ and $c_2$ depend on the width σ and length $t_{sat}$ of the Gaussian pulses. $c_1$ is equal to $\frac{\sigma\sqrt{2\pi}}{t_{sat}}$ and $c_2 = \sqrt{\sqrt{2}}\, c_1$.

Under the assumption of incorporating chemical exchange during the delay $t_d$ which leads to a bi-exponential recovery the steady-state magnetization is given by (52):

$$\frac{M_{wz}^{ss}(\Delta\omega)}{M_0} = \frac{(1-e^{-R_{1w}td}) - R_{1w}cos^2\theta/\overline{R}_{1\rho}(\Delta\omega)(1-e^{-\overline{R}_{1\rho}(\Delta\omega)tsat})}{e^{\overline{R}_{1\rho}(\Delta\omega)tsat} - (1-\frac{\overline{R}ex}{k_{sw}}) \cdot e^{-R_{1w}td}}$$ (Equation 21)

The measured magnetization under non-steady-state conditions is given by (52):

$$\frac{M_{wz}(\Delta\omega)}{M_0} = \left(\frac{M_{initial}}{M_0} - \frac{M_{wz}^{ss}(\Delta\omega)}{M_0}\right) \cdot u^n e^{-\overline{R}_{1\rho}(\Delta\omega)tsat \cdot n} + \frac{M_{wz}^{ss}}{M_0}$$ (Equation 22)

where $M_{wz}^{ss}$ is the steady-state magnetization, *n* the number of pulses,

$u = e^{-R_{1w}td}$ assuming only $R_{1w}$ recovery during the inter-pulse delay or

$u = (1-\frac{R_{ex}}{k_{sw}}).\ e^{-R_{1w}td}$ assuming $R_{1w}$ recovery and chemical exchange during the delay

## 10. Temperature and pH dependence of the CEST signal

Let us now consider the exchange rate itself $k_{sw}$. In aqueous solution $k_{sw}$ can be separated into three components (38):

$$k_{sw} = k_a[H_3O^+] + k_b[OH^-] + k_{buffer} \quad \text{(Equation 23)}$$

where $k_a$ and $k_b$ refer to acid and base-catalysed proton exchange with $H_2O$ being either an acid or base catalyst. $k_{buffer}$ considers proton exchange of the buffering system and depends on the concentration and pH of the buffer. For instance, in a solution of phosphate buffer saline (PBS) the phosphate group protons exchange with water protons, which in turn modulates the CEST contrast due to its presence as one of the components of the pseudo rate constant $k_{sw}$ (e.g. an increase in PBS concentration will quench the CEST effect) (55). The base-catalysed term which dominates the exchange of amide and amine protons depends on pH and temperature T according to the following equation:

$$k_b(pH,T) = k_b(T)\ x\ 10^{pH-pk_w(T)} \quad \text{(Equation 24)}$$

where $k_b(T) = k_b(T_0)exp^{\frac{E_{A,B}}{R(1/T_0-1/T)}}$, $k_b(T_0)$ corresponds to the base-catalysed term at temperature $T_0$, $E_{A,B}$ is the activation energy for a protonated functional group (e.g, $[NH_2]_2^+$) to become deprotonated and release $OH^-$ ions and R=8.314 J/(mol K) is the gas constant. $pk_w(T)$ (i.e. $pH_w = -\log_{10}k_w$ with $k_w = [H_3O^+]\,[OH^-]$ the ionic water product in dilute aqueous solutions) can be expanded using the Van't Hoff equation which describes the dependence of $OH^-$ ions on temperature at a given pH:

$$pk_w(T) = pk_w(T_0) - \left(\frac{\Delta H_R^0}{R}.\ ln10\right).(1/T_0 - 1/T) \quad \text{(Equation 25)}$$

where $\Delta H_R^0 = 55.84 \frac{kJ}{mol}$ is the standard reaction enthalpy for the self-dissociation of water. It is important to note that the buffer-catalysed exchange rate constant $k_{buffer}$ is also pH dependent, and for a given pH value, the individual concentration of the dominant phosphate groups $H_2PO_4^-$ and $HPO_4^{2-}$ can be determined (50). Finally, the temperature dependency of $k_{buffer}$ is described using the Arrhenius equation.

Substituting Equation 25 to Equation 24 and if we assume a mono-exponential dependence of $k_{sw}$ on pH and *T*, the exchange rate constant $k_{sw}(pH, T)$ becomes:

$$k_{sw}(pH,T) = k_h(T)\ x\ 10^{pH-pk_w(T)} + k_{buffer} \approx k_b(T_0).\frac{mol}{l}.10^{pH-14+\frac{E_{A,B}+\Delta H_R^0}{R.ln10}(\frac{1}{T_0}-\frac{1}{T})}$$

(Equation 26)

For the case of a base-catalysed system equation 26 can be written as follows:

$$k_{sw}(pH,T) = k_b(T_0).\frac{mol}{l}.10^{pH-14+\frac{E_{A,B}+\Delta H_R^0}{R.ln10}(\frac{1}{T_0}-\frac{1}{T})} \quad \text{(Equation 27)}$$

If $k_b(T_0)$ and $E_{A,B}$ are known then the exchange rate can be calculated as a function of pH and T. Variation of $k_{sw}(pH,T)$ for a given change in pH or temperature can be written as follows:

$$\Delta k_{sw}(pH,T)/k_{sw}(pH,T) = [(E_{A,B} + \Delta H_R^0)/RT^2]\,\Delta T \quad \text{(Equation 28)}$$

Imaging contrast due to pH alterations has been presented in a number of CEST studies, exploring its effect either on amine or amide exchange (57). Until the advent of CEST-based pH-weighted imaging, the only available NMR approach for measuring intracellular pH in humans was phosphorus spectroscopy. However, because of poor spatial resolution it has always had limited utility in the clinic. The strong pH dependence of the amide protons in tissue in addition to a total amide content in proteins and peptides of over 70 mM (12)(48) provide enhanced sensitivity to probe pH changes in several pathologies noninvasively, including cancer, stroke and hyperglycaemia. An example of pH weighted imaging via CEST in patients with acute stroke is given in Figure 10. According to the authors of this study, pH alteration is considered as one of the most important effects leading to the changes seen in the

CEST effects during ischaemia. The relative importance of pH effects vs. other changes in the molecular environment is still an unresolved issue in the field.

**<<< Insert Figure 10 here >>>**

## Techniques to measure the exchange rates

As seen above, the exchange rates of amide or amine protons are strongly dependent on their molecular environment (30). While pH has a determinant effect, other modifications of the overall environment will also lead to modifications of the exchange rate. For example, the combination of the concentration of various metabolites and their chemical exchange rates can be used to assess different tissue environments such as cancer vs. contralateral region, or ischaemic vs. non-ischaemic tissues (58). Measurements of chemical exchange rates can be also used to select the most appropriate parameters for CEST acquisition (59),(60). This is important because CEST contrast is complex. More precisely CEST contrast varies with labile proton concentration, exchange rate and chemical shift, scales with magnetic field strength, RF irradiation scheme, duration and amplitude of the irradiation pulses as well as the water $T_1$ and $T_2$ relaxation times (40). Thus, it is crucial to isolate CEST properties from the effects of experimental parameters for proper quantification of its physiological underpinnings.

For this purpose, many analysis methods have been developed to enable estimation of exchange rates from CEST MRI data. The Bloch-McConnell equations can be used to fit simultaneously CEST Z-spectra at multiple $B_1$ powers or saturation lengths and an estimate of chemical exchange rate can be obtained (61). Simplified solutions of the BM equations have also been proposed as a less intense computational approach to calculate exchange rates, and they can usually be written in a linear fashion to speed up the fitting procedure. These methods will be introduced later in this article. Importantly, each one of these factors has different dependencies on experimental parameters and different weightings of the experimental results. Furthermore, recent studies employ different types of labelling approaches for saturating the metabolite system and for suppressing the signals originating from overlapping exchanging protons or from slowly exchanging processes such as MT. The

following section aims to introduce the reader to different experimental approaches used for CEST imaging, highlighting existing clinical applications and identifying potential limitations of each method.

## 11. Water exchange spectroscopy (WEX)

### 11.1. Concept

A simple way to measure exchange rates in magnetic resonance spectroscopy is through the assessment of the line-width (LW) of the solute protons, which is directly related to the exchange rate (e.g. when the exchange rate is within a particular window, i.e. not very slow and not very fast), but also to the solute transverse relaxation rate, $T_2$ and field inhomogeneities. This approach, however, is limited to *in vitro* studies and is inappropriate for measuring fast exchange rates (i.e. above 100 Hz) due to severe line broadening. An alternative technique for measuring exchange rates is water exchange spectroscopy (WEX) (62),(63),(64),(65),(66). During WEX, the water magnetization is first labelled and subsequently transferred to the solute protons during a mixing time, here considered as a single pool for simplicity. Then, the water signal is suppressed and the signal from the exchangeable protons is measured. WEX, similar to LW measurements, is less suitable for measuring the exchange rates of fast exchanging protons because the maximum signal intensity requires very short mixing times, which are limited by the hardware used (i.e. very short RF pulse duration is required).

**<<< Insert Figure 11 here >>>**

### 11.2. Pulse sequence

First the water magnetization is selectively excited by the 90° - $G_1$ -180° module and then it is either inverted to –z or put back to the z axis by a second 90° pulse (Figure 11). Following water excitation, the labelled z-water magnetization is transferred to exchanging species during the mixing time $t_m$ and then the exchangeable proton signal is detected after suppressing the background water signal. During the mixing period transfer of magnetization via single or multiple quantum scalar couplings can also occur and can be eliminated by the application of gradient pulses. In addition, water suppression can be performed using

traditional water suppression methods, such as e.g. the WATERGATE approach (63) which consists of a 3-9-19-19-9-3 composite pulse with a pair of gradients around it. It is important to note that partial saturation of water occurs during WATERGATE because the basic pulse sequence is based on a stimulated echo which leads to signal loss due to coherence selection. To avoid this an extra water-selective 90° pulse is added in the WATERGATE period for flipping the water magnetization back to z-axis before gradient dephasing (WEX type II) (63). The phases of the flip-back pulses are adjusted so that the water magnetization is along the z-axis and the exchangeable protons' magnetization is in the xy plane during WATERGATE. For applying this sequence *in vivo* outer volume suppression is combined with PRESS localisation for obtaining a high level of water and lipid suppression. Quantification of the average exchange rate can be performed by fitting the magnetization of the sum of all exchangeable protons at a particular frequency offset and irradiation amplitude as a function of mixing time. The solute signal intensity as a function of mixing time $t_m$ is given as follows:

$$S_s(t_m) = \frac{k_{sw} S_{0s}}{k_{sw} + R_{1s} - R_{1w}} \left[ e^{-R_{1w} t_m} - e^{-(R_{1w} + k_{sw}) t_m} \right] \qquad \text{(Equation 29)}$$

Substantial errors in $R_{1w}$ and $R_{1s}$ do not produce significant errors in quantification of $k_{sw}$. However, when the exchange rate becomes too high the maximum signal intensity will be reached in several milliseconds and short mixing times need to be used. In turn, its utility is therefore limited by the finite duration of the water labelling pulses.

### 11.3. Applications

Using this detection scheme, which does not suppress the signal from exchangeable protons, it is possible to follow the interactions between molecular sites on a time scale of a few milliseconds to a few seconds. More specifically, the WEX experiment was applied to report on exchange effects occurring on a time scale of 2-200 $s^{-1}$ for mobile molecules of any size as well as on intramolecular NOEs within these molecules and intermolecular NOEs occurring during short-lived binding of metabolites to the semisolid matrix (62). At short mixing times, the measured signal is due to the chemical exchange of amide protons of peptides and proteins. Amine exchange under physiological conditions is severely affected by line broadening and chemical shift averaging and therefore it is more difficult to measure by WEX. The effect of intermolecular NOE in proteins is much smaller than that of chemical

exchange and involves mainly aliphatic protons that give signals mostly on the opposite side of the spectrum from the amides; therefore it does not contribute to the measured exchange rate either. For longer mixing times the presence of intramolecular NOEs (i.e. 0.9-4.5 ppm) in the WEX spectra is evident as shown in brain cytosol (62). These spin diffusion processes are mainly visible for the more slowly moving large macromolecules. Additional evidence about confounding general magnetization transfer (MT) processes, of which intramolecular NOEs is one example, can be demonstrated, e.g. from spectra of cell extracts. Indeed, in the original paper (62), the exchangeable peaks at 6.8 and 7.8-8.8 ppm are no longer present, indicating that these protons most likely originate from sidechain and backbone amides of mobile proteins.

Recently, WEX spectroscopy has been demonstrated on a clinical system at low magnetic field strength to determine the exchange rate of guanidinium protons of creatine as a function of pH and temperature (67). WEX spectra were obtained at different pH values and temperatures for different mixing times appropriately distributed to capture the guanidinium WEX signal at its maximum. A Lorentzian lineshape analysis was used to measure the integrated peak area for each mixing time which corresponds to $S_s(t_m)$ in Equation 29. Subsequently, the data were fitted as a function of $R_{1w} + k_{sw}$ to calculate the exchange rate $k_{sw}$. The effect of pH and temperature on exchange rate was calculated using Equation 27 as described in section 10 which demonstrates a monoexponential dependence of the exchange-rate constant $k_{sw}$ of creatine on pH and temperature. In addition, base-catalysed proton exchange was found to be the dominant exchange pathway in creatine followed by a weak contribution of buffer-catalysed proton exchange.

Another study from the same group applied WEX to study the dipeptide carnosine, which was shown to be involved in pH buffering of the muscle and neurotransmitter function (68). The chemical exchange of carnosine occurs in the slow exchange regime and generates a distinct CEST peak at 3.1 ppm.

### 11.4. Disadvantages

Using the WEX experiment to calculate exchange rates suffers from a few drawbacks. First, small changes in the build-up curves have a large effect on the results of the fitting which produces less accurate results. Second, the exchangeable peak intensity after water

suppression will be reduced because of fast exchange with water protons which again introduces errors in the integrated peak area.

## 12. Chemical exchange saturation transfer by intermolecular Double Quantum Coherence (iDQC)

### 12.1. Concept

Similarly to multiple quantum coherences present in NMR, intermolecular Multiple Quantum Coherence imaging (iMQC) corresponds to specific imaging of simultaneous intermolecular spin flips, based on protons located on separate molecules in solution, at various distance from each other. For example, a resonance corresponding to a simultaneous flipping up of a proton on a macromolecule and a flipping down of a proton in the water can be detected because the net number of spin flips is zero. This is because the detection of these signals is made possible through dipolar interaction, which is proportional to the magnetic field strength. In particular, when magnetization isotropy is broken with gradient pulses, the dipole-dipole coupling can increase to produce noticeable effects. Sequences for imaging iMQC consist of an RF pulse followed by a gradient for generating a magnetization helix (i.e. spin magnetization is induced along a helical axis). The measured signal is generated from spins with distance equal to half of the length of the helix. Therefore, by changing the helix length, which is inversely proportional to the duration and strength of the gradient pulse, the interaction distance between spins is altered making this technique of interest for biological and clinical imaging (69),(70),(71). Recently it was shown that the MT effect of immobilised molecules could be enhanced due to its large homogeneous broadening when saturation transfer experiments are combined with the iMQC method, in which the contrast is described by the m-th power where m is the quantum coherence order. Generally, the intensity of the iMQC signals is very low, and decreasing with m, thus, for imaging purposes, only zero-quantum (iZQC) and double quantum (iDQC) have enough signal to be used. A series of experiments have been applied in combination with off-resonance saturation pulses and were compared to standard CEST imaging. In contrast to the conventional single-quantum coherence method (SQC), the very small iMQC signals generally limits the widespread application of the method in MRI.

**<<< Insert Figure 12 here >>>**

### 12.2. Pulse Sequence

A modified COSY (two-dimensional correlated spectroscopy) experiment with an asymmetric z-gradient echo detection sequence in combination with an off-resonance saturation pulse followed by a standard spin-echo imaging sequence was designed for obtaining images based on the intermolecular double quantum coherence method (iMQC) (69) (Figure 12).

### 12.3. Theoretical Description

During an iMQC experiment the off-resonance saturation pulse is followed by an RF pulse applied in the x-direction resulting in magnetization transfer along the y-direction which then evolves under both the gradient effects and the chemical shift during an evolution time $\tau$ as shown in Figure 12. Part of the transverse magnetization is subsequently transferred to the longitudinal direction by a second $\beta$ RF pulse. The final observable transverse magnetization which evolves under the dipolar field $B_d = \mu_0 M_z$ and the second gradient then becomes (70):

$$M_{obs}^{w}(t_{sat},\tau,t) = i^{-(n+1)}\frac{M_z^w(t_{sat})^n}{2^n(n\text{-}1)!}sin^n\alpha.sin^{n\text{-}1}\beta(1-cos\beta)(\gamma\mu_0 t)^{n\text{-}1}e^{n\tau/T_{2w}}e^{-t/T_{2w}}$$

(Equation 30)

Where

$$M_z^w(t_{sat}) = M_0 - \frac{k_{sw}M_s^{ss}}{R_{1w}+k_{ws}}(1-e^{-(R_{1w}+k_{ws})})$$ (Equation 31)

Equation 31 is obtained by solving the BM equations under a weak pulse approximation where the exchangeable proton pool is assumed to be completely isolated and reaches an equilibrium state instantaneously upon irradiation.

If the relaxation effects are neglected

$$M_{obs}^{w} \propto i\frac{M_z^w(t_{sat})^n}{2^n(n\text{-}1)!}sin^n\alpha.sin^{n\text{-}1}\beta(1-cos\beta)$$ (Equation 32)

and in the case of intermolecular double quantum coherence i.e. n=2 Equation 30 becomes

$$M_{obs}^{w} \propto \frac{1}{4}[M_0^w - \frac{k_{sw}M_s^{ss}}{R_{1w}+k_{ws}}(1-e^{-(R_{1w}+k_{ws})})]^2 sin^2\alpha.sin\beta(1-cos\beta)$$

(Equation 33)

It can be seen that the signal is affected mainly by the exchange rate constant $k_{sw}$, the duration of the saturation pulse $t_{sat}$, the saturation efficiency and the flip angles $\alpha$ and $\beta$. The maximum iDQC signal is obtained when optimal flip angles $\alpha$ and $\beta$ are being used and the measured signal can be written as (70):

$$M_{obs}^{w} \propto i\,\frac{3\sqrt{3}}{16}\left[M_0^w - \frac{k_{sw}M_s^{ss}}{R_{1w}+k_{ws}}\left(1-e^{-(R_{1w}+k_{ws})}\right)\right]^2 \qquad \text{(Equation 34)}$$

**<<< Insert Figure 13 here >>>**

### 12.4. Applications

Figure 13 shows a conventional z-spectrum and an iDQC Z-spectrum from samples containing 2 % agar and 0.1 mol of glucose at different saturation lengths (i.e. 0.5, 1.0, 2.0 and 4.0 s) (70). For CEST a standard pulse sequence was used while the iDQC spectrum was obtained by inserting a saturation pulse before the iDQC sequence. When comparing the iDQC with the SQC method it was obvious that an enhanced contrast was obtained with the former technique. For example, the iDQC-CEST signal with a presaturation pulse train of 2 sec was found to be larger than the SQC signal using a saturation pulse length of 4 s. It is worth noting that the observed signal intensity for a conventional SQC experiment decays monotonically due to transverse relaxation. In contrast for iMQC experiments the signal is modulated by Bessel functions; thus, it first increases to a maximum value and then decays to zero (i.e. for iDQC the maximum signal is observed at $2.2\tau$). In addition, the summed rate constant $r_1 = R_{1w} + k_{ws}$ can be calculated by fitting the signal intensity at different saturation lengths using Equation 34.

The iMQC effect has also been exploited in a preclinical system operating at 4.7T for imaging cartilage using solutions of glycosaminoglycans and cartilage tissue (71). In that case, the signal was shown to be equal to $CEST_{iDQC} = 2CEST_{SQ} - CEST_{SQ}^2$ under a weak pulse approximation. Because of the linearity of the CEST effect with agent concentration and by using a polynomial plot the measured signal (i.e. $2p[GAG]-p_2[GAG]_2$) can be expressed as a function of the exchange rate and the relaxation parameters of the sample.

Pulse sequences for measuring exchange rates between proton species

Another application of the iDQC technique combines the iDQC CEST detection with multiple refocusing pulses for improving image SNR (71). Similarly to a CPMG type of experiment, this approach replaces the single refocusing pulse in the detection period with multiple refocusing pulses (MRP), which gives higher refocusing signal intensity. The iDQC-MRP CEST sequence consists of the combination of a standard iDQC-CEST sequence with a nonselective $\pi$ pulse or multiple refocusing pulses equally spaced with a total duration $\Delta$ inserted in the middle of the evolution period $\tau$ (Figure 12). The MRP scheme refocuses the chemical shifts and magnetic field inhomogeneities during the evolution period while it retains the intermolecular dipolar interactions and increases iDQC build-up. The coherence selection in the iDQC sequence is achieved by the application of two gradients with an area ratio of 1:2 and a four-step phase cycling with the phases of the four pulses being (x, -x, y, -y) and for the receiver (x, x, -x, -x). The iDQC-MRP CEST signal is calculated using MTR asymmetry analysis and it is related to the single-quantum coherence signal using the following equation:

$$CEST_{iDQC} = 2\eta MTR_{asym}^{SQC} + (MTR_{asym}^{SQC})^2 \qquad \text{Equation 35}$$

where $\eta$ is the saturation efficiency which depends on the exchange rate and the relaxation parameters as well as on the experimental parameters such as $B_1$ magnitude and duration.

Amine and amide exchange as well as NOE effects were used as representative systems to study the iDQC-MRP CEST technique in creatine and egg samples (69). The SQC sequence was compared to iDQC-MRP by looking at the signal at 1.8 ppm, which was found to be stronger with the latter approach. In addition, the CEST signals were very similar for the iDQC and iDQC-MRP sequences but the SNR was higher with the iDQC-MRP approach. Finally, when MTR asymmetry analysis was calculated the iDQC-MRP method was found to be more sensitive for detecting low concentration solute protons compared to SQC.

### 12.5. Disadvantages

In general the low SNR of iMQC limits its applications in biological tissues (the maximum intensity of the obtained iDQC signal is about 33% of the conventional SQC signal), and therefore severely restricts this method in clinical settings.

## 13. Apparent exchange rate mapping with diffusion MRI

### 13.1. Concept

Filter exchange spectroscopy (FEXI) combines a double quantum filter experiment with a spin diffusion experiment in which molecular exchange and spin diffusion occur before signal detection. The pulse sequence consists of two pulsed gradient spin-echo (PGSE) blocks: the first perturbs the magnetization of the different water compartments according to their different diffusion rates (e.g. intracellular and extracellular) and the second block is used to measure the contributing signals as they return to equilibrium (72),(73),(74),(75). The inherent $T_2$ values of various components i.e. mobile vs. rigid enables the uncoupling of the signals by manipulating experimental parameters such the mixing time $\tau_m$. The few applications of this technique published so far include the measurement of the intracellular water lifetime and the quantification of the cell membrane permeability in yeast cells where the intracellular and extracellular components display a bimodal PGSE signal decay. Recently, the apparent exchange rate (AXR) has been introduced as a model-free measurement of molecular exchange between various compartments (76).

**<<< Insert Figure 14 here >>>**

### 13.2. Pulse Sequence

The FEXI pulse sequence consists of two PGSE blocks separated by a mixing time $\tau_m$ followed by the signal read out (Figure 14). Each PGSE block consists of two gradients with strength g, duration $\delta$ and time between the pulses $\Delta$. The first PGSE block attenuates the signal from species with fast diffusion while the second block is used for signal encoding. A diffusion weighting factor is defined as follows:

$$b = (\gamma g \delta)^2 (\Delta - \delta/3) \qquad \text{(Equation 36)}$$

The signal intensity for a single PGSE experiment in the absence of chemical exchange is defined using the following equation (76),(77):

$$I(b) = I_0 (f_A^{eq} e^{-bD_A} + f_B^{eq} e^{-bD_B}) \qquad \text{(Equation 37)}$$

where $I_0$ is the signal intensity with g=0, $f_{A/B}^{eq}$ are the fractional populations of sites A and B and $D_{A/B}$ are the diffusion coefficients. For instance, site A could represent the intracellular space and site B could be the extracellular medium. During a FEXI experiment a diffusion filter is applied to attenuate the extracellular signal and if a mixing time $\tau_m$ is chosen so that significant chemical exchange occurs between the two sites the signal intensity becomes (76):

$$I(b,\tau_m) = I_f(\tau_m)(f_A(\tau_m)e^{-bD_A} + f_B(\tau_m)e^{-bD_B}) \qquad \text{(Equation 38)}$$

where $I_f(\tau_m)$ is the signal intensity with g=0. Equation 37 is similar to Equation 38 although the value of $f_A$ is modified by the diffusion filter and a first order chemical exchange. More specifically, the fractional populations $f_A^0$ and $f_B^0$= 1-$f_A^0$ evolve towards the equilibrium values as shown below (76):

$$f_A(\tau_m) = f_A^{eq} + (f_A^0 - f_A^{eq})e^{-kt_m}$$
$$f_B(\tau_m) = 1 - f_A(\tau_m) \qquad \text{(Equation 39)}$$

where *k*= $k_{AB} + k_{BA}$.

Equations 38 and 39 are fitted to a two-dimensional array of b and $\tau_m$ which forms the basis of a FEXI experiment. When the condition $k_{AB}f_A = k_{BA}f_B$ is satisfied the intracellular exchange rate constant can be obtained by $k_{AB} = kf_B$. Then the diffusional permeability of a cell can be calculated as P = $k_{AB}.\frac{r}{3}$ where r is the radius of the cell.

Another approach for quantifying the effect of chemical exchange using diffusion gradients is by rewriting Equation 37 in the following form (76),(73),(74):

$$I(b,\tau_m) = I_f(\tau_m)e^{-bADC''(t_m)} \qquad \text{(Equation 40)}$$

where $ADC''(t_m) = ADC[1 - \sigma e^{-AXR.t_m}]$ and $\sigma = \frac{(D_A - D_B)(f_A^{eq} - f_A^0)}{ADC}$ is the filter efficiency with AXR = $k_{AB} + k_{BA}$ the apparent exchange rate constant. When b and $t_m$ are small Equation 40 can be applied to complex multisite systems in a manner analogous to the estimation of ADC in biological tissues.

**<<< Insert Figure 15 here >>>**

### 13.3. Applications

According to the literature, the effects of restricted diffusion are on a time scale of approximately 1ms and those of exchange are in the order of 0.1-1 s. For maximising restricted diffusion and minimising chemical exchange, the values of $\delta$ and $t_d$ need to be on the order of ms. However, for measuring the exchange effect,, the length of $\tau_m$ and its impact on $f_A$ are considered. During a FEXI experiment the signal with short $\tau_m$ consists of the intracellular component and when increasing $\tau_m$ the effect of the extracellular compartment starts to appear. For detecting exchange a significant variation of $f_A(\tau_m)$ needs to be used within the available $\tau_m$ from 10ms to 100ms. Finally, the choice of b values determines the weighting of the filter and the information on exchange is obtained by varying $f_A$ as a function of $\tau_m$ (see Equation 39). For this purpose, the b values are chosen differently from conventional diffusion studies, in which a linear or a quadratic choice of b is employed. To obtain an estimate of $f_A$ data points are collected with b << $1/D_B$ and in the range $1/D_B$ <b < $1/D_A$.

The model-free analysis can be also used for calculating the apparent exchange rate (AXR). AXR can be applied if chemical exchange is sufficiently slow for observing multimodal diffusion in a PGSE experiment. Regarding the experimental parameters, the PGSE filter design is crucial. Its purpose is to maximise the detected difference between fast and slow diffusion modes and to retain adequate SNR for accurate measurements. It has been demonstrated that the model-free analysis in the low range of b values gives similar results to the bimodal analysis (Equation 38) (76). In addition, the optimal $b_f$ is chosen as a compromise between precision and accuracy of the estimated AXR. Finally, any information regarding the equilibrium fractional populations is used for calculating the intracellular lifetime $\tau_A = 1/(f_A AXR)$.

The AXR has been measured in the healthy human brain and in meningioma brain tumour (73),(74) . In fixed monkey brain a tensor-based AXR analysis has been performed (78). Preliminary results showed that the AXR is sensitive to altered gene expression of the urea

transporter (79) and this approach has also been applied to investigate breast tumour cells (80). Another study reported recently aimed to evaluate the effect of transcytolemmal water exchange on the accuracy of the fitted microstructural parameters obtained using quantitative diffusion MRI methods by means of computer simulations and cell culture studies *in vitro* (81). It was demonstrated that transcytolemmal water exchange has minor effects on measuring the cell diameter and the intracellular diffusion coefficient for physiologically relevant models of membrane permeability. However, when using similar models the fractional volume of the intracellular water is significantly underestimated. One major disadvantage of combining transcytolemmal water exchange in quantitative diffusion models is its reliance on high SNR, which is needed for accurate fitting and which results in long scanning times.

To summarise, FEXI complements diffusion MRI and the AXR parameter has been shown to carry additional information about physiological parameters. The AXR is quantified for exchange rates up to approximately 20-40 $s^{-1}$.

### 13.4. Advantages

The measurement of exchange rates is based on restricted diffusion of water molecules within cells, avoiding the use of paramagnetic ions or other shift reagents.

### 13.5. Disadvantages

The lower limit of exchange rate measurements is given by the maximum available gradient strength and gradient slew rate which depends on $\delta$ and $t_d$. In addition, the time scales of restricted diffusion and chemical exchange need to be well separated for resolving the intracellular and extracellular compartments. Therefore, the method can only be used in a restricted set of problems and is not universally applicable.

## 14. Ultrafast CEST imaging

All the methods presented so far have focused on measurements of exchange rates without consideration of time. However, the need to vary the saturation frequency in CE-based experiments will lead to very long scan times, during which, among other issues, stability problems might crop up. A solution to this issue has been presented and is described below.

### 14.1. Concept

A fast method to study MT effects was developed by Swanson and co-workers based on the application of a gradient field simultaneously with the saturation pulse (82). This approach was inspired by ultrafast NMR spectroscopic methods in which spatial encoding of evolution times is used to speed-up multi-dimensional NMR experiments. The same concept was applied in CEST, allowing a Z-spectrum to be acquired in two scans (i.e. with and without RF saturation pulse) by encoding a whole range of frequencies with a single saturation pulse (83),(84),(85),(86). In summary, under the effect of a gradient pulse the resonance frequency of the spins becomes a function of spatial location and the saturation frequency is effectively encoded in space. Then, for encoding the off-resonance frequencies back to frequency information a readout gradient along the same direction with the saturation gradient is applied. A Z-spectrum is obtained as the ratio of the two 1D profiles measured with and without RF saturation. The potential of this method for rapid characterization of homogeneously distributed CEST agents has been shown *in vitro*. However, it remains challenging to expand this method to 2D imaging because the second dimension that would be needed for spatial encoding in this case is already being used to encode the frequencies in the Z-spectrum.

**<<< Insert Figure 16 here >>>**

### 14.2. Pulse sequence

A gradient field is applied simultaneously with the saturation pulse at a certain frequency offset to create a single-shot acquisition (Figure 16). Only protons with spatial positions on-resonance with the RF pulse are directly saturated and the protons in other spatial locations experience the off-resonance irradiation at a frequency offset determined by their distance to the on-resonance slice. Due to this frequency encoding, protons that exchange their magnetization at certain frequency offsets now can exchange it at certain spatial locations. In analogy to a Look-Locker experiment the exchange profile is acquired by interleaving gradient-encoded saturation pulses with EPI images. An incremental saturation is created which enables the acquisition of a QUEST-type experiment (see below).

### 14.3. Underlying physics for using a gradient

The application of an off-resonance saturation pulse simultaneously with a gradient creates a $\Delta B_{sat}(d) = G_{sat}.d$, where d is the distance from the centre of the gradient to the centre of the slice and $G_{sat}$ is the strength of the gradient pulse. This results in a shift of the resonance frequencies in the slice by $\Delta\omega_{sat}(d) = \gamma.G_{sat}.d$ and because the saturation pulse is applied at the same frequency offset the slice experiences an effective irradiation at offsets $-\Delta\omega_{sat}(d)$. In other words, the effect of a saturation pulse will be position-dependent. For example, if the frequency of the saturation pulse is $\Delta\omega_{sat} = 0$ then only protons at $d$=0 experience on-resonance saturation. For all other locations, protons experience off-resonance saturation with a position-dependent frequency offset. Thus, in contrast to the conventional approach, the off-resonance data-points are not generated from additional scans.

### 14.4. Applications

The ultrafast z-spectroscopy sequence was applied in several samples including salicylic acid, 3,5-dibromosalicylic acid, 3-nitrosalicylic acid, 2-cyclohexylamino-benzoic acid, 5-amino-2-benzoic acid, 2-amino-benzoic acid, 4,6-dihydroxyisophthalic acid and 2,5-dihydroxyterephthalic acid for calculating the exchange rates using a series of Z-spectra acquired with different saturation times (85). According to the results of this study the measured exchange rates were slightly faster than those obtained with the conventional QUEST technique, which might be due to the reduced SNR during the image readout.

A modification of the sequence includes the application of a small flip back pulse after the EPI readout to recover the remaining longitudinal magnetization, similarly to the MeLOVARS approach (details of this method can be found in section 35) (85). Moreover, the implementation of a multislice technique has been shown to enable the acquisition of Z-spectra from multiple samples within the same experiment (i.e. each tube was covered by a separate slice) (83). In addition, ultra fast Z-spectroscopy (UFZ) has been extended for fast characterisation of CEST agents by incorporating a slice selection gradient, a gradient echo sampling and gradient pulse timings to avoid signal distortions. In the case of Eu-DOTA-4AmC the chemical shift of the agent decreases as the temperature increases and the bulk water signal becomes broader corresponding to an increase in the exchange rate (84).

Recently, localised CEST information was used to better characterise lesions in tumour patients (87). Using this underlying idea an ultrafast spatially localised CEST-spectroscopic method using point resolved spectroscopy (PRESS), termed UCEPR, was applied in the human brain for studying the APT effect. This method combines the time efficiency of UFZ spectroscopy with the localisation encoding of a PRESS sequence. Although its feasibility was demonstrated in the healthy human brain, the bandwidth of the acquired Z-spectrum depends on the position and the geometry of the selected voxel. In addition, the implemented method was compared with standard CEST techniques and the results were found to be in good agreement. In the case of UCEPR there was an acceleration factor of six-fold compared to imaging but with a reduced SNR.

### 14.5. Advantages

UFZ spectroscopy is robust against spatial variations in the sample shape or solution distribution, which makes this method applicable under severe $B_0$ inhomogeneities. In addition, the acquisition of a Z-spectrum in the presence of a gradient can suppress radiation damping artefacts.

### 14.6. Disadvantages

Similar to other ultrafast methods, UFZ spectroscopy has lower SNR and limited frequency resolution due to water diffusion during RF saturation. Diffusion will lead to broadening that increases linearly as a function of the square root of saturation time. Also, if many samples are scanned simultaneously, UCEPR should be repeated for each one of the samples. Furthermore, in the case of multi-slice acquisitions, the samples should be arranged in a specific way (i.e. a single slice should only cover a single sample), otherwise the obtained z-spectrum will contain contributions from more than one sample.

## 15. Z-spectroscopy with alternating-phase irradiation (ZAPI)

### 15.1. Concept

Z-spectroscopy with alternating phase irradiation (ZAPI) uses phase and/or amplitude modulation of a continuous wave RF pulse to discriminate between different timescales of interactions, based on the differing responses obtained with modulations at different rates (modulation periods) and mean RF powers (defined as the average of the square of the RF

amplitude). ZAPI has been applied on tissue samples and *in vivo* for calculation of the MT effect without any interference from direct water saturation or other long $T_2$ components originating from the exchangeable protons in metabolites and proteins leading to CEST effects (88), (89).

## 15.2. Theory

The effects of a periodically modulated irradiation with a period $\tau$ and RF amplitude $\omega_1$ ($=\gamma B_1$) can be analysed using general solutions of the Bloch equations under two approximations:

1. When the period of modulation $\tau$ is small compared to the inverse of the RF amplitude $\omega_1$ and $T_2$ ($\omega_1\tau \ll 1,\ \tau \ll T_2$) the system will have a coherent response.
2. If $\omega_1 T_2 \ll 1$ the response to the irradiation is incoherent and the system approaches steady state exponentially.

In the incoherent case the z-magnetization of water is described by the following equation:

$$\frac{dM_z}{dt} = R_1 M_0 - M_z\left[\frac{R_1\left(R_1^2+\Delta^2\right)+\omega_1^2 R_2}{R_2^2+\Delta^2}\right] \qquad \text{(Equation 41)}$$

where $M_z$ approaches steady-state depending on the spin-lattice relaxation $R_1$ and the rate constant $\frac{\omega_1^2 R_2}{R_2^2+\Delta^2}$. The rate of saturation is proportional to the square of the RF amplitude and to the ratio $\frac{R_2}{R_2^2+\Delta^2}$, which is the lineshape of the spectrum and corresponds to a Lorentzian function with a width equal to $1/(\pi T_2)$. In the case of solid and semi-solid systems the Lorentzian term is replaced by a Gaussian or a super-Lorentzian lineshape Indeed, when partially ordered materials such as biological tissues are considered, then the integration over all possible dipolar orientations in the semisolid material results in a non-zero time-averaged dipolar Hamiltonian, leading to a Super-Lorentzian lineshape, defined mathematically as:

$w_b(2\pi\Delta) = \int_0^{2\pi} d\theta sin\theta \sqrt{\frac{2}{\pi}} \frac{T_2^b}{|3cos^2\theta\text{-}1|} e^{-2\left(\frac{2\pi\Delta T_2^b}{|3cos^2\theta-1|}\right)^2}$, with $\theta$ = dipole orientation with respect to the external magnetic field (136). In addition, the degree of saturation depends on the average irradiation amplitude, independently of the type of the irradiation pulse, meaning that for a given amplitude any modulation pattern will give the same degree of saturation for a spin pool. In contrast, in the coherent case, alterations in the modulation induce different patterns for MT, chemical exchange and direct water saturation enabling the separation of these processes under specific modulation waveform characteristics.

**<<< Insert Figure 17 here >>>**

In ZAPI the irradiation pulse is applied on resonance with the water frequency and the saturation of the MT/CEST pool is performed by modulating the amplitude or the phase of the RF pulse. More precisely, a square wave modulation is used in which the phase of the RF pulse is changed by 180° every $\frac{\tau}{2s}$. In addition, sinc wave modulation can be applied, and is termed Z-spectroscopy with alternating-phase irradiation and sine modulation (ZAPISM) in which the amplitude of the RF pulse is given by $\omega_1 \sin(2\pi t/\tau)$. In that case, the frequency profile of the acquired z-spectrum displays two off-resonance peaks with their frequency depending on the period of the RF modulation $\tau$ (Figure 17). With careful selection of the irradiation amplitude and modulation frequency, the direct water saturation becomes negligible and only macromolecular protons experience RF irradiation (i.e. during $\tau$ the water magnetization is rotated through a small flip angle away from equilibrium and then returns intact except for slight $T_2$ effects). Overall when comparing ZAPI and ZAPISM with other types of pulsed saturation experiments including trains of binomial and hard pulse for probing MT, it can be seen that ZAPISM discriminates such processes by using a lower $B_1$ and large separation between sidebands. In addition, the SAR calculated for ZAPISM is lower than standard saturation transfer approaches.

### 15.3. Technical considerations

When a ZAPI sequence is used without modulation (i.e. constant phase of the CW pulse) the experiment is simplified to a conventional saturation transfer experiment. However, in the standard ZAPI and ZAPISM sequences the RF phase is inverted by 180° every 100 $\mu s$ $(i.e.\ \tau/2)$ so that the on-resonance signals are saturated for $T_2 << \tau$ but are unaffected for $T_2 >> \tau$. In addition, ZAPI can be considered a dual off-resonance irradiation with side lobes falling at $\pm 1/2\tau$ (i.e. when $\tau$ decreases the off-resonance frequency increases). The MT/CEST contrast can be estimated using the ZAPI sequence by acquiring data with different modulation frequencies while measuring the difference compared with a CW experiment for the same irradiation amplitude. Two factors need to be considered: $B_1$ must be high enough to produce the desired MT effect and $\tau$ must be long enough (i.e. longer than the longest macromolecular / exchangeable proton $T_2$ but shorter than the $T_2$ of the unsaturated components / solvent) so that the exchangeable pool is saturated effectively. In the brain, an

appropriate compromise for conventional MT is around τ=200 $\mu s$. Shortening $\tau$ results in a stricter $T_2$ filter and reduces the level of saturation since fewer protons in macromolecules are saturated. By changing $\tau$ from 200 $\mu s$ to 50 $\mu s$ MT in muscle decreases by 50 % while in the brain the decrease is about 70%.

### 15.4. Applications

ZAPI contrast was applied to ischaemia for investigating the time course and magnitude of changes in an infarcted brain by comparing ipsi- and contralateral values. The performance of ZAPI was compared with other imaging contrasts such as $T_{1\rho}$ and $T_{2\rho}$(89). In summary, the results included a decrease in the diffusion tensor by 42%, indicative of severe ischaemia, in addition to a reduction in CBF values by 76%. $T_1$ and $T_{1\rho}$ increased linearly as a function of time as well as $T_2$ but the onset of the increase was reported only after an hour following the start of ischaemia. In the acute phase of ischaemia ZAPI contrast was elevated during the early minutes and the magnitude of ZAPI change was different when compared to conventional MT. Early ZAPI signal changes mostly reflect macromolecular $T_2$ distribution rather than net water accumulation because the direct saturation lines were narrower, and the effects of relaxation rates were less prominent than those observed in conventional MT experiments at the corresponding off-resonance frequency.

### 15.5. Disadvantages

Errors in RF modulation will cause changes in the pattern of sidebands resulting in artefacts in the images. In addition, $B_1$ inhomogeneities will lead to different MT weighting introducing bias to the measurements. While ZAPI has been applied to detect macromolecular protons with short $T_{2s}$ its feasibility for probing exchangeable protons has not been demonstrated. The $T_2$ differences between exchangeable protons in mobile structures and water will be longer and this method might not be optimal in that case.

## 16. Alternative Interpretation of the ZAPI method

### 16.1. Concept

An alternative interpretation can be given of the use of sine and cosine modulated pulses introduced above in the ZAPI sequence, where the phase of the square pulse was modulated by 180° every 1/Ω with Ω being the modulation frequency. According to this alternative interpretation, the sequence is tuned so that the CEST contrast is isolated from asymmetric

MT contrast because of the uniformity of the MT contribution over a broad range of frequencies. In particular, if the duration of the off-resonance pulse is much shorter than the time scale of MT, the MT contrast becomes uniform over a large frequency range and can therefore be cancelled. Unlike the ZAPI technique which uses frequency modulated pulses to probe MT contrast here the simultaneously applied two-frequency RF irradiation is used to create similar baseline MT contrast aiming to isolate the contribution of exchangeable protons in mobile molecules (with long $T_2$) from the one created from large immobile molecules (with very short $T_2$).

### 16.2. Theoretical description of simultaneous two-frequency RF irradiation

Following a sequence similar to the previously introduced ZAPI technique, Provotorov's theory can be used to describe a system undergoing chemical exchange upon double off-resonance RF irradiation. However, the interpretation is then reframed based on the spin-density formalism. The complete mathematical framework is given below.

While the Bloch-McConnell equations describe the evolution of the magnetization in the presence of chemical exchange and relaxation, they do not address dipolar interactions between spins . In such cases a thermodynamic theory can be adapted in which two reservoirs of spins, evolving through a conventional Zeeman and dipolar Hamiltonian, respectively, are connected through the application of weak RF irradiation. The kinetic equations describing the saturation of such a strongly coupled system (i.e. local dipolar field $\omega_{loc}$ >> $\omega_1$) in terms of polarization can be written as follows if $\omega_0 \gg \omega_1$ (127):

$$\frac{dPs}{dt} = -W\left(P_s - \frac{\Delta\omega}{\omega_{loc}}P_d\right) - \frac{P_s - P_{s,0}}{T_{1,s}} \qquad \text{(Equation 88)}$$

$$\frac{dPd}{dt} = W\frac{\Delta\omega}{\omega_{loc}}\left(P_s - \frac{\Delta\omega}{\omega_{loc}}P_d\right) - \frac{P_d}{T_{1,d}} \qquad \text{(Equation 89)}$$

where $Ps$ and $Pd$ are the spin and dipolar polarizations for N spins with $Ps = \left(\frac{2}{N}\right) tr\{S_z\rho\}$ and $Pd = \left(\frac{2}{N}\right)\left(\frac{tr\{H_d\rho\}}{\omega_{loc}}\right)$, $\rho = \beta_z(t)\omega_o S_z - \beta_d(t)H_d$ is the spin density operator, $H_d$ is the dipolar Hamiltonian, $S_z$ is the z component of the spin angular momentum, $\omega_o$ is the resonance frequency, $\beta_z$ and $\beta_d$ are the Zeeman and dipolar spin temperatures,

$W = \pi\omega_1^2 g(\Delta\omega)$ is the amplitude of the RF field and $g(\Delta\omega)$ is the absorption lineshape. $T_{1,s}$ and $T_{1,d}$ are the spin-lattice relaxation rates for Zeeman and dipolar spins and $P_{s,0}$ is the thermal equilibrium polarization.

Under two-frequency irradiation, Provotorov's theory as described above can be expanded by an additional weak RF irradiation (127). In this thermodynamic interpretation, using the spin density formalism, one can regard the Zeeman and dipolar spin reservoirs evolving under their respective Hamiltonians as separate subsystems and can treat a weak RF irradiation as a thermal contact between these subsystems. If the difference between the two irradiation frequencies of the applied RF pulse is much larger than its amplitude then each frequency contributes to the kinetics in the same way while the cross-effect between the two RF components is equal to zero. The kinetic equations under two-frequency irradiation (i.e. $W, W'$) can be written as(127):

$$\frac{dPs}{dt} = -W\left(P_s - \frac{\Delta\omega}{\omega_{loc}}P_d\right) - W'\left(P_s - \frac{\Delta'\omega}{\omega_{loc}}P_d\right) - \frac{P_S - P_{S,0}}{T_{1,s}} \qquad \text{(Equation 90)}$$

$$\frac{dPd}{dt} = W\frac{\Delta\omega}{\omega_{loc}}\left(P_s - \frac{\Delta\omega}{\omega_{loc}}P_d\right) + W'\frac{\Delta\omega}{\omega_{loc}}\left(P_s - \frac{\Delta'\omega}{\omega_{loc}}P_d\right) - \frac{P_d}{T_{1,d}} \qquad \text{(Equation 91)}$$

The eigenvalues of equations 90 and 91 (i.e. $Ps$ and $Pd$) describe the dynamics of the system.

However, the equations above do not include any exchange term. As such, to build a two-pool model for MT, these equations can be modified by adding an additional Zeeman polarization $P_I$ without any dipolar couplings to either of the two other pools to account for MT, and by including in addition a first order exchange terms (127):

$$\frac{dPs}{dt} = -W\left(P_s - \frac{\Delta\omega}{\omega_{loc}}P_d\right) - W'\left(P_s - \frac{\Delta'\omega}{\omega_{loc}}P_d\right) - \frac{P_S - P_{S,0}}{T_{1,s}} + k_{I\to S}P_I - k_{S\to I}P_S$$

(Equation 92)

$$\frac{dPd}{dt} = W\frac{\Delta\omega}{\omega_{loc}}\left(P_s - \frac{\Delta\omega}{\omega_{loc}}P_d\right) + W'\frac{\Delta\omega}{\omega_{loc}}\left(P_s - \frac{\Delta'\omega}{\omega_{loc}}P_d\right) - \frac{P_d}{T_{1,d}} \qquad \text{(Equation 93)}$$

Pulse sequences for measuring exchange rates between proton species

$$\frac{dP_I}{dt} = -WP_I - W'P_I - \frac{P_I - P_{I,0}}{T_{1,I}} + k_{S\to I}P_S - k_{I\to S}P_I \qquad \text{(Equation 94)}$$

From these kinetic equations, it is possible to separate MT effects from chemical exchange processes if the former are homogeneous along the irradiating frequency dimension. In the following, applications of the double-frequency / ZAPI pulse sequence to achieve uniform MT are presented and interpreted using the above theory.

### 16.3. Pulse sequence

To achieve a uniform magnetization transfer effect, a single shaped RF pulse is multiplied by a cosine term with a modulation frequency $f_m$ for creating the two frequency components of the RF irradiation. The frequency difference between the two is twice the modulation frequency $f_m$ and each component carries half of the total irradiation power. Ideally, by using a two-frequency irradiation scheme CEST effects are induced in the same way as the conventional single irradiation CEST techniques while the MT effects become uniform (Figure 18). This requires the frequency separation between the two irradiations ($2xf_m$) to be large enough so that the CEST pool is saturated while at the same time simultaneous irradiation of other exchangeable protons except those in semi-solids is avoided. Since most of the diamagnetic intrinsic exchangeable protons resonate within 6ppm, a good choice for the frequency separation would be 12 ppm.

A uniform MT ratio is then calculated as (128):

$$\text{uMTR}(f_m) = 1 - M_{z,sat}(f_0 = 0, f_m)/M_0 \qquad \text{(Equation 95)}$$

where $M_0$ is the water signal without RF irradiation and $M_{z,sat}$ is the water signal under cosine-modulated RF irradiation with frequency offset $f_0$.

Similarly to conventional CEST experiments, the water signal is plotted against the frequency offset of the cosine-modulated RF irradiation to create a Z-spectrum. The displayed frequency positions are calculated using the frequency offsets plus or minus the modulation frequency. Unlike a CEST Z-spectrum the water signal here displays two dips at the positive and negative modulation frequencies which can also be used to create a $B_0$ map based on the frequency difference between the two peaks, in a manner very similar to ZAPI. Finally, the

flatness of the Z-spectrum at zero frequency reflects the uniform saturation of MT within the $\pm 6$ ppm range where most diaCEST effects are detectable. Beyond these frequencies complete saturation of MT effects is not possible and *in vivo* MT has a super-lorentzian shape, which means that different MT effects will be induced depending on position on that shape.

Figure 18 shows a spectrum obtained using a uMT CEST acquisition with a cosine-modulated RF irradiation at the modulation frequency $f_m$. If the offset of the two-frequency components is set to the frequency of the exchangeable protons a CEST effect is created since one of the RF components hits the CEST pool. However, when the RF frequency is moved to the downfield resonances no CEST pools contribute to the measured water signal while the contribution of MT is uniform. To compensate for the experimentally observed direct water saturation, another experiment is performed at a symmetric frequency around the water signal.

In that case, the asymmetry of the uniform MT is defined as (128):

$$\mathrm{uMTR_{asym}}(f_0, f_m) = [M_{z,sat}(f_m - f_0, f_m) - M_{z,sat}(-f_m + f_0, f_m)] / M_0$$

(Equation 96)

where the frequency positions of the cosine-modulated RF irradiation at the frequency offset $f_0$ is $f_0 \pm f_m$.

In summary, two-frequency CEST contrast is obtained by combining two acquisitions of two-frequency RF irradiation in such a way that the irradiation frequencies are symmetric between the two acquisitions and one of the four frequency positions is at the CEST resonance frequency.

**<<< Insert Figure 18 here >>>**

### 16.4. Applications

Validation of the double flip angle irradiation approach was performed by means of simulations and by applying this imaging scheme in the brain and knee (128),(129). An example of simulated spectra consisted of a 5 s long RF irradiation scheme with $\omega_1 =$

$\omega'_1$=100 Hz and with the frequency separation between the two frequency components set at 2000, 5000 and 10000 Hz, respectively (Figure 19). The resulting Z-spectra at $f_0$ were obtained with RF irradiation applied at $f_0 + f$ and $f_0$-$f$ where $f = (\Delta\text{-}\Delta')/2$ is the frequency separation between the two components and $f_0 = (\Delta + \Delta')/2$. For analysis, the distances between the two frequency components and the chemical shift of the exchangeable protons (i.e. 3.2 ppm) were compared. Interestingly, it shows that the appearance of the CEST peak depend on the frequency difference between both saturation frequencies compared to the chemical shift difference between the CEST agent and water. For example, if the frequency difference between both saturation bands is larger than twice the chemical shift, the two CEST peaks will be observed on opposite sides in the asymmetry plots (see Fig 19(g) and 19(h)).

Several samples including a CS solution, gelatine and a bovine cartilage piece were used to validate the results of numerical simulations (127). Uniformity of the MT effect is obtained when the two frequency components affect the spectrum of the CEST agent simultaneously. Later work involved the application of conventional CEST and uMT to healthy volunteers and multiple sclerosis (MS) patients. MS lesions in the brain lead to longer $T_2$ and reduced MT effects possibly because of demyelination (128).

### 16.5. Disadvantages

The implementation of the two-frequency irradiation scheme requires many digitization steps of the cosine modulation irradiation to approximate a continuous variation of the RF amplitude with time, which makes this approach prone to digitization and truncation errors. In addition, due to hardware constraints there is a limitation of the maximum number of digitised steps. Finally, there are SAR constraints when this scheme is applied *in vivo*.

**<<< Insert Figure 19 here >>>**

## 17. Frequency exchange spectroscopy (FLEX)

### 17.1. Concept

An ‘exchange-rate filtered’ approach which allows for the detection of backbone protons from proteins without the need for saturation transfer or additional post-processing has been

designed and named “frequency-labelled exchange transfer” (FLEX) (90),(91),(92). In this method, a series of label-transfer modules (LTMs) selectively label exchangeable solute protons by encoding their chemical shift through the chemical evolution of the magnetization of the solute protons in the xy plane. Then the longitudinal water magnetization which contains chemical shift information is detected directly on the water resonance. The amplitude of the FLEX signal as a function of the number of LTM modules can be used to extract the amplitude of each exchangeable proton resonance as well as the signal decay rate constant $k_{sw}$ during the evolution time by time-domain fitting.

**<<< Insert Figure 20 here >>>**

### 17.2. Pulse sequence

The pulse sequence consists of a series of pairs of binomial excitation pulses followed by delays. Specifically, a short selective 90° pulse is applied on resonance with the exchangeable (or solute) protons and excites them over a range of frequencies (Figure 20). Then a delay termed $t_{evol}$ follows during which the signals of the solute protons are frequency encoded, after which A selective 90°-x pulse then flips the ‘frequency labelled’ magnetization back to the longitudinal axis. After storage of the frequency information in the form of longitudinal magnetization by the 90°-x pulse, a waiting period, $t_{exch}$ allows chemical exchange of the labelled solute magnetization with the solvent. Here, $t_{exch}$ can also be used as an exchange rate filter since the size of the water z-magnetization (i.e. the build-up of the signal) is proportional to the exchange rate of the labile proton pool and to the longitudinal relaxation time of water $T_{1w}$. The whole preparation period involves the application of multiple modules for accomplishing measureable changes in the water signal. In addition, other types of labelling schemes can be used instead of saturation transfer, including inversion transfer of the solute magnetization or dephasing transfer accomplished by a gradient pulse (11).

The FLEX signal is collected as a function of the temporal variables $t_{evol}$ and $t_{exch}$. More specifically, a FLEX experiment requires multiple acquisitions with different evolution times for obtaining a signal, equivalent to an artificial “FID”, which contains the multiple frequency components measured through the water signal (i.e. this “FID” corresponds to the signal intensity as a function of LTMs). Separation of the different frequency components included in this “FID” can be done through a Fourier transform akin to what would happen

with a real FID signal. Other types of deconvolution techniques might also be used in this case, as well as all pre-processing techniques available in NMR, such as exponential line-broadening and time domain analysis. The magnitude of the FLEX contrast for each exchangeable proton group as a function of the evolution time $t_{evol}$ = t is described by the proton transfer rate (PTR) under the assumption of negligible direct water saturation during the RF excitation (90):

$$PTR_s = x_s\lambda_s(1 - e^{-k_{sw}t}x)\sum_{i=1}^{n} e^{\left\{-1+\frac{i-1}{n}\right\}t_{sat}/T_{1w}} \qquad \text{(Equation 42)}$$

where $x_s$ is the fractional concentration of the solute (s) protons and $\lambda_s$ is the labelling efficiency of the pair of excitation pulses. The summation term shows that the water magnetization after the first LTM module (i.e. i=1) will decay mainly with $T_{1w}$; however, after n modules $T_{1w}$ decay will hardly affect the measured signal. For multiple exchanging solute protons, the total water signal can be written as a convolved time-domain "FID" (90):

$$I_w = \sum PTR_s\, e^{-(k_{ex}+1/T_{2s}^*).t_{evol}}.\cos(\Delta\omega_{so1}.t_{evol}) \qquad \text{(Equation 43)}$$

where $T_{2s}^*$ is the effective transverse relaxation time of solute protons and $\Delta\omega_{so1}$ is the chemical shift difference between the labelled protons and the offset frequency of the labelling pulse. It can be seen that the time domain FLEX signal (Equation 43) is modulated by the superposition of frequencies from the components (s), each having a frequency determined by $\Delta\omega_{so1}$ and a decay rate proportional to $(k_{ex} + 1/T_{2s}^*)$.

Time-domain analysis of the exponential decay term can thus be performed for removing signal from water protons that have also been excited by the pair of RF pulses or any rapidly decaying components or MT from semi-solid protons if $\frac{1}{T_{2s}^*} > (k_{ex} + 1/T_{2s}^*)$ (93). The exchange rate of the solute protons can be obtained by fitting the normalised signal decay (obtained by dividing the FLEX time-domain signal to the average water signal without LTMs) for each pool at different evolution times using Equations 42 and 43 and assuming $T_{2s}^* \approx 0$.

The FLEX analysis does not involve asymmetry analysis and the length of the scan time depends on the number of points sampled for the artificial FID (i.e. number of evolution time points). In addition, the scanning time depends on the recycle delay between subsequent scans, the length of the exchange time and the number of LTMs. The main complication when applying this sequence with a non-imaging readout is radiation damping, which drives the system quickly back to equilibrium with a characteristic reduction in the apparent $T_{1w}$. There are a few ways of reducing radiation damping, including the dephasing of the water magnetization when it is not detected or minimising water excitation by using far off-resonance carrier frequency or by using narrower excitation pulses. Other options would be to detune the receiving coil during evolution, but this is more difficult to achieve on MRI scanners as opposed to laboratory NMR spectrometers. Another problem might be the minimum allowed evolution time $t_{evol}$ for observing fast exchangeable protons through the encoding of the early part of the FID due to hardware constraints on MRI scanners (in particular due to a limited max $B_1$ field).

**<<< Insert Figure 21 here >>>**

## 17.3. Applications

### 17.3.1 Using FLEX to separate magnetization transfer from chemical exchange processes in ParaCEST agents

ParaCEST agents have great potential for targeting tissue properties of biological importance such as enzyme activity, pH and temperature. In addition, they have several favourable technical features such as the large chemical shift difference between the exchanging proton pools and free water. As a consequence, saturation pulses can be applied far away from the water resonance eliminating direct water saturation. However, the detection of fast exchanging agents with conventional RF irradiation requires high $B_1$ amplitude (for sufficient labelling efficiency), which leads to strong MT effects for i*n vivo* applications. The FLEX technique has been shown to separate MT effects from exchange transfer in a rapidly exchanging paraCEST system (i.e. EuDOTA-$(glu)_4$ with a resonance frequency of 50 ppm from water at 37 °, and with an exchange rate of the water molecules to the bound water of $19 \times 10^3\ s^{-1}$) by combining both fast labelling through the use of selective excitation pulses and separation of the MT effects using time-domain analysis (Figure 21) (91). The decay rate of the FLEX FID is caused by the combined effects of exchange rate and transverse

relaxation $T_2$*. The time-domain fit yielded an exchange rate of 21 x $10^3$ $s^{-1}$ for EuDOTA-$(glu)_4$ in 4% agarose and 19 x $10^3$ $s^{-1}$ for EuDOTA-$(glu)_4$ in solution.

### 17.3.2. In vivo imaging of paraCEST agents using FLEX

Previous studies using FLEX demonstrated the possibility of using time-domain analysis to separate MT effects from exchange transfer in solutions of paraCEST agents.This approach has shown the utility of FLEX-MRI to monitor the distribution of paraCEST agents in mice bladder and kidney (92). *In vivo* the signal of EuDOTA-$(glu)_4$ was obscured by the broad MT component at 50 ppm; however, time-domain analysis yielded the three main signal components, as in Fig. 21: a broad MT component coming from semi-solid bound water (see section 5), the paraCEST component and the free water signal. The FLEX method also allowed measurements of the exchange rates by fitting the time-domain signal for the individual components. The exchange rate of EuDOTA-$(glu)_4$ was found to be 14.3 x $10^3$ $s^{-1}$ for bladder and 15.3 x $10^3$ $s^{-1}$ for kidney. The PTR maps obtained in the same structures were very homogeneous demonstrating that the paraCEST agent is uniformly distributed in urine. However, PTR intensity reflecting the FLEX signal at 50 ppm was found to be higher in the bladder mainly because of its large aqueous content and the relatively low macromolecular proton concentration.

### 17.3.3. Detection of rapidly exchanging compounds using on-resonance FLEX

Rapidly exchanging protons can be detected using the on-resonance version of the FLEX sequence, which requires the application of the excitation pulses directly at the water resonance (94). Validation of this approach has been shown for the intermediate to fast exchange regime by measuring exchange rates up to 10,000$s^{-1}$. A disadvantage of this approach is the generation of multiple types of water echoes when a large number of on-resonance RF pulses (i.e. $\omega_{rf} = 0$) are applied in the presence of spatial field inhomogeneities, thereby causing the water magnetization to be slightly off-resonance in parts of the image and evolving in between the excitation pulses.

## 17.4. Advantages

Unlike saturation transfer experiments where the length of the RF pulse is long enough to achieve a steady-state saturation, frequency labelling with the FLEX sequence is on the order of microseconds, enabling imaging of rapidly exchanging protons on molecules. The chemical evolution principle used here allows simultaneous labelling of multiple exchanging

components. In addition, variation of the time parameters in the pulse sequence enables exchange rate filtering. Finally, the use of time domain analysis offers the possibility of separating different magnetization transfer mechanisms based on the speed of the decay of the time domain signal.

### 17.5. Disadvantages

Sensitivity could be lost when the train of excitation pulses labels a significant fraction of the water pool. A compromise between excitation efficiency and direct water saturation effects requires lengthening of the RF pulses which reduces the detection efficiency of rapidly exchanging protons (fast exchanging protons exchange their magnetization before they can be labelled). In addition, in the case of diaCEST species, the application of RF pulses is closer to the water resonance causing unavoidable direct water effects. Compared to standard CEST acquisition, the FLEX sequence requires a large number of LTM modules to produce modulations in the water signal, which increases the SAR up to six times compared to the conventional CEST approach. A few technical considerations arise when the FLEX sequence is applied *in vivo*. The duration of the excitation pulses is strongly linked with their irradiation amplitude. Thus, shorter pulses will require higher $B_1$ amplitude resulting in SAR constraints and an increase in the contribution of the semisolid MT to the measured signal. Furthermore, for studies of humans, hardware limitations also impact the choice of the excitation pulses' duration. Finally, similarly to saturation transfer approaches, $B_0$ and $B_1$ inhomogeneities modulate the water signal in an independent manner, leading to artefacts.

## 18. A STEAM inversion transfer technique to measure the exchange rates on the downfield part of the proton spectrum.

### 18.1 Concept

Measurements of exchange rates have been facilitated by using a short TE sequence (stimulated echo acquisition mode (STEAM)) in which a non-water suppressed metabolite cycling (MC) preparation was combined with an inversion transfer technique (138).

### 18.2 Pulse sequence

The concept behind metabolic cycling (MC) consists of selectively inverting the resonance frequencies either upfield or downfield from the water peak using band-pass inversion pulses, and was originally introduced to detect metabolites without the need for water suppression, through the subtraction of two measurements, with and without the application of these inversion pulses (140). In this particular implementation, the MC sequence, followed by a short TE STEAM-based voxel-selection readout, was preceded by a frequency selective pulse for inverting the water resonance with varying inversion delay times. For quantification, the water-reference signal was measured without MC or water suppression. The exchange rates were calculated by modelling the longitudinal magnetization of the inverted water using the Bloch-McConnell equations for a two-pool system. For modelling the initial magnetization was set to zero, followed by a subsequent inversion pulse and recovery. Four equal components were modelled to account for exchange and recovery during the STEAM sequence.

### 18.3 Applications

The sequence was implemented in human brain studies using a 9.4 T whole body MRI scanner. Using, separation of high field peaks in the 8.2 to 8.5 ppm region (amide protons) was possible and exchange rates were found to vary from 0.74 to 13.8 Hz (138). Many of the downfield resonances were found to be quite broad, indicating several underlying components, with potentially different exchange rates for different components. For simplification, in this study, only single peaks with visible resonances yielded measurable quantities. Additionally, occipital and white matter regions yielded similar exchange rates, which the authors attributed to similar proportions of metabolites and macromolecular molecules in both regions. The 5.8 ppm peak, which was previously tentatively assigned to urea by the same group, had a mean exchange rate of 7.4 Hz. Limited contributions were found from metabolites in the upfield as compared to the downfield region of the spectrum.

### 18.4 Advantages

An in-depth investigation of downfield metabolites as well as comparison of downfield and upfield concentration was possible using this scheme with increased peak separation because of the use of an ultra-high field human MRI scanner.

### 18.5 Limitations

The measurable exchange rates were limited by several factors, including TE, and the global water inversion pulse duration. In particular, these limitations permitted detection of only slowly exchanging protons since fast exchanging spins would no longer be refocused and could therefore not be detected. Additionally, faster exchange species require shorter inversion times which were not achievable because of hardware limitations. Finally, the duration of this experiment was relatively long, which limits its application only to research settings.

# Measuring exchange rates with methods based on solutions of BM equations

The next series of sequences and methods reviewed here focus on quantification. They are mostly used with an imaging readout, and include various techniques to estimate the main parameters underlying the BM equations.

## 19. Quantifying exchange rates in CEST using the saturation time and saturation power dependencies of the CEST effect

### 19.1. Concept

The dynamic behaviour of a CEST experiment is analogous to those of off-resonance $T_{1\rho}$ and $T_{2\rho}$ experiments and only differs in the initial orientation of the z-magnetization. Thus, a simple way to describe saturation transfer experiments is by adapting the theoretical calculations used for $T_{1\rho}$ experiments and designing techniques to measure exchange rates by exploiting the effect of the exchange rate on the water signal intensity as a function of saturation time and saturation power.

### 19.2. Pulse sequence

The pulse sequence generally used consists of a continuous wave saturation pulse followed by a fast read-out (61),(43).

### 19.3. Theory

The solution of the BM equations yields the water magnetization as a function of the experimental RF saturation parameters such as the frequency offset of the RF irradiation, the intensity $\omega_1$ and the pulse length $t_{sat}$. In the case of sufficient separation between the water and the exchangeable proton resonances (i.e. no direct saturation of the water resonance when irradiating the exchangeable proton pool) the water signal intensity at steady-state conditions can be described as follows (61):

$$\text{PTR} = MTRasym = \frac{S_w(-\Delta\omega_{sw}) - S_w(+\Delta\omega_{sw})}{S_{0w}} = \frac{k_{sw}.\alpha.x_{CA}}{k_{sw}.\alpha.x_{CA}+R_{1w}} \left[1 - e^{-(R_{1w}+k_{sw}.\alpha.x_{CA})t_{sat}}\right]$$

(Equation 44)

where $x_{CA}$ is the fractional concentration of the exchangeable protons relative to water proton concentration (111 Mol), $t_{sat}$ is the saturation time and $\alpha$ is the efficiency for saturating the exchangeable spin system given as follows (95):

$$\alpha = \frac{\frac{\Delta\omega_{sw}^2}{\Delta\omega_{sw}^2+\omega_1^2}k_{sw}+R_{2b}}{k_{sw}+R_{2b}}\frac{\omega_1^2}{\omega_1^2+k_{sw}(k_{sw}+R_{2b})} \approx \frac{\omega_1^2}{\omega_1^2+k_{sw}(k_{sw}+R_{2b})} \quad \text{(Equation 45)}$$

where $\omega_1 = \gamma.B_1$ is the irradiation amplitude, $k_{sw}$ is the exchange rate constant and $R_{2b}$ is the transverse relaxation rate of the CEST pool.

A simplified form of Equation 44 is obtained under steady state conditions, i.e. when $t_{sat} \to \infty$: $MTRasym = \frac{k_{sw}.\alpha.x_{CA}}{k_{sw}.\alpha.x_{CA}+R_{1w}}$ (as per Equation 44) which in turn is fitted at several irradiation amplitudes $\omega_1$ to calculate an exchange rate. This approach is known as quantification of exchange rates at various saturation powers (termed QUESP). Alternatively, exchange rates can be quantified by varying the saturation time $t_{sat}$ (termed QUEST experiment) at constant saturation power $\omega_1$ (see Equation 44) (61). Note that in that case, the complete solution for α, as shown in equation 45, needs to be used for accurate estimates.

Initially, quantitative CEST experiments were performed in fully relaxed systems or systems which are close to a full steady state in order to estimate physiological parameters such as the pH (i.e. the case of the original QUEST/QUESP theory). It is possible, however, to speed-up the acquisition and reduce scanning time by using an arbitrary initial magnetization, i.e. $Mi \neq Mo$. In that case, the expression for MTRasym is replaced by the following:

$$\text{MTRasym} = \frac{S_W(-\Delta\omega_{SW})-S_W(+\Delta\omega_{SW})}{S_{0w}} = \frac{k_{sw}.\alpha.x_{CA}}{k_{sw}.\alpha.x_{CA}+R_{1w}} + \left(\frac{M_i}{M_0}-1\right).e^{-R_{1w}t_{sat}} - \left(\frac{M_i}{M_0}-\frac{R_{1w}}{R_{1w}+k_{sw}.\alpha.x_{CA}}\right).e^{-(R_{1w}+k_{sw}.\alpha.x_{CA})t_{sat}} \quad \text{(Equation 46)}$$

Note that Equation 46 is equal to Equation 44 when $M_i = M_0$.

The QUEST/QUESP formulas as described above are only valid in the narrow-peak limit, i.e. $\Delta\omega_{sw} \ll \Gamma$ where Γ is the $B_1$-dependent width of the CEST peak defined as follows (95):

$$\Gamma = 2.\sqrt{\frac{R_{2s}+k_{sw}}{k_{sw}}.\omega_1^2 + (R_{2s}+k_{sw})^2} \qquad \text{(Equation 47)}$$

Outside of this regime accurate estimates can be only obtained by numerically fitting the BM equations, and applications of the theory given above would yield erroneous measurements of exchange rates.

### 19.4. Applications

Initial experimental work for calculating exchange rates via MRI was performed for the amide protons of poly-L-lysine (PLL) and of SPD-5 dendrimers (61). The exchange rates were calculated using QUEST and QUESP techniques with the experimental parameters designed to fulfil the following criteria: (1) $k_{sw} \ll \omega_1$, (2) $k_{sw} \ll \Delta\omega$. However, the QUESP and QUEST formulas were later rewritten for providing more precise estimates of exchange rates in the case of weak labelling (i.e. $k_{sw} \ll \omega_1, \alpha < 0.5$) and non-equilibrium initial magnetization conditions (43). The revisited theory was validated by calculating the exchange rates of EuDOTAM-Gly, which possess amide protons resonating at 55 ppm (40). A series of multi-$B_1$ experiments were performed and the measured exchange rates were determined to be 3000 Hz at 13.4 °C and 7000 Hz at 23.8 °C respectively. It is worth noting that when the original QUEST/QUESP formulas were used, the exchange rates of EuDOTAM-Gly were underestimated by 2000 and 3200 Hz respectively. Another point of importance is that a QUESP analysis can be applied without *a priori* knowledge of the concentration of the CEST agent, in contrast to QUEST where the effect of concentration cannot be uncoupled from alterations in exchange rate at a single irradiation amplitude.

In the following, a few technical considerations are discussed concerning exchange rate selectivity and optimal sampling of the experimental parameters. In principle QUESP requires the collection of z-spectra at various $B_1$ values. However, bias is introduced in the measurements when low $B_1$ is used for calculating the exchange rates of fast exchanging species or high $B_1$ in the case of slowly exchanging protons. Therefore, as a rule of thumb the labelling efficiency $\alpha$ needs to be sampled by acquiring experimental data at various irradiation amplitudes and at least one irradiation amplitude should produce $\alpha$ =0.5 (i.e. $B_1 = k_{sw}/\gamma$, with $\alpha$ defined in Equation 45) while the saturation time $t_{sat}$ should cover the saturation build-up below $2T_{1w}$.

## 20. QUEST and QUESP with pulsed irradiation

### 20.1. Theory

A challenge for CEST imaging is its lack of specificity for separating exchanging protons from different molecules, mainly because exchange processes are detected indirectly through the water signal. In addition, if chemical exchange is sufficiently fast, it causes coalescence of the rapidly exchanging protons with water. Thus, the irradiation scheme needs to be optimised to minimise direct water saturation and to increase exchange rate selectivity by using pulsed-CEST approaches (53),(96),(97). These strategies however are more complex *in vivo* because of other contributing factors such as magnetization transfer from lipids, proteins, metabolites and other semisolid structures.

To measure the exchange rate in a pulsed CEST experiment (see section 7) the magnetization transfer ratio asymmetry (MTRasym = $\frac{M_{sat}(-\Delta\omega)}{M_0}$ - $\frac{M_{sat}(+\Delta\omega)}{M_0}$ ) can be used under different $B_1$ amplitudes for steady-state conditions (using Equations 18, 19) or various saturation times $t_{sat}$ of a train of RF pulses applied at a given $B_1$ amplitude (using Equation 20) (54).

For $M_{sat}(-\Delta\omega)$: $\overline{R_{1\rho}} = \overline{R_{eff}} = R_{1w} + (R_{2w}-R_{1w}) \cdot c_1 \frac{\omega_1^2}{\omega_1^2+\Delta\omega^2 \cdot c_2^2}$ (Equation 48)

and for $M_{sat}(+\Delta\omega)$:

$$\overline{R_{1\rho}} = \overline{R_{eff}} + \overline{R_{ex}} = R_{1w} + (R_{2w}-R_{1w}) \cdot c_1 \frac{\omega_1^2}{\omega_1^2+\Delta\omega^2 \cdot c_2^2} + x_s k_{sw} \cdot c_1 \frac{\omega_1^2}{\omega_1^2+k_{sw}^2 \cdot c_2^2}$$

(Equation 49)

where $c_1 = \frac{\sigma\sqrt{2\pi}}{t_{sat}}$ and $c_2 = c_1\sqrt{\sqrt{2}}$ and $\sigma$ is a factor that depends on the width and the length $t_{sat}$ of the Gaussian pulses, and the overbars represent the average values over time.

A solution to these equations can be found under certain conditions and assumptions.

Assuming steady state conditions:

1st assumption: no exchange during the interpulse delay

Pulse sequences for measuring exchange rates between proton species

MTRasym = $\frac{R_{1w}}{M_0}$ [ $\frac{1}{\beta}$ - $\frac{1}{\overline{R_{ex}}+\beta}$] where $\beta = \overline{R_{eff}} + R_{1w}(1 - DC)$ and DC is the duty cycle defined as:

$$DC = t_{sat}/(t_{sat} + t_d) \quad \text{(Equation 50)}$$

2nd assumption: $R_{1w}$ recovery and exchange during interpulse delay

$$\text{MTRasym} = \frac{R_{1w}(t_d+t_{sat})\cdot(\overline{R_{ex}}.t_{sat}+ \overline{R_{ex}}/k_{sw}(1-R_{1w}t_d))}{(\overline{R_{eff}}.t_{sat}+R_{1w}t_d)\cdot(\overline{R_{eff}}\cdot t_{sat}+\overline{R_{ex}}\cdot t_{sat}+ \overline{R_{ex}}/k_{sw}(1-R_{1w}t_d))}$$

(Equation 51)

where $\overline{R_{eff}} = R_{1w} + (R_{2w}\text{-}R_{1w}) \cdot c_1 \frac{\omega_1^2}{\omega_1^2+\Delta\omega^2\cdot c_2^2}$ and $\overline{R_{ex}} = x_s k_{sw} \cdot c_1 \frac{\omega_1^2}{\omega_1^2+k_{sw}^2\cdot c_2^2}$

Assuming non-steady-state conditions:

$$\text{MTRasym} = \frac{M_{initial}}{M_0} \cdot u \cdot e^{-\overline{R}_{eff}(\Delta\omega)t_{sat}\cdot n}\left(1 - e^{-\overline{R}_{ex}(\Delta\omega)t_{sat}\cdot n}\right) + \text{MTR}_{\text{asym}}^{ss} + u \cdot e^{-\overline{R}_{eff}(\Delta\omega)t_{sat}\cdot n}\left(\frac{M_{WZ}^{SS}(\Delta\omega)}{M_0} \cdot e^{-\overline{R}_{ex}(\Delta\omega)t_{sat}\cdot n} - \frac{M_{WZ}^{SS}(-\Delta\omega)}{M_0}\right)$$

(Equation 52)

where $u$ and $\text{MTR}_{\text{asym}}^{ss}$, $M_{wz}^{ss}(\Delta\omega)$, $M_{wz}^{ss}(-\Delta\omega)$ are substituted using Equations 48-50 and Equations 21 and 22.

### 20.2. Advantages

For limited scanning time where only a certain number of offsets can be acquired, the rederived QUESP formula (43) yields exchange rate measurements close to the full BM fit (41).

### 20.3. Disadvantages

QUEST and QUESP techniques suffer from the relatively small sensitivity of the exchangeable peak due to the low concentration of the contrast agents which is further reduced at high exchange rates because of the broadening of the resonance. It is however relatively straightforward to measure the exchange rates *in vitro*, where the only competing

effects are direct water saturation which depends on the frequency difference between the solute proton and water, the exchange rate, the saturation bandwidth, and the water linewidth. In contrast, when these measurements are performed *in vivo*, other contributing factors such as MT and exchange relayed NOEs compete with the CEST effect. Finally, in the case of a QUESP experiments, the maximum allowed irradiation amplitude for *in vivo* applications is limited by the maximum deposition of RF into the body, estimated by the calculated SAR level.

## 21. The Omega plot method

### 21.1. Concept

A modified version of QUESP was developed for measuring the exchange rates of paraCEST agents such as EuDOTA-$(gly)_4^-$ and EuDOTA-$(gly\ OEt)_4^{3+}$ by fitting CEST spectra collected at a single frequency offset with various irradiation amplitudes (98).

### 21.2. Theory

The rate of loss of the water magnetization under continuous RF irradiation depends on the exchange rate constant $k_{sw}$ , the saturation of the exchanging protons (i.e. 1-$\frac{M_Z^{SS}}{M_{z0}}$) and the longitudinal water relaxation $R_{1w}$. The latter term does not play any role however under steady-state conditions, and a set of equations can then be written as followed. Let us consider the product of $k_{sw}.\left(1-\frac{M_Z^{SS}}{M_{z0}}\right)$, which can be described by (98):

$$R_{CEST} = \left(1-\frac{M_Z^{SS}}{M_{z0}}\right).k_{sw} = \frac{\omega_1^2/k_{sw}^2}{1+\omega_1^2/k_{sw}^2} \qquad \text{(Equation 53)}$$

After a long pre-saturation period, the water magnetization reaches steady-state and becomes equal to $\left(1-\frac{M_Z^{SS}}{M_{z0}}\right).R_{1w} = \frac{c}{[\mathrm{H_2O}].}\frac{M_Z^{SS}}{M_{z0}}R_{CEST}$, which can be written using Equation 53 as follows (98):

$$\frac{M_Z^{SS}}{M_{z0}\text{-}M_Z^{SS}} = \frac{[\mathrm{H_2O}]}{c}k_{sw}.R_{1w}\left(\frac{1}{k_{sw}^2}+\frac{1}{\omega_1^2}\right) \qquad \text{(Equation 54)}$$

With *c* = concentration of exchangeable protons.

A plot of $\frac{M_Z^{SS}}{M_{Z0}\text{-}M_Z^{SS}}$ versus $\frac{1}{\omega_1^2}$ produces a line with a slope of $\frac{[H_2O]}{c}\ k_{sw}.R_{1w}$ and a y-axis intercept of $\frac{[H_2O]}{c\ k_{sw}}\ .R_{1w}$ while the x-axis intercept at $\frac{M_Z^{SS}}{M_{Z0}\text{-}M_Z^{SS}}$ = 0 provides a direct read out of the exchange rate (see Figure 22).

### 20.3. Application

The Omega plot technique has been mainly applied to calculate the exchange rates at which water leaves the co-ordination sphere of $Eu^{3+}$ of EuDOTA-$(gly)_4^-$ and EuDOTA-(gly OEt)$_4^{3+}$. Due to hyperfine shift effects of the paramagnetic $Eu^{3+}$ the bound-water shifts from 57 ppm at 4 °C to 38 ppm at 38°C. In addition, the bound-water exchange rate changes about one order of magnitude over this temperature range. The calculated lifetimes $\tau_M$ of EuDOTA-$(gly)_4^-$ were found to be 896 $\mu s, 880\ \mu s, 641\ \mu s, 210\ \mu s\ and\ 93\ \mu s$ for 4°C, 10 °C, 15 °C, 25 °C and 37 °C respectively (98).

### 21.4. Advantages

The omega plot method gives the exchange rates independently of the concentration of the agent presented in solution. Additionally, this method cancels spillover effects, since it is based on the corrected inverse metric approach of a steady state Z-spectrum.

### 21.5. Disadvantages

When measuring the exchange rates *in vivo* other contributing factors such as MT and exchange relayed NOEs compete with the CEST effect. In addition, for *in vivo* measurements, the mathematical equations need to be rewritten while considering MT (i.e. to be extended from a two-site exchange model to a three-site exchange model). Finally, similarly to a QUESP experiment, the maximum allowed irradiation amplitude for *in vivo* applications is limited by the maximum deposition of RF into the body, estimated by the calculated SAR level.

## 22. The reciprocal linear QUEST analysis method

### 22.1. Concept

Several methods have been developed to estimate exchange rates from CEST data, including the fitting of z-spectra with BM equations which is the gold standard technique, or indeed the use of QUEST and QUESP approaches for obtaining faster estimates of exchange rates. Here,

the reciprocal QUEST/QUESP method is reviewed which recasts the original QUEST/QUESP formulas in a linear form and has been shown to yield exchange rates with an improved accuracy (99),(100).

## 22.2. Theory

Under steady-state conditions, i.e. $t_{sat} \rightarrow \infty$, Equation 44 simplifies to:

$$\frac{S_W(-\Delta\omega_{SW})\text{-}S_W(+\Delta\omega_{SW})}{S_{0W}} = \frac{k_{SW}.\alpha.x_{CA}}{k_{SW}.x_{CA}+R_{1W}} \qquad \text{(Equation 55)}$$

Then the ratio of CEST measurements at a short saturation time $t_{sat} \ll \infty$ relative to a very long saturation time is only dependent on an exponential term as shown below (99):

$$\frac{[\frac{S_W(-\Delta\omega_{SW})\text{-}S_W(+\Delta\omega_{SW})}{S_{0W}}]_{t_{sat}\ll\infty}}{[\frac{S_W(-\Delta\omega_{SW})\text{-}S_W(+\Delta\omega_{SW})}{S_{0W}}]_{t_{sat}\rightarrow\infty}} = 1 - e^{-(R_{1W}+k_{SW}.\ x_{CA})t_{sat}} \qquad \text{(Equation 56)}$$

which can be written as follows (99) :

$$\frac{1}{-\ln\left[1-\frac{[\frac{S_W(-\Delta\omega_{SW})\text{-}S_W(+\Delta\omega_{SW})}{S_{0W}}]_{t_{sat}\ll\infty}}{[\frac{S_W(-\Delta\omega_{SW})\text{-}S_W(+\Delta\omega_{SW})}{S_{0W}}]_{t_{sat}\rightarrow\infty}}\right]} = \frac{1}{(R_{1W}+k_{SW}.\ x_{CA})t_{sat}} \qquad \text{(Equation 57)}$$

The original QUEST method employs fitting of the exponential term of Equation 56 at various saturation times. In contrast, the Reciprocal Linear QUEST (RLQUEST) method uses a plot of Equation 57 to determine the exchange rate.

A linear version of QUESP can be also obtained by rearranging the formulae for the omega-plot method, which works under steady-state conditions, to obtain Equations 58 and 59 (100):

$$\frac{M_Z^{SS}}{M_{Z0}\text{-}M_Z^{SS}} = \frac{R_{1W}k_{SW}}{x_{CA}}\left(\frac{1}{k_{SW}^2} + \frac{1}{\omega_1^2}\right) \qquad \text{(Equation 58)}$$

which is a linear equation if x = $\frac{1}{\omega_1^2}$ and y = $\frac{M_Z^{SS}}{M_{Z0}\text{-}M_Z^{SS}}$

Pulse sequences for measuring exchange rates between proton species

$$\frac{M_{Z0}\text{-}M_Z^{SS}}{M_Z^{SS}} = \frac{x_{CA}k_{SW}}{R_{1W}}\text{-}k_{SW}^2\left(\frac{M_{Z0}\text{-}M_Z^{SS}}{M_Z^{SS}}\ \frac{1}{\omega_1^2}\right) \qquad \text{(Equation 59)}$$

$$\omega_1^2\frac{M_Z^{SS}}{M_{Z0}\text{-}M_Z^{SS}} = \frac{\omega_1^2 R_{1W}}{x_{CA}k_{SW}} + \frac{R_{1W}k_{SW}}{x_{CA}} \qquad \text{(Equation 60)}$$

The chemical exchange rate is measured using Equation 60 (a plot of $\frac{M_Z^{SS}}{M_{Z0}\text{-}M_Z^{SS}}\ versus\ \frac{1}{\omega_1^2}\ should\ be\ linear\ with\ a\ slope\ of\ \frac{R_{1W}k_{SW}}{x_{CA}}$)

## 22.3. Applications

Numerical simulations and experimental data obtained from iopromide were used to validate the reciprocal linear QUEST technique (RL-QUEST). More precisely, the exchange rate constants of the amide protons resonating at 4.2 and 5.6ppm were found to be 133 $s^{-1}$ and 1131 $s^{-1}$ respectively using a BM fit. In contrast, the exchange rate constants of amide protons at 5.6ppm were underestimated at 800 $s^{-1}$ using both the QUEST and RL-QUEST methods (99). This might be due to the missing $\alpha$ factor in Equations 55 and 57. Equations 59 and 60 were also validated in the same samples. According to the results a deviation from linearity was observed at high saturation powers because the chemical shift difference between the water and the CEST agent was much larger than the saturation power. Additionally, a deviation from linearity was observed at very low powers because of insufficient saturation of the exchanging protons. In comparison with the omega plot method Equations 59 and 60 produced more accurate estimates of fast exchange rates. However, the omega plot was more accurate and precise in estimating the exchange rates of the slowly exchanging amide protons of iopromide.

## 22.4. Advantages

Fits of RL-QUEST are much faster than nonlinear fitting methods such as the standard QUEST, since RL-QUEST has a zero y-intercept. It can thus be performed with only two CEST measurements, which then also reduces acquisition time as compared to non-linear QUEST, as well as simplifying its data analysis. For accurate estimates of exchange rates with RL-QUEST a minimal saturation power is required for optimal saturation efficiency.

### 22.5. Disadvantages

Similarly to QUEST, the RL-QUEST approach requires the concentration of the CEST agent to be provided for the fit, which can be challenging for *in vivo* MRI studies, especially if the concentration of the solute is changing during the measurement e.g. due to pathological changes.

## 23. Quantification of the chemical exchange rate $k_{ws}$ with RF saturation time dependent ratiometric analysis QUESTRA

### 23.1. Concept

Another linear variant of the QUEST method is QUESTRA, in which the ratio of water signals in a CEST / Z spectrum at both positive and negative frequencies is evaluated. Simulations showed that only one CEST spectrum is required for QUESTRA analysis; thus it can be much faster than RL-QUEST (101). However, calculation of the exchange rates based on one experimental dataset is more prone to errors. Therefore, multiple saturation times improve the quality of the fitting.

### 23.2. Theory

Because of the equal relaxation recovery and spillover effects of both label (at the resonance frequency of the exchangeable protons) and reference scans (at the opposite frequency to that of the exchangeable proton), the MTR asymmetry ratio can be used to cancel out these effects. It can be shown that QUESTRA approaches steady state exponentially at a rate equal to the reverse exchange rate constant $k_{ws}$ and simplifies the QUEST analysis by improving its precision and reproducibility (e.g. in the presence of RF irradiation, spillover effects render QUEST analyses susceptible to errors). QUESTRA addresses this by taking the ratio at which both CEST signals, represented by MTRlabel and MTRref, approach their steady-state, which is defined as follows (101):

$$QUESTRA\,(tsat) = \frac{\left[1-\frac{MTR_{label}(tsat)}{MTR_{label_{ss}}}\right]}{\left[1-\frac{MTR_{ref}(tsat)}{MTR_{ref_{ss}}}\right]} = e^{-(R_{1w}+k_{sw}.\,x_{CA})t_{sat}}/e^{-R_{1w}t_{sat}} = e^{-k_{sw}.\,x_{CA}t_{sat}}$$

(Equation 61)

$$k_{sw} = \frac{-1}{t_{sat}x_{CA}}.\ln\,(QUESTRA)$$

(Equation 62)

### 23.3. Pulse sequence

The QUESTRA pulse sequence requires a long relaxation delay and a CW RF saturation module of adjustable duration $t_{sat}$.

### 23.4. Applications

Simulated data were used to evaluate QUESTRA in comparison with the QUEST method, demonstrating its improved performance in measuring exchange rates. More precisely, QUEST analysis was found to be sensitive to changes in $T_{1w}$ and $T_{2w}$, $B_1$ and chemical shift while QUESTRA was more robust provided that the RF irradiation amplitude and chemical shift offsets were not too small (i.e. less than 0.3 $\mu T$ and less than 1.00 ppm from the water peak at 4.7T) (101).

In the same study, the QUESTRA approach has also been applied for calculating the exchange rates of gel samples containing creatine at various pH values. A linear relationship of the CEST contrast with saturation times was observed. Again, the contrast was found to be insensitive to sample parameters such as $T_{1w}$, $T_{2w}$ and irradiation amplitude $B_1$ while chemical exchange in creatine was found to be base-catalysed (101).

### 23.5. Disadvantages

QUESTRA does not account for overlapping CEST effects or asymmetric effects such as the endogenous MT observed *in vivo*.

**<<< Insert Figure 22 here >>>**

## 24. Quantification of exchange rates using Magnetic Resonance Fingerprinting

### 24.1. Concept

A CEST magnetic resonance fingerprinting (MRF) method has also been implemented for quantifying the exchange rates and volume fractions of amine, amide and semi-solid protons in healthy rat brains. To extract this information a multi-power acquisition was used in which the signal trajectories were matched to a dictionary of simulated signals using BM equations (102), (103). This approach offers a few advantages compared to other techniques including sensitivity, specificity and speed (104). However, it also suffers from serious drawbacks such

as the relatively poor fingerprinting trajectory efficiency which can cause incorrect quantification of exchange rates or agent concentration in soluble samples (105).

### 24.2. Pulse sequence

The MRF method uses variations of the image acquisition parameters to generate unique signal trajectories for different tissues. The experimentally measured trajectories are then matched to a simulated dictionary of signals created using the BM equations. In one study, for example, a CEST-MRF acquisition protocol consisted of single-slice, single-shot gradient echo EPI images, with the saturation pulse applied at 3.0ppm off-resonance and the irradiation amplitude pseudo-randomly varied to produce data in a range between 0-6 $\mu T$ *in vitro* or 0-4 $\mu T$ *in vivo* (Figure 23) (103).

**<<< Insert Figure 23 here >>>**

### 24.3. Applications

Exchange rate measurements of L-arginine samples prepared at various concentrations and pH values were obtained using CEST-MRF and QUESP analysis. It was confirmed again that chemical exchange in L-arginine is base-catalysed and the calculated exchange rates were in good agreement for both methods (102). An average exchange rate constant of amide protons in the rat brain cortex was measured to be 36.6 $s^{-1}$ and the semi-solid proton pool volume fraction in white matter was 11.2 % compared to 7.6 % for grey matter. Another application of MRF is the MRF-SPEM technique which uses a reduced dataset by separating the exchange rates in subgroups (137). Similarly to other MRF techniques, the RF saturation frequency offset, saturation amplitude, duration, and repetition time are varied here throughout the acquisition; however, an additional dataset with far off-resonance frequency is acquired to correct for magnetization transfer effects when calculating the exchange rate of amide protons. Calculation of the exchange rates was performed by fitting MRF-SPEM data using a two or a three pool BM equation model. The APT signals were found to be higher in grey matter than in white matter, presumably as a result of the higher content of mobile proteins and peptides. The apparent exchange rates of amide protons of grey and white matter were around 162 Hz and 365 Hz respectively (137).

### 24.4. Advantages

The acquisition time is significantly reduced compared to standard CEST acquisition (~ 2 mins for 30 iterations of saturation power). Moreover, the dictionary correlation plots showed that the CEST-MRF method provides improved discrimination of proton exchange rates and volume fractions compared to a single Z-spectrum. Compensation for $B_0$ field inhomogeneity could also be achieved by incorporating a range of $B_0$ shifts into the simulated dictionary.

### 24.5. Disadvantages

Because of the random selection of irradiation amplitudes, relatively poor discrimination of exchange rate and concentration was observed in correlation plots, implying strong similarities between signals arising from different tissues. In addition, the quality of the estimated parameters is strongly affected by the SNR level, which is generally very limited for all CEST-based methods. Finally, MRF requires a large multi-parametric fitting procedure for calculating the exchange rates. Therefore, improved discrimination should be obtained by optimizing the hyperspace of acquisition parameters to find those which most facilitate the discrimination.

## 25. Ratiometric approach to determine pH

### 25.1. Concept

A standard approach to make use of the pH-sensitivity of CEST to assess pH is to create a calibration curve of pH versus exchange rate. However, several parameters need to be measured such as the exchange rate and the $T_1$ value of the water protons. Alternatively, the ratio of the saturated magnetization from two or more exchange sites with different chemical shifts and pH dependencies in a single molecule could be used for eliminating the effects associated with $T_{1w}$, agent concentration and number of exchange sites on the agent. It is important to note that this method can be applied either to intrinsic (diaCEST) agents or to exogenous (diaCEST or paraCEST) contrast agents.

### 25.2. Theory

Under steady-state conditions, the water magnetization in a CEST experiment can be written as follows - see Equations 53 and 58 (106):

Pulse sequences for measuring exchange rates between proton species

$$\frac{M_z^{ss}}{M_{z0}} \approx \frac{1}{(1 + k_{ws}T_{1w})} \qquad \text{(Equation 63)}$$

where $k_{ws} = k_{sw}\,[Agent][n]$ is the exchange rate constant multiplied by the agent concentration and the number of exchange sites on the agent.

When two sites are present in a single molecule the ratio of the saturated water magnetization from both sites can be written using the following equation (106):

$$\frac{M_Z^{Site\,2}(M_{Z0}\text{-}M_Z^{ss})^{Site\,1}}{M_z^{Site\,1}(M_{Z0}\text{-}M_z^{ss})^{Site\,2}} = \frac{k_{sw}^{Site\,1}\,[Agent]^{Site\,1}n^{Site\,1}}{k_{sw}^{Site\,2}\,[Agent]^{Site\,2}n^{Site\,2}} \qquad \text{(Equation 64)}$$

A plot of Equation 64 as a function of pH could be used for measuring the pH of a solution containing the agent.

### 25.3. Applications

Validation of the proposed theory was performed in 5,6-dihydrouracil (106). This agent produces two peaks at resonance frequencies of 2.67 and 5.00 ppm. The pH dependence is different for each site although both sites exhibit a base-catalysed exchange effect. The same principle was explored in a more recent study where the CEST effect of amide and guanidyl protons was used to enhance pH-sensitivity (107). Guanidyl protons are abundant in arginine side chains of proteins and exchange with water protons at a much faster rate than the amide protons. Acidification under physiological conditions reduces the exchange rate of amide protons with water resulting in decreased CEST signal. At the same time the CEST effect between guanidyl protons and water depends strongly on the selection of irradiation amplitude. The pH enhanced image can be obtained as $\{S_{sat}(\alpha.B_1, 3.6\,ppm)\text{-}S_{sat}(B_1, 2.0\,ppm)\}/S_0$ where $\alpha$ is the ratio of the RF powers used for saturating the protons at 3.6 and 2.0 ppm. The pH enhanced contrast was evaluated in protamine samples (i.e. simple protein molecules) which contain both amide and guanidyl protons and *in vivo* validation was performed via inhalation of 10 % of $CO_2$ which yields a pH drop of 0.1-0.2 in the healthy rat brain or by scanning rats following MCAO, where a pH drop from 0.5 to 1 unit is observed in the ischaemic core (107). According to the findings of this study, the pH enhanced contrast outperforms the results of MTR asymmetry analysis by cancelling competing effects and minimising $B_0$ artefacts.

In another study, the ratio of CEST contrast from amide and amine protons was used to measure absolute pH using the CEST effect at 2.75 and 3.5 ppm. Similarly to the two previous studies, the amide and amine effects change in opposite directions with increasing pH; thus the ratio of amide/amine protons is sensitive to pH (108). Amine/amide concentration independent detection was then defined as:

$$\text{AACID}=\frac{M_Z^{SS}(3.5\ ppm)xM_Z^{SS}(6.0\ ppm)\text{-}M_Z^{SS}(2.75\ ppm)}{M_Z^{SS}(2.75\ ppm)xM_Z^{SS}(6.0\ ppm)\text{-}M_Z^{SS}(3.5\ ppm)} \qquad \text{Equation 65}$$

The AACID analysis was based on three assumptions: (1) the ratio of exchangeable amide and amine protons remains constant throughout the brain during ischaemic conditions, (2) AACID is a linear function of pH *in vitro* and *in vivo*, (3) direct water saturation has a negligible effect on the AACID ratio (108). Application of AACID in stroke showed that the quantitative resolution of pH measurements is 0.14 pH units in contrast to MTR asymmetry analysis which detects pH changes of 0.2 pH units and greater. Another application of AACID was to detect tumour-selective acidification after ionidimine injection (LND), an anticancer agent which limits aerobic respiration in cancer cells by blocking pyruvate transport from the cytoplasm leading to energy depletion (109). LND treatment was shown to cause the amide peak to decrease significantly in tumour regions while amine protons resonating at 2.0 ppm and 2.75 ppm do not change substantially after LND treatment, which could help in localising brain cancer and monitoring tumour response to chemotherapy (109).

### 25.4. Advantages

The ratiometric approach is insensitive to variations in tissue $T_1$, $T_2$, macromolecular concentration, and concentration of amine and amide protons. Therefore, the obtained AACID contrast most likely reflects changes in chemical exchange rates which can be used for quantitative pH measurements. Finally, for calculating AACID three images are collected which further reduces the required scanning time.

### 25.5. Disadvantages

This ratiometric analysis enables calculation of pH via the CEST signal from multiple molecular sites; however, the soluble agent concentration needs to be known. *In vivo* applications of this approach might be more challenging because the concentration ratio of the two proton sites is unknown. In addition, one major limitation of this technique is the

high concentration of CEST agents required to generate large enough effects which in turn increases the toxicity of any injected agents *in vivo*. Furthermore, in the case of the ratiometric analysis used by Jin et al (107) and McVicar et al (108), the produced pH maps were obtained at high magnetic field. For the widely available clinical systems working at lower magnetic field, this method will be functioning at its limits because the amine/guanidinium proton exchange approaches the fast exchange regime for both species while direct water saturation will also be increased.

# Measuring exchange rates through relaxation

## 26. pCEST: Positive contrast using Chemical Exchange Saturation Transfer

### 26.1. Concept

Generally, the CEST contrast is negative i.e. upon application of off-resonance saturation pulses the water signal decreases in the presence of chemical exchange. However, positive exchange contrast can also be generated using a modification of the off-resonance spin lock experiment with simultaneous background signal suppression. This method requires the application of an inversion pulse followed by a series of saturation pulses in a way similar to the ASL concept of background suppression using multiple inversion pulses. Detection of a small but positive contrast is generally easier, and can be separated from other sources of signal reduction. In addition, a reduction in background noise and related physiological artefacts can lead to an increase in confidence in the generated CEST signal, of interest in this case.

### 26.2. Pulse sequence

Positive CEST contrast is generated by acquiring data using on-resonance and off-resonance RF irradiation. Here, an apparent relaxation constant is measured which is referred to as $R_{1\rho app}$ (110):

$$R_{1\rho app} = R_{1A}cos^2\theta + (R_{2A} + R_{ex})sin^2\theta \qquad \text{(Equation 66)}$$

where $R_{ex} = \frac{p_A p_B \Delta\omega^2 k_{sw}}{\Delta_B^2 + \omega_1^2 + k_{sw}^2}$.

A schematic of positive contrast generation using pCEST is shown in Figure 24. Following inversion, the magnetization returns to its steady state with rates that are different for RF ON (application of RF saturation pulses on resonance with the exchangeable protons) and RF OFF (control experiment with the RF saturation pulses moved to the opposite frequency side of the water peak at the same absolute offset as the exchangeable proton resonance). At $T_{null}$ ( $t_{sat} = T_{null}(\text{RF OFF})$) the signal with RF OFF is null, but the signal with RF ON is small and positive resulting in true positive contrast with CEST. The positive CEST contrast is defined as (110):

$$\text{pCEST} = \frac{M_z(RF\ ON) - M_z(RF\ OFF)}{M_0} \times 100\% \qquad \text{(Equation 67)}$$

It is worth noting that the steady-state magnetization is smaller when the RF is applied on-resonance compared to the off-resonance case because it depends on $p_B$, $\tau_B$, $R_{1A}$, $R_{1B}$, and $\omega_1$.

**<<< Insert Figure 24 here >>>**

### 26.3. Applications

$R_{1app}$ has been measured in a solution of EuDOTA(glyOEt)$_4^{3+}$(110). In the case where $\Delta_{RF} >> \omega_1$, (far off-resonance), $R_{1\rho app}$ is equal to the off-resonance $R_{1\rho}$ and when $\Delta_{RF} = 0$, $R_{1\rho app}$ (on resonance with water) is equal to $R_{2\rho}$. Moreover, the apparent rate $R_{1\rho app}$ increases if the frequency of the RF field approaches the frequency of the exchangeable protons and if $\omega_1$ is increased. It should be noted that pCEST requires experimental optimisation for identifying the maximum signal difference between on and off-resonance irradiation (i.e for the PARACEST solutions the optimum $\omega_1$ was 46 $\mu T$) because the null time depends on $T_1$, field strength, and direct saturation effects.

In the presence of MT, pCEST produced lower signal intensity. This is consistent with previous work showing that the presence of MT results in a decreased CEST effect because of the slower water/proton exchange in gels. Furthermore, the presence of an additional pool of protons (i.e. macromolecular protons) results in more exchange pathways and alters the dynamics of the system.

### 26.4. Disadvantages

In analogy with CEST experiments, pCEST is sensitive to $B_0$ and $B_1$ inhomogeneities. In addition, spatial variations of $T_1$ over the measured object may reduce the effectiveness of the background suppression, even when using an adiabatic pulse. Finally, to calculate $R_{1\rho app}$, multiple saturation durations are required resulting in six times longer acquisition times compared to the conventional CEST technique.

## 27. Using the dependence of the transverse relaxation rate on chemical exchange

### 27.1. Concept

$T_2$ agents are primarily paramagnetic substances. $T_2$ relaxation occurs via dipolar interactions between the water molecules and the paramagnetic centre. Recently, it was shown that diaCEST agents such as glucose also generate $T_2$ contrast. The magnitude of $T_2$ enhancement depends on the regime of chemical exchange and its magnitude reaches a maximum when $k_{sw}=\Delta\omega$. In the case of glucose, chemical exchange between water protons and OH groups of glucose reduces the measured $T_2$ values.

### 27.2. Pulse sequence

$R_2$ measurements can be obtained using a Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence (111). The basic experiment consists of a spin echo sequence with a 90°pulse for creating a transverse magnetization component $M_{xy}$ followed by a number of spin-echoes (delay-180°-delay) which show the decay of $M_{xy}$. This sequence is repeated n times while incrementing the number of spin-echo blocks. In general, an MLEV phase cycling is applied on the refocusing pulses for pathway selection (135). Signal acquisition is performed using a standard imaging readout (Figure 25).

**<<< Insert Figure 25 here >>>**

### 27.3. Applications

The exchange contribution to $R_2$ can be analysed using the Swift-Connick equation (112): $R_{ex} = k_{sw}\rho_B \frac{R_{2B}^2 + R_{2B}k_{ex}+\Delta_\omega^2}{(R_{2B}+k_{sw})^2+\Delta_\omega^2}$ where $R_{2B}$ is the transverse relaxation rate of the agent and $\rho_B$

is the mole fraction of the exchangeable protons. When $\Delta\omega \gg R_{2B}$ the Swift-Connick equation can be approximated by

$$r_{2ex} = \frac{\rho_B}{[glucose]} \frac{k_{sw}\Delta_\omega^2}{k_{sw}{}^2+\Delta_\omega^2} \qquad \text{(Equation 68)}$$

In the case of glucose, a linear relationship between glucose concentration and $R_2$ was observed. The exchange rate and the chemical shift can be estimated by fitting the relaxivity values of glucose at various concentrations. According to the results of the study proposing this method to assess exchange rates, chemical exchange in glucose is in the intermediate to fast exchange regime (17),(32).

### 27.4. Advantage

Spin-echo pulse sequences already exist in clinical MR systems and can be used directly to calculate the exchange rate through $T_2$ measurements.

### 27.5. Disadvantage

To be able to detect any variations in $T_2$ due to chemical exchange at clinical field strength large original $T_2$ values are required, limiting its application field to liquid-like tissues.

## 28. PRO-QUEST (PROgressive saturation for Quantifying Exchange rates using Saturation Times)

### 28.1. Concept

PRO-QUEST (PROgressive saturation for Quantifying Exchange rates using Saturation Times) provides measurements of exchange rates within a timeframe appropriate for clinical studies. Here, the off-resonance saturation pulses are interleaved with the acquisition of exchange-weighted images in a Look-Locker scheme. Exchange-weighted contrast is obtained by calculating the exchange relaxation rate from two independent acquisitions: one with a CEST RF pulse and the second without a CEST RF pulse. Validation experiments were conducted in solutions of various amino acids and in rat brain and the calculated exchange rates were in good agreement with standard QUEST/QUESP techniques (113), (41).

### 28.2. Pulse sequence

PRO-QUEST was implemented by inserting an off-resonance saturation module consisting of a single RF pulse before each readout module of a Look-Locker sequence so that the saturation effects progressively accumulate, while the measured signal is recovering due to longitudinal relaxation (Figure 26). To get accurate estimates of exchange rates, PRO-QUEST is applied with and without saturation modules. The longitudinal magnetization during a Look-Locker experiment is given below (113),(114):

$$M_{zd}(n\tau) = \frac{1-[(\cos\vartheta)^{n-1}\, e^{-(n-1)(\tau R_1)}]}{1-[(\cos\vartheta)\, e^{-(\tau R_1)}]} M_{zd}(\tau) + \mathrm{Meq}\,(1-e^{-(t_d R_1)})[\,(\cos\vartheta)^{n-1} e^{-(n-1)(\tau R_1)}]$$

(Equation 69)

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

The longitudinal water magnetization after n off-resonance saturation pulses ($M_{zsat}$) can be described as follows (113):

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

$$+ \mathrm{Meq}\,(1-e^{-(t_d R_1)})[(\cos\vartheta)^{n}\; e^{-n\left(\tau R_1 - t_{sat}(R_1 - R_{1\rho})\right)}] \qquad \text{(Equation 70)}$$

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

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 $\varphi$ being the angle between the effective field and the z-axis, $\Omega$ is the frequency offset with respect to water (i.e $\omega_{rf}$) and $\omega_1=\gamma B_1$ is the amplitude of the RF field.

The exchange rates are calculated using the following equation:

Pulse sequences for measuring exchange rates between proton species

$$\bar{R}_{1\rho} = \frac{1}{t_{sat}}\int_0^{t_{sat}} R_{1\rho}(t)\, dt = R_1 + \frac{1}{t_{sat}}\int_0^{t_{sat}} (R_2\text{-}R_1)\frac{\omega_1^2(t)}{\delta^2+\omega_1^2(t)}\, dt$$

$$+ \frac{1}{t_{sat}}\int_0^{t_{sat}} \rho_B\ k_{ex}\frac{\omega_1^2(t)}{k_{ex}^2+\omega_1^2(t)}\, dt = \overline{R_{eff}} + \overline{R'_{ex}} \qquad \text{(Equation 71)}$$

by substituting $\bar{R}_{1\rho}$ to Equation 70.

**<<< Insert Figure 26 here >>>**

### 28.3. Applications

The calculated exchange rates of metabolites such as glutamate, glutamine, taurine and alanine using PRO-QUEST were found to be within the intermediate-to-fast exchange regime (113). In addition, when the same sequence was applied in healthy and infarcted rats after 24 hours an improved imaging specificity to ischaemic acidification during stroke was obtained relative to standard APT-weighted imaging (113). Recently this sequence has been applied on a 3T MR scanner and according to the findings a true Rex remains elusive due to the combined R2 effects because of the relative small frequency separation between exchangeable protons and the water peak, which leads, even for amides, to a large direct water saturation effect (139).

### 28.4. Advantages

A reduction of scan time from 58 to 16min was 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 be obtained by repetition of the sequence without off-resonance saturation pulses.

### 28.5. Disadvantage

Sensitivity to $B_0$ imperfections could be enhanced as a single off-resonance frequency is used. Use of PRO-QUEST as a quantitative measurement method at clinical field strengths might be limited due to the small frequency separation between exchangeable groups and water at 3T.

# Pulse sequences that improve exchange rate sensitivity by suppressing competing effects

Amide protons resonate at 3.5 ppm downfield of water and their slow chemical exchange rate is compatible with saturation schemes at irradiation levels that can be used for human applications. Currently, APT imaging is being trialled in the clinic to map pH for predicting progression to infarction in stroke. Furthermore, APT contrast has been found to correlate with tumour grade, and enables the differentiation between tumour recurrence and radiation necrosis (27). However, *in vivo* APT imaging can be misinterpreted due to magnetization transfer effects involving semisolid protons in membranes and macromolecules that are induced by the RF irradiation required to generate the APT contrast. In addition, other sources of errors are introduced by overlapping CEST resonances at 3.5 ppm as well as from aliphatic protons on the opposite side of the Z-spectrum, when generating the contrast using a simple asymmetry analysis. This section describes the development of pulse sequences that aim to suppress these confounding effects in order to achieve an increased reliability in APT imaging.

## 29. Variable delay multiple pulsed saturation technique (VDMP)

### 29.1. Concept

This method describes a fast way of obtaining exchange-weighted contrast without the need to collect full Z-spectra. The VDMP sequence is similar to a pulsed-CEST experiment where a train of selective saturation pulses is interleaved with interpulse delays and the z-component of the water magnetization is detected using a fast MRI read-out. Here, the interpulse delay (i.e. the time from the end of the saturation pulse until the start of the next saturation module) is varied and the build-up curves obtained as a function of the mixing time can be used as an exchange-rate filter to distinguish exchange processes with different rates (115),(116),(117),(118). Similar to pulsed-CEST techniques the original VDMP approach was aimed at maximising CEST contrast while neglecting the interference of slowly exchanging protons mainly from semi-solid compounds or fast exchanging species such as amines and hydroxyls. More specifically, the MT contribution can be suppressed by acquiring two images with two different mixing times in which the MT intensity is the same (see Figure 27). The VDMP difference image is then obtained by subtracting the two

images;i.e. the first image is collected with a delay equal to zero (reference image) and the second image with a delay optimised to a specific exchange process.

### 29.2. Pulse sequence

**<<< Insert Figure 27 here >>>**

The pulse sequence consists of a block of four 180° pulses with a pulse width $t_{sat}$ and inter pulse delay $t_{mix}$ followed by a fast MRI readout (Figure 27). A phase cycle scheme together with the application of crusher gradients is used for destroying any residual transverse magnetization due to inaccuracies in flip angles and stimulated echoes.

For the on-resonance version of the VDMP sequence, a train of high-$B_1$ binomial pulses is applied on the water resonance (118). Then by varying the inter-pulse delay (i.e. mixing time) the exchangeable proton pools with different exchange rates can be distinguished and quantified based on their individual characteristic build-up patterns. It is worth noting that the on-resonance VDMP sequence is similar to the on-resonance FLEX sequence with zero evolution time between the pulses and longer pulse duration for labelling fast-exchanging protons. The measured signal is excitation-based and not frequency-selective as in FLEX; thus, the signal contains contributions from all the fast exchanging protons.

The on-resonance VDMP sequence is slightly modified and consists of two hard pulses of high-power with alternating phase. The phases of these RF pulses are cycled between each pulse to suppress any residual magnetization in the transverse plane and stimulated echoes as is done in the off-resonance version of VDMP .

A VDMP experiment requires the collection and subtraction of two images obtained at a particular frequency offset with the same irradiation amplitude and with two interpulse delays (i.e. the minimum allowed interpulse delay and an optimised delay to maximise the CEST effect or to minimize the contribution of MT). If $t_{sat} + t_{mix}$ is much smaller than the longitudinal relaxation time of the water $T_{1w}$, the proton transfer ratio (PTR) is obtained by summing the magnetization after n VDMP modules (117):

$$PTR = x.\lambda.\eta.\beta \qquad \text{(Equation 44)}$$

where $\eta = \sum_{i=1}^{n} e^{-nt_{mix}/T_{1w}}$ and $\beta = 1 - e^{-k_{sw}.t_{mix}}$ , $x$ is the concentration ratio between exchangeable protons and water protons, $\lambda$ is the saturation efficiency, and $\beta$ is the exchange transfer efficiency which describes the build-up process of the signal.

The VDPM sequence is optimised by varying the irradiation amplitude or by modulating the interpulse delay. For instance, different saturation efficiencies of the CEST pulses (e.g. obtained by varying the irradiation amplitude or duration) affect semisolid or mobile structures differently because their intrinsic $T_2$s are different. In short, the saturation efficiency is proportional to the irradiation amplitude and the absorption lineshape of protons which in turn is proportional to $T_2$ (116), (117). For semisolid components, the $T_2$ will always be shorter than the duration of the applied RF pulse (lasting generally a few ms). Thus, this system will be partially saturated compared to mobile proteins or small peptides with longer $T_2$s. Moreover, variations of the RF amplitude induce different sensitivities for detecting mobile proteins and lipids. For instance, when a weak irradiation amplitude is used, the signals from amide and aliphatic protons are detected as separate resonances. However, by increasing the saturation power the contribution from species with shorter $T_2$ values increases, resulting in a single broad line.

The build-up of the signal in a VDMP experiment contains contributions from the saturated exchangeable protons as well as the recovery of the magnetization due to $T_{1w}$. *In vivo* the measured build-up curves also contain signals from semisolid compounds or fast exchanging species. The concept in VDMP is that not only the shape and the amplitude of the irradiation pulse can be used to provide exchange-weighted contrast but also the interpulse delay. Initially, the primary consideration when choosing an interpulse delay in CEST studies is to avoid mean RF absorption by the body (SAR) constraints in clinical systems. However, when $t_{mix}$ is varied in a systematic fashion, chemical exchange processes can be distinguished from other saturation transfer phenomena. For instance, slowly exchanging protons require long interpulse delays and the build-up curves first increase and then later gradually decrease as $t_{mix}$ increases. In contrast, the rapidly exchanging protons (> 500 $s^{-1}$) have transferred almost

all of their magnetization during the labelling pulses, so their build–up curves will start at a maximum and then decrease as $t_{mix}$ increases (see Figure 28).

## 29.3. Applications

### 29.3.1. Slowly exchanging protons such as APT and relayed NOEs

Several model systems were used to validate the developed VDMP-CEST sequence including bovine serum albumin (BSA) in solution, cross-linked BSA obtained by heating the solution to high temperature, and L-glutamic acid. In addition, VDMP-CEST was applied *in vivo* on healthy rat brain (117). The optimised pulsed sequence was used as a $T_2$ filter by choosing the proper irradiation amplitude and as a magnetization transfer filter by adjusting the interpulse delay and the number of off-resonance saturation pulses.

### 29.3.2. Separation of fast and slowly exchanging protons using off-resonance VDMP

The VDMP sequence was initially used for detecting the slowly-exchanging signals of amide and aliphatic protons by applying weak saturation pulses (116). For this purpose, an off-resonance approach was used to separate fast exchanging from slowly exchanging protons. In short, the off-resonance VDMP build-up curves are fitted using a three-pool BM model in which the final observed z-magnetization after a train of n RF pulses can be calculated using a recursive method in which

$$\begin{cases} \Delta M_{s,slow}(t) = \overline{\gamma_{slow}}\, x_{slow} e^{-\Lambda(t_{mix}+t_p)},\ \Delta M_{s,fast}(t) = \overline{\gamma_{fast}}\, x_{fast} e^{-\Lambda(t_{mix}+t_p)} \\ \text{and} \\ \Delta M_w^n(t) = \Delta M_w^{n-1}(t) e^{-\Lambda(t_{mix}+t_p)} \end{cases} \qquad \text{(Equation 73)}$$

Here, $\overline{\gamma_{fast}}\, x_{fast}$ and $\overline{\gamma_{slow}}\, x_{slow}$ are the fractions of the solute magnetization originating from fast and slowly exchanging protons and $\Lambda$ is defined as follows:

$$\Lambda = \begin{bmatrix} R_{1w} + k_{ws,slow} + k_{ws,fast} & -k_{sw,slow} & -k_{sw,fast} \\ -k_{ws,slow} & R_{1s,slow} + k_{sw,slow} & 0 \\ -k_{ws,fast} & 0 & R_{1s,fast} + k_{sw,fast} \end{bmatrix} \qquad \text{(Equation 74)}$$

where $k_{ws,slow}$ and $k_{ws,fast}$ are the transfer rate constants for the slowly and fast transferring protons respectively.

In the case of slowly exchanging protons, $\overline{\gamma_{slow}}$ is equal to the saturation efficiency $\alpha$ while for fast exchanging species there is an additional factor $\eta$ that corresponds to the enhancement factor resulting from one labelling pulse so that $\bar{\gamma} = \alpha.\eta$.

### 29.3.3. On-resonance VDMP

The on-resonance VDMP sequence was validated *in vitro* and *in vivo* in healthy rat brains. *In vitro* applications of this sequence showed that the slowly exchanging protons of BSA (cross-linked and solution) require the application of only a few VDMP modules because of the efficient saturation through $T_2$ dephasing (118). In the case of fast exchanging species, and when $T_1$ and back-exchange effects are neglected, the amplitude of the transfer depends on the product of the saturation efficiency and concentration. In turn, saturation efficiency is a complex function of $T_2$ and chemical exchange rate. Thus, the obtained VDMP contrast of fast exchanging protons occurs during the labelling pulses and there is no new saturation transfer of protons during the mixing time. When the same mechanism was applied *in vivo*, the signals of fast exchanging protons were suppressed by using a few RF pulses, so that the detected exchanging components were primarily from myelin lipids and larger proteins found in the brain.

### 29.3.4. Clinical translation

The VDMP sequence has been optimised for providing clean APT/rNOE-CEST images of the human brain at 7T (117). The optimised sequence consisted of two scans with different interpulse delays while maintaining magnetization transfer levels identical. As expected, the VDMP Z-spectra in grey and white matter showed very different MTC intensities; however, the MT contribution becomes negligible when subtracting the normalised VDMP images at $t_{mix}$= 0 and 100 ms. The APT map obtained using this analysis showed higher signal intensity in GM than in WM possibly due to the higher mobile protein content in grey matter. In addition, after MTC and DS cancellation the signal from amide protons is only a few percent. In contrast, the intensity of rNOE-CEST is much higher and iso-intense throughout the brain. This indicates that the fractions of mobile macromolecules with rNOE signals are equally distributed in grey and white matter.

Pulse sequences for measuring exchange rates between proton species

**<<< Insert Figure 28 here >>>**

### 29.4. Advantages

Several favourable features make VDMP-CEST an ideal choice for *in vivo* applications. First, recording a full Z-spectrum is not necessary and the collection of two images at two different inter-pulse delays provides clean APT and rNOE-CEST images. Secondly, the VDMP-CEST difference image has negligible direct saturation effects when the saturation offset is much larger than the bandwidth of the saturation pulses. Another advantage of VDMP-CEST is that the difference spectrum is quite broad around the amide and aliphatic proton resonances making it insensitive to $B_0$ homogeneities.

## 30. Detection of amide protons using chemical exchange rotation transfer (CERT)

### 30.1. Concept

Another way of detecting amide protons has been introduced based on chemical exchange rotation transfer (CERT) (119),(120),(121),(122),(123). This approach avoids experimental complications of standard saturation transfer techniques by creating label and reference scans using two different acquisitions each with a different off-resonance irradiation flip angle ($\theta$=90° and 180°). The pulse sequence is optimised so that saturation transfer effects are the same; however, different rotation effects exist between the two scans and this is what is being measured. Instead of calculating the MTR asymmetry between two frequency offsets the contrast here is calculated by subtracting the signals at different irradiation flip angles. The APT contrast probing rotation transfer of solute is used for imaging endogenous proteins and peptides, tissue microenvironment and pH *in vivo*.

### 30.2. Pulse sequence

**<<< Insert Figure 29 here >>>**

### 30.3. Basic principles

In principle, rotation transfer contrast results from modifying the flip angles of the irradiation pulses in a pulsed CEST experiment while maintaining constant averaged irradiation power

by increasing the duration of the irradiation pulse (Figure 29). This will then result in almost identical saturation efficiencies between the two acquisitions obtained at two different flip angles $\theta$ but with different solute rotation effects. In the case of amide protons and when the timing of the RF preparation (pulse duration $t_p$ and interpulse delay $t_d$) is greater than the chemical exchange rate, isolation of rotation transfer effects is performed by subtracting signal obtained using $\theta$=180° from signal obtained using $\theta$=90°. To maintain constant averaged power between the two acquisitions for creating equal saturation transfer effects, the repetition time between the two irradiation pulses $t_{pd}$ ($t_{pd} = t_p + t_d$) as a function of the applied flip angle $\theta$ is calculated using the following relationship (120):

$$t_{pd} = \sqrt{\frac{p_2}{dc}} \frac{\theta}{\gamma . p_1 B_{1average}} \qquad \text{(Equation 75)}$$

where $p_1$ is the ratio of the average amplitude to the maximum amplitude of the irradiation pulse and $p_2$ is the ratio of the average of the square of the amplitude to the square of the maximum amplitude of the irradiation pulse. Here $B_{1average}$ is defined as the square root of the mean square irradiation amplitude over the entire pulse train.

Further analysis (as detailed below) utilises the ratio of images obtained at multiple flip angles $\theta$ to eliminate contributions of MT and direct water saturation for enhancing proton selectivity and proving robust APT contrast.

### 30.4. Derivation of metrics to calculate the rotation transfer effect

An empirical equation was derived to describe the CEST contrast in the case of rotation transfer using a three-pool model (i.e. water, amide protons and MT) with a symmetric MT effect under different conditions (i.e. $f_s, T_{1s}, T_{2s}, T_{1w}, T_{2w}, B_0, k_{sw}, f_m$) (119):

$$\text{CEST contrast} = \Phi_1(\theta, f_s, k_{sw}) . \Phi_2(f_s, T_{1s}, T_{2s}, T_{1w}, T_{2w}, B_0, k_{wm}, f_m, k_{sw}) \qquad \text{(Equation 76)}$$

where $\Phi_1$ characterises the oscillation of the CEST effect as a function of $\theta$ and $\Phi_2$ is the magnitude of the CEST contrast.

Initially, a CEST contrast ratio (CCR) reflecting contrast specific to $k_{sw}$ was defined as follows (119):

$$CCR = \frac{2\ x\ contrast\ (180°)}{contrast(360°)+contrast(540°)} \qquad \text{(Equation 77)}$$

According to Equation 76 under constant averaged $B_1$ the chemical exchange contrast depends on, among other things, $k_{sw}$ and $T_{2s}$. However, dividing the contrast at 180° by the averaged sum of the contrasts at 360° and 540° makes CCR independent of $T_{2s}$ and creates a contrast specific to $k_{sw}$ (see Equation 77) – indeed, the division $\frac{2\ x\ contrast\ (180°)}{contrast(360°)+contrast(540°)}$ cancels the $\Phi_2$ term while leaving the $\Phi_1$ which is independent of the relaxation terms. It is worth noting that when $k_{sw}$ is in the range of 10-100$s^{-1}$ and that CCR is a monotonic function.

The magnetization transfer double angle ratio $MTR_{double}$ can also be defined based on the subtraction of two images acquired following a CEST off-resonance irradiation only on one side of the water peak at different flip angles $\theta$ but constant averaged $B_1$ amplitude (120):

$$MTR_{double} = \frac{S_{360°}(3.5ppm)\text{-}S_{180°}(3.5ppm)}{S_0} \qquad \text{(Equation 78)}$$

It is important to point out that $MTR_{double}$ isolates only the effects of solute rotation. Here $S_{360°}(3.5ppm)$ and $S_{180°}(3.5ppm)$ have different rotation effects but similar saturation transfer effects.

$MTR_{double}$ as defined in Equation 78 assumes that the signal components add linearly which is often not warranted *in vivo*. Thus, application of this approach cannot fully correct direct water saturation and magnetization transfer effects (Figure 30). In addition, signal scales with the relaxation time of the water $T_{1w}$ and quantification of APT contrast might lead to misleading results for many pathologies. A variant of $MTR_{double}$ termed $MTR_{double,\ cpw}$ could be used instead and it is defined as follows (121):

$$MTR_{double,\ cpw} = \frac{S_{360°}(3.5ppm,dc_2)\text{-}S_{180°}(3.5ppm,dc_1)}{S_0} \qquad \text{(Equation 79)}$$

To calculate $MTR_{double,\ cpw}$ the duty cycle termed dc is varied as the applied saturation angle θ changes while the pulse width remains constant. This approach is assumed to increase exchange rate sensitivity while the two pulse trains used to calculate $MTR_{double,\ cpw}$ have similar bandwidths for avoiding direct water saturation. Note that this change in duty cycle makes the present CERT method closer to the multiple-pulses saturation (VDMP) technique (see section 29). However, the basic principles of the two techniques are different. In the original VDMP approach, different delay times are employed to create the same direct saturation and MT effects, while with CERT the same direct saturation and MT effects are produced by using the same average irradiation power.

Recently another metric has been defined when CERT is combined with a previously published approach called apparent exchange-dependent relaxation (AREX) for removing direct water saturation effects, semi-solid MT and $T_{1w}$ contaminations (121):

$$AREX_{double,cpw} = R_{1obs}\left(\frac{S_0}{S_{180^\circ}(dc_1)} - \frac{S_0}{S_{360^\circ}(dc_2)}\right) \qquad \text{(Equation 80)}$$

where $R_{1obs} = 1/T_{1obs}$ and $dc$ is the duty cycle defined as the ratio of the pulse duration to the pulse repetition time. $AREX_{double,cpw}$ combines the use of a single frequency offset with the inverse subtraction approach. In this way direct water saturation, semi-solid MT and relayed NOE effects are cancelled out by AREX while the CERT approach is used as an exchange rate filter to remove contributions from nearby fast exchanging protons. According to numerical simulations of a 2-pool system without MT and for low power irradiation $AREX_{double,vdc}$ (3.5ppm) = $f_s(\eta_{180^\circ}-\eta_{360^\circ})$where $\eta_{360^\circ}$ and $\eta_{180^\circ}$ are the labelling efficiencies of the measurements $S_{180^\circ}(dc_1)$ and $S_{360^\circ}(dc_2)$ respectively. Different duty cycles are used for the $180^c$ and $360^c$ pulses so that $\eta_{180^\circ}-\eta_{360^\circ}$ is maximised. At the same time the signals $S_{180^\circ}(dc_1)$ and $S_{360^\circ}(dc_2)$ are obtained under the same irradiation power so that direct water saturation and semi-solid MT effects are the same in both cases.

**<<< Insert Figure 30 here >>>**

## 30.5. Applications

Validation experiments of the so-called multi-angle ratiometric approach (i.e. normalization of the pulsed-CEST contrast with a contrast obtained at a control off-resonance irradiation flip angle $\theta$ under the same irradiation amplitude $B_1$) were initially performed in samples containing creatine in a mixture with agar. As expected, CCR displayed a monotonic relationship with $k_{sw}$ which can be explained by the effect of the rotation of the solute magnetization on the measured water signal. Quantification of the exchange rates in creatine samples was done by using a steady-state continuous wave saturation scheme at two different irradiation amplitudes. The measured exchange rates of creatine in 3 % agar at pH=5.6, 6.0 and 6.4 were found to be 32, 57 and 84 $s^{-1}$ respectively. The optimised averaged $B_1$ amplitude and duty cycle were chosen to be $2\mu T$ and 30% (119).

### 30.5.1. Calculation of $MTR_{double}$ in samples and in healthy rat brain

Conventional early CEST experiments typically used $MTR_{asym}$ to detect changes in solute content or to calculate the exchange rate between solute and water. For ideal conditions the magnitude of $MTR_{asym}$ changes linearly with changes in solute proton concentration $f_s$ and exchange rate $k_{sw}$. However, non-ideal conditions such as $B_0$ shift, the presence of an asymmetry due to macromolecules and lipids add a significant error to $MTR_{asym}$ that depends strongly on the sample characteristics. More precisely, these non-ideal effects diminish or reverse the $MTR_{asym}$ by competing against the CEST effect. $MTR_{double}$ is obtained at a single frequency offset making the contrast independent of $B_0$. In addition, the plot of $MTR_{double}$ vs $k_{sw}$ or $f_s$ is roughly linear with $k_{sw}$ and $f_s$ under non-ideal conditions, allowing for a more robust measure of the sample parameters (119). Experimental results obtained from healthy rats and egg white showed that $MTR_{double}$ has a strong exchange rate selectivity for $k_{sw} < \gamma . B_{1average}$. Thus, amide exchange is filtered from fast exchanging amines without assuming symmetric MT effects (119).

### 30.5.2. *In vivo* application of CERT to map APT and NOE in a glioma rat model

In vivo experiments show that the CERT APT contrast in a rat tumour model is greater (3.1 %) than in normal tissue (2.5%) (123). In addition, the NOE effect at -1.6ppm shows substantial image contrast within the tumour while the NOE peak at -3.5ppm displays no contrast. Unlike $MTR_{asym}$, the CERT approach has a decreased signal strength (i.e. contrast between tumour and normal tissue at the amide resonance is 4.3% for $MTR_{asym}$ and 0.4 % for $MTR_{double}$) (123).

### 30.5.3. CERT imaging of intermediate-exchanging amines at 2 ppm

CERT imaging was shown to have a strong dependence on exchange rate, allowing a more specific exchange rate filtering of the signal in addition to the chemical shift information. Early studies used the $MTR_{double}$ metric with constant duty cycle for detecting slowly exchanging amide protons (120),(119). However, further developments have been aimed at using CERT for filtering exchanging protons with intermediate exchange rates by varying the duty cycle of the acquisition scheme while keeping the pulse width constant. In addition, CERT analysis was combined with AREX for removing MT and $T_{1w}$ contaminations. $AREX_{double,cpw}$ was applied to isolate contrast arising from creatine from that due to other brain metabolites. In particular, $AREX_{double,cpw}$ at 2ppm has a lower value in tumours in line with previous MRS measurements showing that the concentration of creatine declines in tumours (122), if one expects the principal signal at 2ppm to be coming from creatine. However, a major disadvantage of this approach is the contamination from other intermediate-exchanging amines in protein side-chains at 2 ppm as well as the reduced contrast obtained by this method *in vivo*.

In another study $AREX_{double,cpw}$ was applied in rat brains bearing 9L tumours. Simulations were performed and demonstrated its insensitivity to $T_1$, $T_2$, $f_m$, MT and nearby amines (121). Further experimental work on creatine, egg white and glutamate samples showed limited baseline contributions to $AREX_{double,cpw}$ with the contrast being proportional to amide concentration and amide-water exchange rate. In the same study, $AREX_{double,cpw}$ has also been compared with standard APT analysis such as MTR asymmetry and the three-point method termed APT* showing a better suppression of confounding factors (121).

## 30.6 Disadvantages

The magnitude of $MTR_{double}$ is half of that of $MTR_{asym}$. Therefore, subtle signal changes might not be detectable using $MTR_{double}$ due to insufficient SNR. In addition, this method is based on the dependence of the CEST effect on irradiation flip angle and is therefore sensitive to $B_1$ inhomogeneities similarly to conventional CEST. The experimental and theoretical work on $MTR_{double}$ showed that the $B_{1average}$ determines the exchange rate sensitivity. At high $B_{1average}$ or at low frequency offsets the adiabatic condition of the irradiation pulses is no longer valid. Thus, direct rotation of the water signal further

complicates the analysis and induces an enhanced effect on $MTR_{double}$. Finally, only spins with relatively slow exchange rates show a rotation effect when irradiated at their resonance frequency, limiting this method to applications of slowly exchanging protons.

## 31. Saturation with frequency alternating RF irradiation (SAFARI)

### 31.1. Concept

The SAFARI approach is based on the idea that under saturation conditions and once the amide protons have been fully saturated, increasing the irradiation power will not change the amide transfer effect (124),(125). Therefore, APT becomes independent of power while the MT and direct saturation effects vary mostly linearly with RF amplitude. To isolate the APT effect, four measurements are required with off-resonance irradiation: one at the amide proton frequency (i.e. +3.5 ppm), the second on the opposite frequency (i.e. -3.5 ppm) and the third and fourth with both frequencies alternating during one saturation (i.e. between +3.5 ppm and -3.5 ppm and between -3.5 ppm and +3.5 ppm). Indeed, it was shown to be necessary to acquire two SAFARI images with reverse ordering of the pulse frequencies to ensure identical RF power was deposited on the positive and negative frequencies. The calculated $MTR_{SAFARI}$ ratio isolates the CEST effect from both direct water saturation and MT effects even in the presence of $B_0$ inhomogeneities.

### 31.2. Pulse sequence

The SAFARI sequence consists of a pulsed-irradiation module with the off-resonance frequencies of the saturation pulse alternated between +3.5 ppm (amide proton resonance) and -3.5 ppm (aliphatic proton resonance) at a power level sufficient to completely saturate the amide protons (Figure 31). The measured signal can be written as a combination of CEST, MT and direct water saturation effect as follows (124):

$$S_{SAT}(\text{SARARI}) = S_0 - \text{CEST} - P*W(\omega_s + \delta_{B_0}) - P*W(-\omega_s + \delta_{B_0}) - P*MT(\omega_s + \delta_{B_0} + \delta_{MT}) - P*MT(-\omega_s + \delta_{B_0} + \delta_{MT})$$

(Equation 81)

where $S_0$ is the signal without the application of saturation pulses, $\delta_{B_0}$ is the $B_0$ inhomogeneity, $\delta_{MT}$ is the centre of MT asymmetry, $\omega_s$ is 3.5 ppm, P is the irradiation amplitude and W represents the direct water saturation effect.

If the same number of saturation pulses is applied in a regular pulsed CEST experiment without alternation of the sign of the offset, the total power deposited at the amide resonance will be double compared to the SAFARI scan; however, the APT effect will remain unaffected. The signal upon saturation at the amide resonance and on the opposite side is written as follows (124):

$$S_{SAT}(+\omega_s) = S_0 - CEST - 2P*W(\omega_s + \delta_{B_0}) - 2P*MT(\omega_s + \delta_{B_0} + \delta_{MT}) \quad \text{(Equation 82)}$$

$$S_{SAT}(-\omega_s) = S_0 - 2P*W(-\omega_s + \delta_{B_0}) - 2P*MT(-\omega_s + \delta_{B_0} + \delta_{MT}) \quad \text{(Equation 83)}$$

Instead of using $MTR_{asym}$ analysis, the $MTR_{SAFARI}$ ratio is calculated by acquiring four images: one with saturation pulses applied at the amide proton resonance, the second with saturation pulses alternating between +3.5 ppm and -3.5 ppm, one control image at -3.5 ppm and another SAFARI image with RF pulses alternating between -3.5 ppm and +3.5 ppm. In the original publication (124), the off-resonance irradiation scheme consisted of 3 s Blackman-shaped RF pulses with pulse width 9 ms and interpulse delay 6 ms. Crusher gradients were applied between RF pulses to dephase any residual transverse magnetization.

The calculated $MTR_{SAFARI}$ ratio is calculated as follows (124):

$$MTR_{SAFARI} = 2*MTR(SAFARI) - MTR(+\omega_s) - MTR(-\omega_s) =$$
$$2*[1 - S_{SAT}(SAFARI) / S_0] - [1- S_{SAT}(+\omega_s) / S_0] - [1- S_{SAT}(-\omega_s) / S_0] = CEST \quad \text{(Equation 84)}$$

**<<< Insert Figure 31 here >>>**

### 31.3. Applications

The performance of SAFARI was tested in humans by comparing $MTR_{asym}$ and $MTR_{SAFARI}$ with and without $B_0$ correction (124). *In vivo* $MTR_{SAFARI}$ maps displayed positive contrast indicating that the SAFARI technique could be used to separate APT from MT effects. Furthermore, it became obvious that $MTR_{asym}$ and $MTR_{SAFARI}$ displayed different behaviours with increasing irradiation power. This could be explained by the fact that MT asymmetry is a function of saturation power and as the power decreases MT effects contribute less to the $MTR_{asym}$; thus, the resulting subtraction becomes less negative. However, $MTR_{SAFARI}$ only generates contrast relevant to amide exchange and not MT contributions. Thus, it is maximised at low irradiation amplitudes where amide contrast is maximal. Finally, from these initial experiments, it was obvious that the SAFARI technique was successful in saturating amide protons even when the amide frequency was shifted by at least 100 Hz due e.g. to $B_0$ inhomogeneities.

In another publication, another application included the effects of amide and aliphatic protons in human glioma at 3T by comparing the result of MTR asymmetry to SAFARI imaging. One of the primary findings was that the contrast between glioma and normal brain tissue is dominated by broad macromolecular magnetization transfer asymmetry, rather than chemical exchange of mobile protons. The measured MTRasym was significantly higher in glioma patients (~ 0.17%) and the contributions from other sources including aliphatic protons was 1.03% (125). In contrast to MTRasym, the SAFARI technique was unsuccessful in discriminating tumour regions from normal tissue. This might be because the increase in signal due to the APT effect is counterbalanced by a decreased signal in the aliphatic range, which could result in a lack of contrast.

### 31.4. Advantages

$MTR_{SAFARI}$ was found to be more robust in the presence of $B_0$ inhomogeneity, which minimises the need for $B_0$ correction. In addition, the positive SAFARI contrast indicates that the confounding MT asymmetry effect is removed, thereby allowing accurate quantification of the APT effect.

### 31.5. Disadvantages

In summary, a SAFARI experiment is successful when the water saturation is small and the exchangeable protons are almost fully saturated, limiting applications for the detection of only slowly exchangeable species (exchange rate constant below 200 $s^{-1}$). In principle, other exchangeable protons can also contribute to the measured signal, such as the CEST signal coming from amine protons with nearby frequencies as well as from exchangeable protons at the opposite frequency offsets. An additional contribution from such protons to the $MTR_{SAFARI}$ signal should therefore be expected.

## 32. Transfer rate edited experiment for selective detection of CEST

### 32.1. Concept

A transfer rate edited (TRE) CEST experiment consists of a variable number of discrete label transfer modules (LTMs) that label solute protons prior to the detection of the water signal. Transfer rate editing is accomplished by combining two independent methods, namely, a water band excitation pulse (excitation labelling) and a frequency selective continuous wave (CW) labelling (saturation labelling) (126). The initial excitation pulse creates a single quantum coherence for equalising the energy states of all the exchanging protons in the sample and the longer-duration CW pulse selectively labels these protons for enhanced sensitivity.

### 32.2. Theory

A TRE-CEST experiment contains two different types of labelling (Figure 31) (126):

a. An excitation labelling consisting of a composite excitation pulse (e.g. a binomial P1331 pulse, as used in the original publication) with a frequency-band-stop region centred at the water resonance. Because this water editing pulse simultaneously labels protons at many frequencies it cannot be used on its own to generate a Z-spectrum and the saturation field strength is the same for all the frequency offsets without weighting of any particular frequency. The magnetization under an excitation pulse of a proton species i is given as shown below, assuming a negligible T1 effect during RF pulse applications:

$$S_{ext} = \sum_i [x_i](1 - e^{-(t_{sat} \cdot k_{swi})}) \qquad \text{(Equation 85)}$$

where $x_i$ is the concentration of protons and $k_{swi}$ their exchange rate. The summation yields the average signal from all the protons in the sample, and the overall effect of the excitation pulse can be described as a scaling factor of the Z-spectrum which prepares the z-magnetization before the application of the off-resonance saturation pulse. Excitation labelling simply sets up the spin magnetization of the system prior to the start of the frequency encoding CW saturation pulse.

b. A longer duration CW saturation pulse which produces saturation labelling that grows linearly with the rate of magnetization transfer and saturation time. The signal intensity generated by CW labelling of proton species i is given:

$$S_{CW,i} = [x_i].k_{swi}.t_{sat} \quad \text{(Equation 86)}$$

Each of these schemes contributes an amount of signal to the overall water magnetization produced by the saturation of the spin system (Figure 32), while the total signal generated by a TRE-CEST module is the sum of signals produced by the excitation pulse $S_{ext}$ and saturation pulse $S_{CW}$ independently:

$$S_i = \sum_i S_{ext,j}\left(1-\delta_{j,i}\right) + S_{cw,i} \quad \text{(Equation 87)}$$

where $\delta_{j,i}$ is the Kronecker delta function where $S_{ext,j}$ drops to zero for j=i proton species. The product $k_{swi}.t_{sat}$ determines the weighting of the TRE-CEST experiment. When $k_{swi}.t_{sat}$ becomes very large, either due to a rapid rate of magnetization transfer or because of the use of a very long saturation pulse $t_{sat}$, Equation 86 becomes $S_i = [x_i].k_{swi}.t_{sat}$= $S_{CW,i}$. In the case where $k_{swi}.t_{sat}$ is very small, $S_i \approx \sum_i S_{ext,j}$, which is equal to the excitation signal of all the solute protons in the sample. Thus, by changing the duration of the labelling period $t_{sat}$ the properties of the experiment will be different and therefore, the amount of signal amplification can be tuned to probe a given rate of magnetization transfer. The signal amplification produced by TRE-CEST after N LTMs is given by (126):

$$S_{i,Net} \approx \sum_{i=1}^{N} S_{i,n}.e^{-\left[\frac{N*n}{N}\right].R_1.T} \quad \text{(Equation 88)}$$

where $R_1$ is the spin lattice relaxation rate of water and $S_{i,n}$ is the signal generated by the nth module. The difference in MTR generated by the TRE-CEST experiment is a linear function

of the number of the applied LTMs in the sequence. Therefore, this linear attenuation could be used to suppress more slowly exchanging protons. This is done by optimising TRE-CEST to suppress a given signal by choosing the number of cycles N that will maximise the signal loss experienced by slowly exchanging protons, compared to the signal loss experienced by faster exchanging protons.

**<<< Insert Figure 32 here >>>**

### 32.3. Applications

The TRE-CEST sequence has been applied in BSA and egg white samples for removing slowly exchanging protons arising from NOE-mediated transfer while maintaining the more rapid components of APT arising from chemical exchange of amide protons (126). The dependence of the signal on the product $k_{swi}.t_{sat}$ has also been explored by varying the number of LTMs modules of constant duration $t_{sat}$ while keeping the total labelling time of the experiment equal to the time of a CW CEST experiment $T_{total}$ (i.e. $t_{sat}(N) = T_{total} / N$). By comparing the amplitude of the signal of the TRE-CEST experiment to that of an equal duration CW experiment the transfer rate attenuation of TRE-CEST as a function of N can be described. The TRE-CEST profile of protons with fast exchange rates shows a minimal dependence on the duration $t_{sat}$(N) while the profiles of slower signals are influenced by the $t_{sat}$(N) through an exponential decay.

Experimental validation of TRE-CEST in BSA samples shows that the exchange rate of amide protons is between 20 and 35 $s^{-1}$. There is a systematic deviation from the general BM simulations indicating the presence of many distinct proton species that are resonating with different rates in BSA samples (126).

### 32.4. Advantages

TRE-CEST uses both a CW pulse and a number of discrete LTM modules to provide additional information about overlapping signal components that would not be distinguishable when using a CW pulse. Therefore, TRE-CEST effectively eliminates signals from slowly exchanging protons and improves the detectability of faster exchanging components such as amide protons.

Pulse sequences for measuring exchange rates between proton species

### 32.5. Disadvantages

While this method holds promise for imaging amide protons it has not been demonstrated apart from laboratory samples. In addition, the irradiation scheme combining a water band-stopped excitation pulse and a frequency selective continuous wave pulse would lead to substantial RF deposition in tissues in clinical settings.

## Detection of chemical exchange effects through different labelling schemes and pulse shapes

## 33. On resonance paramagnetic chemical exchange effect (OPARACHEE)

### 33.1. Concept

Exogenous paramagnetic agents have a few advantages that could be used in the clinic. First, PARACEST agents allow the use of faster exchanging species without violating the slow-to-intermediate exchange condition (e.g. they possess spins that resonate relatively far away from the water signal, such as those in lanthanide complexes). Second, because the applied RF irradiation for inducing a CEST effect is further away from the bulk water, there are fewer direct saturation effects. A major issue when visualising PARACEST agents is the strong RF field required to induce a measurable effect, in addition to their inherent toxicity that limits the total agent amount that can be used. Therefore, their use in human applications is currently limited. One way of avoiding the issues related to high-power RF pulses is to design RF pulses with modulated amplitude and phase to enhance the chemical exchange effect while maintaining a low deposition power in the tissues. An example is the OPARACHEE approach which utilises a WALTZ pulse train for imaging PARACEST species (130),(131), (132).

### 33.2. Pulse sequence

OPARACHEE consists of an on-resonance pulse scheme in which the water signal is continuously undergoing 360° rotations due to the application of a train of composite low power WALTZ-16 RF pulses. Being far off-resonance, the exchangeable protons from the contrast agent do not experience this rotation; the result is a relative decrease in water Z-magnetization, much like a conventional CEST experiment, via exchange processes related to T2-based techniques (see Section 27). This technique is promising for detecting rapidly

exchanging protons such as hydroxyls and amines without *a priori* knowledge of their resonance frequency since they are not directly excited. At the same time the sensitivity to discriminate exchangeable protons in different chemical environments is lost when using this approach, because it is not frequency selective.

For MRI experiments a standard spin-echo sequence was employed with a WALTZ-16 pulse train applied very far off-resonance (RF off) and with the same sequence applied on resonance with water (130), (131), (132). The amplitude of the WALTZ-16 pulse train remains constant, while the phase of the individual pulses changes between 0° and 180°. The first application of OPARACHEE used 96 WALTZ-16 pulses of 2.5 ms duration and with a total train length of 240 ms (131).

### 33.3. Applications

Simulations were performed to investigate the performance of OPARACHEE in the absence of chemical exchange for various RF amplitudes and frequency offsets. Ideally, it is desirable to have 100% of the total water intensity after the experiment (i.e. the train of WALTZ-16 pulses corresponds to a complete 360° rotation of the water magnetization without any additional influence) (130). In practice this is not achieved and the resulting signal intensity is about 93% as a result of $T_2$ relaxation during the WALTZ-16 train. Experimental results in TmDOTAM and DyDOTAM solutions demonstrated that a low power WALTZ-16 pulse train can be used to visualise concentrations as low as 30 $\mu$M of the agents, and that the signal intensity decreases linearly with agent concentration (130).

The performance of the technique has been tested *in vivo* by detection of TmDOTA-4AmC- in mouse kidneys, and the magnitude of the effect was compared to the bolus of the injected agent concentration (131). A signal reduction in the water peak due to the presence of the agent was observed at 3 minutes after injection, which corresponds to the maximal concentration of the agent in the kidney. After that a clearance of the agent is observed and the signal intensity starts to return to baseline levels (i.e. prior to the injection of the agent). The observed drop in signal intensity scales with the amount of PARACEST agent. The WALTZ spin echo sequence (131) was found to be sensitive enough to detect 2mM or more of the agent in mouse kidneys and the maximum effect was observed in the papilla where the average minimum signal intensity was 45% for 2mM and 84% for 20 mM bolus

concentration of the agent. The detectable limit of agent concentration is determined by the SNR of the WALTZ-SE images, which is influenced by relaxation and magnetization transfer effects (i.e. shorter relaxation and MT effects result in a higher detection limit *in vivo*).

### 33.4. Advantages

OPARACHEE utilises a low-power WALTZ-16 pulsed train, which is less affected by SAR restrictions compared to standard PARACEST techniques. In addition, knowledge of the resonance frequency of the contrast agent is not necessary because the pulses are applied on-resonance with water.

### 33.5. Disadvantage

One major disadvantage that can limit the potential application of this sequence is the signal sensitivity to short relaxation times. This is particularly problematic in those tissues, where $T_1$ and $T_2$ are short..

## 34. Length and offset varied saturation scheme (LOVARS)

### 34.1. Concept

Another approach to detect chemical exchange is by acquiring a set of images with variable saturation duration ($t_{sat}$) and offset ($\Delta\omega$) of the saturation pulse, termed CEST phase mapping, using a length and offset varied saturation (LOVARS) scheme (133),(134). In this sequence, the cosine modulation of the water signal creates different phases that allow separation of the three main contributors: chemical exchange, magnetization transfer and direct water saturation. Either fast Fourier transform, or general linear model analysis can be applied to decompose the modulation patterns into separate sources of water signal loss.

### 34.2. Pulse sequence

In the absence of chemical exchange, the longitudinal magnetization describes the direct saturation effect and it is a function of the water relaxation rates, frequency offset $\Delta\omega$ and irradiation amplitude $\omega_1$. In addition, the chemical exchange, direct saturation and magnetization transfer effects behave differently with saturation time $t_{sat}$. If direct water and MT effects are assumed to be symmetrical around the water resonance a LOVARS acquisition scheme requires the collection of images as described below (Figure 33) (133):

$$\left[S_{1,}S_{2,}S_{3,}S_{4}\right] = [S(\text{-}\Delta\omega, t_{sat2}), S(\text{-}\Delta\omega, t_{sat1}), S(+\Delta\omega, t_{sat2}), S(+\Delta\omega, t_{sat1})]$$

(Equation 89)

where $S(\text{-}\Delta\omega, t_{sat2})$ is the image obtained by saturation on the opposite side of the exchangeable proton frequency with a saturation length $t_{sat2}$, while $S(+\Delta\omega, t_{sat1})$ is the image obtained at the frequency offset of the exchangeable protons at a saturation time $t_{sat1}$. Assuming that the signal loss for direct water saturation and MT is the same for $-\Delta\omega$ and $+\Delta\omega$, the LOVARS responsive function is written as follows (133):

$$S_{LOV} = A_0 + A_1.\cos[\pi/2(m\text{-}1).\phi] + A_2.\cos[\pi(m\text{-}1)] \qquad \text{(Equation 90)}$$

where m is the number of the acquired images, $\cos[\pi/2(m\text{-}1).\phi]$ represents the asymmetric and $\cos[\pi(m\text{-}1)]$ the symmetric component of the measured signal. For example, agents resonating very close to the water frequency will have a large symmetric component due to direct water saturation. $A_1$ and $A_2$ are the amplitudes of the two cosine functions and $\phi$ is the phase of the cosine modulation.

One way of analysing the data is by taking the Fourier transform of the LOVARS time series. One LOVARS module consists of the acquisition of four images, and after discrete Fourier transform analysis, the measured signal is related to $MTR_{asym}$ as follows (133):

$$L = \frac{1}{S_0}\{[S(\text{-}\Delta\omega, t_{sat2})\text{-}S(+\Delta\omega, t_{sat2})] \text{-i } [S(\text{-}\Delta\omega, t_{sat1})\text{-}S(+\Delta\omega, t_{sat1})]\} = MTR_{asym}(t_{sat2})\text{-}i\ MTR_{asym}(t_{sat1}) \qquad \text{(Equation 91)}$$

The relative phase is defined as tan $(\phi) = MTR_{asym}(t_{sat1})/MTR_{asym}(t_{sat2})$. The two saturation lengths $t_{sat1}$ and $t_{sat2}$ are chosen with CEST contrast $(t_{sat1})$ > CEST contrast $(t_{sat2})$ while MT/DS contrast $(t_{sat1})$ < MT/DS contrast $(t_{sat2})$.

**<<< Insert Figure 33 here >>>**

### 34.3. Applications

Samples containing L-arginine and agar were scanned using the LOVARS scheme. The optimum saturation lengths were found to be $t_{sat1} = 4\ s$ and $t_{sat2} = 1.5\ s$. In L-arginine the signals at $S(+1.8\text{ppm})$ and $S(\text{-}1.8\text{ppm})$ increased with $t_{sat}$ while the overall $MTR_{asym}$ for agar was zero. At $t_{sat1} = 4\ s$ the $MTR_{asym}$ of L-arginine was maximum while in agar the signal reached a plateau after 1.5 s. Using the LOVARS scheme several maps are obtained: a real component, an imaginary component and a phase map. The imaginary component dominates in L-arginine whereas in agar the real and imaginary components display the same magnitude. In addition, the phase map was found to be insensitive to $B_0$ inhomogeneity, concentration and exchange rate. This is because tan $(\phi) = MTR_{asym}(t_{sat1})/MTR_{asym}(t_{sat2})$, with ϕ as defined in Equation 90, which cancels these factors. *In vivo* validation of the LOVARS sequence was performed in mice bearing tumours at the amide proton resonance with $t_{sat1} = 3\ s$ and $t_{sat2} = 0.8\ s$. According to the results of this study the LOVARS phase map was found to be significantly different between tumour and control tissue with four times larger CNR compared to conventional $MTR_{asym}$ analysis with the same acquisition time.

### 34.4 Advantages

Several advantages can be listed for the LOVARS scheme. First, CEST contrast is better defined on LOVARS phase maps compared to standard $MTR_{asym}$ maps. Secondly, LOVARS maps are less sensitive to $B_0$ inhomogeneities. Finally, *in vivo* application to mouse gliosarcomas showed a clear separation of tumour and normal tissue with a CNR four times higher than conventional CEST imaging with the same acquisition time (133).

## 35. Multi-echo length and offset varied saturation scheme (MeLOVARS)

### 35.1. Concept

This method is based on the idea of Look-Locker $T_1$ mapping and works by collecting all the saturation images within one repetition time. Instead of applying a read-out after the saturation train, here multiple signal read-outs are applied during the saturation preparation, similarly to the PRO-QUEST method (see section 28). This technique employs a CW pulse for speeding up the measurements of exchange rate in several CEST compounds.

Pulse sequences for measuring exchange rates between proton species

### 35.2. Pulse sequence

The MeLOVARS pulse sequence divides the saturation pulse into N submodules, each with a length of $t_{sat}$/N and in between the modules a low flip angle $\vartheta$ pulse is applied followed by a flip back pulse $-\vartheta$ for returning the magnetization back to the z-axis (Figure 34) (134). After the read out the transverse magnetization decays to $M_n^{x,\vartheta} e^{-\frac{2TE}{T_2^*}}$ and the longitudinal magnetization after n modules becomes:

$$M_n^{z,-\vartheta} = M_n^{z,sat}[1 - sin^2\theta(1 - e^{-\frac{2TE}{T_2^*}})] \quad \text{(Equation 92).}$$

The CEST contrast is quantified using $MTR_{asym}$ analysis defined as

$$MTR_{asym,n} = [S_n^\theta(-\Delta\omega)^n - S_n^\theta(+\Delta\omega)^n] / S_{0,n}^\theta \quad \text{(Equation 93)}$$

where $S_{0,n}^\theta$ is the image with the same readout (flip angle $\vartheta$) without the saturation pulse.

**<<< Insert Figure 34 here >>>**

### 35.3. Applications

MeLOVARS has been applied for imaging the endogenous APT contrast in mice bearing tumours. CNR was increased by the square root of the number of modules. In addition, images obtained at different saturation times allowed a more stable fit of the 4-pool BM equations *in vivo*. It should be noted that when applying multiple read-outs there is a decay factor which depends on $T_2^*$ $and$ $TE$ (134). For *in vivo* APT imaging an optimal read-out flip angle $\theta = 25°$ was calculated for minimizing $T_2^*$ effects. However, *in vitro* the optimised sequence consisted of the collection of 8 images with $t_{sat}$=0.5-4.0s while *in vivo* Z-spectra were collected using 5 modules with saturation lengths ranging between 0.5-4 s (134).

### 35.4. Disadvantages

The optimised flip angle $\theta$ is small which reduces image SNR. Also, this method is prone to the effects of $B_1$ inhomogeneities.

## Conclusion

As this paper clearly states, there are many ways to take advantage of chemical exchange. We are not at the end of what can be said on the topic, and many new methods are continuously being introduced, allowing us to highlight more precisely aspects of the physical phenomenon, or to improve the detection power of the technique. Chemical exchange effects are small and while CEST provides a sensitivity enhancement mechanism to detect exchangeable protons over direct detection such as used in conventional NMR, the signal obtained is still much lower than that provided by water imaging such as in conventional MRI. Therefore, we encourage anyone so inclined to dive into this fascinating field to first study what is the most appropriate technique to use, balancing positive and negative aspects prior to starting work on a clinical or laboratory-based application of chemical exchange transfer mechanisms.

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## Figure legends

**Figure 1:** Principles of CEST imaging. (a)-(b) Exchangeable protons are saturated using frequency selective RF pulses and saturation is transferred to water with an exchange rate ksw. After a period, this effect is visible on the water signal. (c) At 0 ppm the water protons are saturated directly. (d) The plot of normalised water intensity as a function of irradiation frequency generates the so-called Z-spectrum. Note that the direct saturation effect is also called spill-over effect and represents the largest dip in a Z-spectrum. Smaller dips in the Z-spectrum correspond to exchange processes of small solutes with water protons at their corresponding frequency offset

**Figure 2:** Classification of Chemical Exchange. (a) proton exchange, (b) molecular exchange, (c) proton and molecular exchange, (d) compartmental exchange and (e) molecular and compartmental exchange (reproduced with permission from (19)).

**Figure 3:** Molecular motion timescales accessible through NMR/MRI experiments
The CEST experiment is sensitive to proton exchange with an exchange rate constant from 50000 $s^{-1}$ to as slow as 1 $s^{-1}$ (reproduced with permission from (20)).

**Figure 4:** Representative Z-spectrum from a mouse brain scanned on a 9.4 T MRI scanner. Characteristic features of the Z-spectrum are the direct water saturation around the water resonance (i.e. 0 ppm), amide proton exchange at 3.5 ppm and Nuclear Overhauser Effect (NOE) at a range between –2.0 and –5.0 ppm.

**Figure 5:** Exchange regimes. (a) Exchange between two sites with spin population $P_A$ and $P_B$ (b) A discrete chemical shift difference $\Delta\omega$ between the signals of water and the exchangeable protons is required for CEST imaging. For this to be true, $k_{sw} \leq \Delta\omega$, so that the CEST agent and the water give two separate signals (adapted from (33)).

**Figure 6:** Two-pool exchange model. Pool s represents the exchangeable solute protons and pool w consists of bulk water. Saturation transfer to the water pool has a rate constant $\boldsymbol{k_{sw}}$.

**Figure 7:** Simulations of saturation efficiency. (a) Dependence of the saturation efficiency on the exchange rate. Maximum saturation efficiency (α = 1) is obtained for exchange rate constants < 100 $s^{-1}$. α reduces dramatically when the exchange rate increases. (b) Plot of the product of the exchange rate and the saturation efficiency (α . $k_{sw}$) versus $k_{sw}$. Theoretically, acceptable saturation efficiency can be achieved at power levels up to 5 μT for exchange rate constants up to 10000 $s^{-1}$ (reproduced with permission from (6)).

**Figure 8:** Spin Lock Pulse Sequence. (A) The pulse sequence consists of a SL module followed by a SE–EPI read-out. The water magnetization is flipped by a $\theta$ pulse applied on-resonance and then locked by a SL pulse applied at an offset $\Omega$ with an angular frequency $\omega_1$ and locking time *TSL*. Finally, after the locking pulse the magnetization is flipped back to the *z*-axis for imaging. **(B)** During an off-resonance SL experiment, the magnetization is locked along $B_{\text{eff}}$ ($B_{\text{eff}} = \sqrt{B_1^2 + \frac{\Omega^2}{\gamma}}$) (adapted with permission from (35)).

**Figure 9:** Magnetization trajectories of a proton pool. (a) During a CEST experiment and (b) during a SL experiment. The arrows show the initial water magnetization in both cases (reproduced with permission from (51)).

**Figure 10:** Representative MRI scans. Arterial Spin Labelling (ASL), Apparent Diffusion Constant (ADC) and pH-weighted imaging from patients with acute ischaemic stroke (reproduced with permission from (57)).

**Figure 11:** WEX pulse sequence is used to observe exchangeable protons from water to metabolites in cells (a) and in vivo (b). Solid black boxes are 90º RF pulses. The pulse sequence consists of three periods: 1. Selective labelling of water magnetization, 2. transferring of the of water magnetization to other metabolites during the mixing time, 3. selective suppression of water magnetization during WATERGATE. The pulse phases $\phi_1$ and $\phi_2$ are {x, -x} and {-x, x} respectively. In (b) the three 90º RF pulses are slice-selective sinc pulses. $G_{c.s}$ and $G_{s.s}$ stand for gradient pulses for coherence selection and slice selection, respectively. In the first interval a selective 180º Gaussian-shaped RF pulse is applied at the water frequency while the 180º pulse in the third interval are selective on the exchangeable protons. Single-voxel localization is achieved by three orthogonal slice-selective gradients.

**Figure 12:** CEST pulse sequences for different coherence levels (a) Single Quantum Coherence (SQC) CEST. $\Delta$ = 0 ms, TE = minimum, (b) Conventional intermolecular Double Quantum Coherence iDQC CEST, (c) iDQC Multiple Refocusing Pulses (MRP) CEST. In (b) and (c), a pair of asymmetric z-gradients is used for the coherence selection. The iDQC-MRP sequence (c) inserts n $\pi$ refocusing pulses into the detection period.

**Figure 13:** Z-spectra containing 2 % agar and 0.1 mol of glucose obtained using conventional single quantum coherence and intermolecular double quantum coherence CEST experiments. (reproduced with permission from (69))

**Figure 14:** Pulse sequence diagrams of (a) a pulsed gradient spin echo (PGSE) and (b) Filter Exchange Spectroscopy (FEXI) experiments. In both figures $\delta$ is the gradient pulse duration, $\Delta$ is the time between the start of the two gradient pulses, g is the gradient strength and TE is the echo time during which the magnetization is subjected to $T_2$ relaxation. In (b) $g_f$ is the gradient strength during the 'diffusion filter', $t_m$ is the mixing time, and $t_z$ is the time interval for z-storage of the magnetization.

**Figure 15:** Determination of the apparent exchange rate (AXR) of molecular exchange between tissue microenvironments. White dots are water molecules located in microenvironments with fast and slow local diffusivity. ADC is the population-weighted average of the diffusivity contributions from all the microenvironments. When a diffusion filter is applied (gray line) a reduction of the observed apparent diffusion coefficient ADC' to ADC(1-$\sigma$) is observed at $t_m$=0. When $t_m$ is increased molecular exchange leads to reappearance of MR-visible water in high diffusivity regions. The rate at which ADC' approaches the equilibrium value ADC is the AXR. AXR is higher for small cells (b) than larger cells (c) due to differences in intracellular surface-to-volume ratios, as well as differences in aquaporin (AQP) expression, membrane proteins serving as active water transport across the cell membranes, determining in large part water cell membrane permeability. (reproduced with permission from (73)).

**Figure 16:** The gradient encoded CEST sequence (b) is implemented by removing or altering the elements displayed with yellow colour in (a) and all elements that have to be inserted in (b) are shown in blue. The modifications include removal of the phase encoding gradients

and the phase loop, replacing the low flip angle excitation pulse by a 90º pulse to achieve maximum signal, and inserting a CEST gradient along the read direction while the saturation pulse is switched on. In addition, it is necessary to put a delay $t_d$ after the saturation pulse.

**Figure 17:** ZAPI and CW Z-spectra from boiled egg white. The root mean square saturation amplitude $\gamma B_1$ was varied from 25 to 100 Hz. Both CW and ZAPI follow the same MT Z-spectrum envelope, with direct saturation dips for water (side bands) observed on top of this envelope. Other long-$T_2$ components such as amides and aliphatic protons are observed a few ppm from water: on-resonance for CW and at the side band frequencies for ZAPI. (reproduced with permission from (89)).

**Figure 18:** Schematic of the uMT technique. (a) The frequency positions of the pre-saturation RF irradiation in the uMT method, relative to the water, CEST, NOE and MT pools. The distance between two frequency components is fixed $2f_m$ is fixed and the water signal is measured against the middle of the two frequency positions. (b) A typical z-spectrum obtained with the uMT method (reproduced with permission from (128)).

**Figure 19:** Simulated Z-spectra and asymmetry curves from the two-pool model based on Provotorov's theory. Z-spectra were generated by numerically calculated Pi after a long RF irradiation (i.e. in the steady state). The black and red lines represent the MT and CEST cases. (a) Z-spectra after 5s long single frequency RF irradiation, (b)-(d) Z-spectra after 5 s long two-frequency RF irradiation. The distance between the two frequency components is (b) 2kHz, (c) 5 kHz, and (d) 10 kHz respectively. In figure (a) the spike at 1500 Hz represents the CEST effect, while in figures (b)-(d), the Z-spectra are generated when the frequency separation of the RF pulse is increased. It is worth noting that the CEST effect is still visible, independently of the difference between both saturation frequencies. The asymmetry curves are shown in (e)-(h). When the distance between the two frequency components is smaller than twice the chemical shift of the CEST agent with respect to water, the two CEST peaks will be observed on the same side of the Z-spectrum relative to the zero frequency. If now the distance is larger than twice the chemical shift, the two CEST peaks will be observed on opposite sides in the asymmetry plots (g) and (h) (reproduced with permission from (127)). For more details regarding the simulation parameters please refer to original publication.

**Figure 20:** FLEX pulse sequence. The FLEX sequence consists of a series of n label-transfer modules (LTMs) in which exchangeable solute protons are frequency labelled during an evolution time $t_{evol}$ and subsequently transferred to the water during the exchange period $t_{exc}$. $O_1$ is the offset frequency of the labelling RF pulses and it is selected based on the combined requirement of minimal water excitation and sufficient saturation efficiency for the exchangeable protons.

**Figure 21:** CEST and FLEX spectra of 10 mM EuDOTA-(gly)$_{-4}$ mixed with 4 % agarose in tris buffer. (a) CEST spectrum and MTR asymmetry analysis in red indicating a 3% CEST asymmetry effect. The paraCEST peak in the Z-spectrum is located at 50 ppm. (b) FLEX artificial "FID" for 500 LTMs as a function of $t_{evol}$ (c) FLEX spectrum obtained by Fourier transform of the FLEX "FID". (d) Time domain analysis of the FLEX FID showing three signal components: red line; paraCEST agent bound water, blue line; free water, green line; semi-solid bound water. (e) Corresponding FLEX spectra generated by Fourier transform of the individual components in (d). (f) The residual plot obtained by calculating the deviation of time-domain data points in (d) from fitted curve. (g) The residual spectrum corresponding to the Fourier transform of (f). (reproduced with permission from (91)).

**Figure 22:** MRI techniques for the quantification of exchange rates. (a) (left) measurements of exchange rate as a function of saturation time (QUEST), (right) measurements of the exchange rate as a function of saturation power (QUESP), (b) (left) an omega plot $M_z/(M_0 - M_z)$ versus $1/\omega_1^2$ for 5 mM, 10 mM and 20 mM Eu(DOTA-(glyOEt)$_4$)$^{3+}$ in water at pH 7 and 25 °C, (right) QUESTRA analysis: label and reference scans show linear dependence with saturation time ($T_s$). Figure reproduced with permission from (101).

**Figure 23:** The CEST fingerprinting pattern. (a) Acquisition sequence diagram. In each subsequent acquisition block, CEST saturation powers $B_1$ and CEST saturation times $t_{sat}$ are varied in a pseudorandom pattern. (B), (C) Examples of average CEST saturation power $B_1$ and CEST saturation time $t_{sat}$ series.

**Figure 24:** (a) A schematic of a pulse sequence utilised for saturation transfer experiments ($T_{pw}$ is the pulse length). (b) A schematic of a pulse sequence utilised for apparent relaxation time measurements and for the creation of the positive CEST effect (pCEST); $\pi$ is an

inversion pulse. (c) the difference in the apparent relaxation constants results in a small positive signal at $T_{null}$.

**Figure 25:** Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence for measuring the transverse relaxation rate $T_2$. A train of 180 pulses is applied to repetitively refocus the signal and reduce diffusion effects due to inherent magnetic field inhomogeneities.

**Figure 26:** PRO-QUEST Pulse sequence diagram. An initial 90° saturation pulse(s) is 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 (b) 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 (a) to obtain the exchange-related relaxation rate.

**Figure 27:** The VDMP sequence consists of a train of Gaussian 180º pulses followed by an MRI readout (a multi-spin echo sequence). The pulse width is $t_p$ and the interpulse delay $t_{mix}$. Cyclops phase cycling is applied together with crusher gradients during $t_{mix}$ to suppress residual transverse magnetization during the preparation scheme.

**Figure 28:** Bloch simulations. (a) Effects of mixing time and exchange rate constant (log plot) on the normalised CEST signal intensity $S(t_{mix})/S(t_{mix}=0)$ for the VDMP sequence. (b) Projections of the VDMP-CEST signal ratio from panel (a) as a function of mixing time for four exchange rates corresponding approximately to typical rNOE (16 $s^{-1}$), APT (29 $s^{-1}$), MTC (60 $s^{-1}$) and fast exchange (1000 $s^{-1}$). (c) ΔVDMP as a function of exchange rate constant at $t_{mix}$ = 80 ms. (d) Z-spectra showing water direct saturation at 0 and 80 ms mixing time and ΔVDMP with water $T_1$ relaxation times of 1.5 and 2.5 s respectively (reproduced with permission from (117)).

**Figure 29:** Diagram of pulsed-CEST imaging sequence. The irradiation train consists of a series of Gaussian pulses with flip angle $\theta$ (being either multiple of 90° or 180°) and duration $t_p$ followed by interpulse delay $t_d$. The saturation train lasts 3-4 s before the excitation pulse.

**Figure 30:** Simulated (a) and experimental data (b) for various flip angles $\theta$. A dip and peak occur at around 180° and 360°, respectively, representing the maximum and minimum transfer effects. The arrow shows the approximate contributions of direct water and macromolecular saturation, solute saturation and solute rotation on a PLL sample with pH=6.7. (reproduced with permission from (120)).

**Figure 31:** Pulse sequence for APT-SAFARI imaging with a pulsed off-resonance saturation module followed by an EPI read-out (adapted from (124)). Each scan consists of acquiring four images:
a) Standard CEST image with saturation at the labile proton frequency (i.e. 3.5 ppm),
b) SAFARI image with saturation alternating between the label and control frequencies,
c) Standard CEST control image with saturation at the control frequency (i.e. -3.5 ppm),
d) SAFARI image with saturation alternating between the control and label frequencies.

**Figure 32:** (a)TRE-CEST pulse sequence with N label transfer modules (LTMs) applied prior to detection of the water signal. The pulse flip angle is $\alpha = 11.25°$ and the inter-pulse delay is $t_{del} = 1/(2.\Delta\omega)$ where $\Delta\omega$ is the frequency offset of the slow exchanging resonance in Hz relative to the water frequency. (b) Bloch simulation of z-spectra generated by TRE-CEST (shown in red) and by using continuous wave saturation CEST (shown in black) (reproduced with permission from (126)).

**Figure 33:** Illustration of the LOVARS acquisition scheme. a. The variation in saturation image LOVARS collection pattern is displayed, with both saturation offset $\Delta\omega$ and length $t_{sat}$. b. The resulting signal patterns produced on PLL or 4% agar. c. The LOVARS signal patterns for L-arginine. The time domain data is transformed to the LOVARS frequency domain, generating the new LOVARS parameters for each voxel: real, imaginary and phase values. (reproduced with permission from (133)).

**Figure 34:** Acquisition scheme for MeLOVARS consisting of N saturation modules with interleaved readouts (reproduced with permission from (134)).

## Tables

**Table 1:** Endogenous exchangeable proton groups. Properties of the main exchange groups used to produce CEST contrast (reproduced from (15)).

| | Amide protons | Amine protons | Hydroxyl protons |
|---|---|---|---|
| Chemical shift | 3.5 ppm | 1.8 - 3.00ppm | 0.5 – 1.5 ppm |
| Exchange rate constant ($k_{sw}$) | 10-100 $s^{-1}$ | >500 $s^{-1}$ | 500 – 1500 $s^{-1}$ |
| Endogenous metabolites | Multiple amino acids, protein backbone, etc… | Glutamate (Glu), creatine (Cr) | Glycosaminoglycans (GAG), glycogen, myoinositol (MI), glucose |
| $CEST_{asym}$ @ 7T and physiological conditions | 1-4 % | 7-10 % | 2-8 % |
| Sensitivity to pH | Yes | Yes | Yes |
| CEST applications | Cancer/Stroke | Skeletal muscle and myocardial muscle energetics, cancer metabolism (Cr), neuropsychiatric disorders (Glu) | Osteoarthritis (GAG), neurological disorders (MI), cancer metabolism (glucose) |

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Pulse sequences for measuring exchange rates between proton species

## Glossary

AACID: amine/amide concentration independent detection
AD: Alzheimer's disease
ADC: apparent diffusion coefficient
APT: Amide proton transfer
AREX: apparent exchange dependent relaxation
ASL: arterial spin labelling
ATP: Adenosine Triphosphate
AXR: apparent exchange rate
BM: Bloch-McConnell equation
BPP: Bloembergen, Purcell and Pound
BSA: Bovine serum albumin
CA: contrast agent
CBF: cerebrospinal fluid
CE: chemical exchange
CERT: chemical exchange rotation transfer
CEST: chemical exchange saturation transfer
CPMG: Carr-Purcell-Meiboom-Gill
CW: continues wave
DC: duty cycle
diaCEST: diamagnetic CEST agents
DS: direct saturation
EPI: Echo planar imaging
FEXI: filter exchange spectroscopy
FLEX: frequency-labelled exchange transfer
GAG CEST: glycosaminoglycan CEST
GE: gradient echo
GluCEST: glutamate mediated CEST
glucoCEST: glucose mediated CEST
glycoCEST: glycogen mediated CEST
iDQC: intermolecular double quantum coherence
iMQC: intermolecular multiple quantum coherence

Pulse sequences for measuring exchange rates between proton species

LL: look-locker
LOVARS: length and offset varied saturation scheme
LTM: Label transfer modules
LW: linewidth
MCAO: middle cerebral arterial occlusion
MeLOVARS: multi-echo length and offset varied saturation scheme
MICEST: myo-inositol CEST
MRF: magnetic resonance fingerprinting
MRP: multiple refocusing pulses
MS: multiple sclerosis
MT: magnetization transfer
MTC: magnetization transfer contrast
MTRasym: magnetization transfer asymmetry
NMR: Nuclear magnetic resonance
NOE: Nuclear Overhauser Enhancement effect
OPARACHEE: On resonance paramagnetic chemical exchange effects
paraCEST: paramagnetic CEST contrast agent
pCEST: positive CEST
PGSE: pulse gradient spin echo
PRESS: point resolved spectroscopy
PRO-QUEST: progressive saturation for quantifying exchange rates using saturation times
PTE: proton transfer enhancement
PTR: Proton transfer ratio
QUESP: quantification of exchange rates at various saturation powers
QUEST: Quantifying exchange rates based on saturation transfer
QUESTRA: Quantification of chemical exchange rate with saturation time dependent ratiometric analysis
RLQUEST: reciprocal linear QUEST
SAFARI: saturation with frequency alternating RF irradiation
SAR: specific absorption rate
SL: spin lock
SNR: signal to noise
SQC: single quantum coherence
TRE-CEST: transfer rate edited CEST sequence

Pulse sequences for measuring exchange rates between proton species

UCEPR: ultrafast chemical exchange using point resolved spectroscopy

UFZ: ultra fast z-spectroscopy

uMT: uniform magnetization transfer

VDMP: variable delay multiple pulsed saturation technique

WEX: water exchange spectroscopy

ZAPI: Z-spectroscopy with alternating phase irradiation

ZAPISM: Z-spectroscopy with alternating phase irradiation and sine modulation