NMR magnetization-transfer analysis of rapid membrane transport in human erythrocytes

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Abstract Nuclear magnetic resonance (NMR) magnetization-transfer (MT) experiments provide a convenient tool for studying rapid sub-second membrane-transport processes in situ in metabolically active cells. These experiments are used with membrane-permeable substances when separate (resolved) NMR signals are observed from their populations inside and outside the cells. Here, we provide a description of the theory and practice of the most common NMR MT experiments that have been used to study membrane-transport processes in human erythrocytes (red blood cells; RBCs). The procedures, involved in the analysis of the experimental data for defining mechanisms of transport, and for estimating values of kinetic parameters in the corresponding mathematical models, are given special attention.

Keywords NMR · Magnetization transfer · Membrane transport · Human erythrocyte · Equilibrium exchange · Michaelis-Menten kinetics

Abbreviations

B-M	Bloch–McConnell
DST	Differential saturation transfer
EXSY	Exchange spectroscopy
FID	Free induction decay
Ht	Haematocrit
IT	Inversion transfer

MT	Magnetization transfer
NMR	Nuclear magnetic resonance
NOE	Nuclear Overhauser effect
RBC	Red blood cell
RF	Radio-frequency
ST	Saturation transfer

Introduction

The ability of cells to selectively bind to and exchange various molecules via their membranes is fundamental to all forms of life. Abnormalities, associated with membrane-transport processes, are linked to many diseases. Knowledge of the thermodynamics and kinetics that describe membrane-transport processes provides insights into molecular-level mechanisms that operate in normal and disease states of cells. Better understanding of these processes is the key to developing new ways of treating diseases that are associated with membrane transport. Such treatments might be via drugs that are designed to target specific proteins, which play an important role in membrane-transport reactions (Kell and Oliver 2014).

Nuclear magnetic resonance (NMR) spectroscopy provides convenient and powerful tools to probe molecular processes in a wide range of cellular systems including those in vivo. NMR methods are based on the magnetic labelling of nuclear spin populations by radio-frequency (RF) electromagnetic pulses without affecting physico-chemical equilibria. When the spin-bearing molecules undergo physical translocation, they can be tracked via their NMR signals, thus allowing measurements of the transmembrane fluxes of solutes without the requirement of physical separation of the cells from their bathing medium. Using NMR methods, it is possible to quantify the kinetics of membrane-transport processes in metabolically active cells

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over a range of time scales from milliseconds to hours (Hills and Belton 1989; Kirk 1990; Kuchel 2015).

Members of the general class of NMR experiments, called magnetization-transfer (MT) experiments, are especially suitable for studying the kinetics of rapid reactions that are under equilibrium exchange. In most cases, MT methods rely on the magnetic resonance frequency (chemical shift) of nuclear spin(s) in the solute of interest being different on either side of a cell membrane. Such differences in resonance frequency can arise naturally, by virtue of the distinct physico-chemical properties of the cytosol and the extracellular medium, such as pH (Labotka 1984; Stewart et al. 1986), magnetic susceptibility (Durrant et al. 2003; Kirk and Kuchel 1988a; Kuchel et al. 2003), ionic or hydrophobic interactions (Chapman and Kuchel 1990; Kuchel et al. 2015; Wittenkeller et al. 1992), extent of hydrogen bonding (Kirk and Kuchel 1988b; Maciel and Natterstad 1965; Potts et al. 1990; Potts and Kuchel 1992; Xu et al. 1991), or the presence of chelators and ‘shift reagents’ (Boulanger et al. 1985; Chu et al. 1984; Gupta and Gupta 1982; Puckeridge et al. 2012).

The MT methods are suitable for studying membrane transport that is sufficiently fast that ‘nuclear spin memory’ (magnetization) is not lost due to relaxation processes (characterized by the time constant T_1), yet it must be ‘slow on the NMR time scale’ so that the resonance frequencies of the two sites are not averaged to one value leading to a single peak in the spectrum. Thus, MT techniques are applicable when unidirectional membrane-transport rate constants are in the range of the value of $1/T_1$, and in many cases this is $0.1\text{--}50\text{ s}^{-1}$.

The theory and practice of NMR MT techniques, when applied to cellular systems to study enzyme kinetics and membrane transport, has been discussed in detail before (Kuchel 1990; Kuchel and Chapman 1983; Kuchel et al. 1994). However, the aim of the present mini-review is to give a more general description of how membrane transport in human erythrocytes can be quantitatively characterized using MT experiments. Additionally, we introduce more recent examples and the associated twists in the theory pertinent to the analysis of the data.

Transmembrane exchange of a solute in RBCs

The human RBC has no organelles; hence, its plasma membrane is the only structural barrier to the exchange of hydrophilic solute (or water) molecules into and within it. This simplifies the description of solute distribution in suspensions of RBCs to transport between only two compartments: extra- and intracellular, as illustrated in Fig. 1. However, note that, for excessively hydrophobic solutes, it might be necessary to also consider their distribution into the phospholipid bilayer, e.g. Eytan and Kuchel (1999).

Fig. 1 Kinetic scheme illustrating transmembrane exchange of a solute in a red blood cell. The semi-permeable plasma membrane of the cell separates the solution into extra- and intracellular compartments. S_o and S_i represent populations of a membrane-permeable hydrophilic solute S outside and inside the cells, respectively. k_{oi} and k_{io} are the unidirectional rate constants for the influx and efflux of S , respectively

In the absence of any active transporter-proteins, the probability of a solute or water molecule crossing from one side of a membrane to the other is directly proportional to its concentration (more strictly, *activity*) in each compartment. The two unidirectional fluxes, as shown in Fig. 1, expressed per unit of *aqueous* volume of the sample V , can be defined as follows:

$$\frac{J_{oi}}{V} = k'_{oi}[S_o] \quad (1)$$

$$\frac{J_{io}}{V} = k'_{io}[S_i] \quad (2)$$

where J_{oi} and J_{io} are the influx and efflux of the solute, respectively (units; mol s^{-1}). $[S_o]$ and $[S_i]$ are the molar concentrations of solute S in the extra- and intracellular compartments, respectively (units; mol L^{-1}); k'_{oi} and k'_{io} are the *intrinsic* rate constants for influx and efflux, respectively (units; s^{-1}).

At equilibrium, the solute is distributed between the two compartments according to the electrochemical potential difference across the membrane, which is expressed mathematically as the Nernst equation (Stein and Litman 2015):

$$zF(\psi_o - \psi_i) = RT \ln \left(\frac{[S_i]^{eq}}{[S_o]^{eq}} \right) \quad (3)$$

where R is the universal gas constant (units; $\text{J K}^{-1} \text{mol}^{-1}$); T is the absolute temperature (units; K); z is the electrical charge, carried by the solute molecule (a positive or negative integer); F is the Faraday constant (units; $\text{J V}^{-1} \text{mol}^{-1}$); ψ_o and ψ_i are the electrical potentials (units; V) outside and inside the cells, respectively; and $[S_i]^{eq}$ and $[S_o]^{eq}$ are the respective concentrations of the substrate inside and outside the cells at equilibrium.

When electrochemical equilibrium pertains, there is no *net* flux of the solute between the compartments. However, the unidirectional fluxes between the two compartments continue and are equal in both directions. Thus,

$$k'_{oi}[S_o]^{eq} = k'_{io}[S_i]^{eq} \quad (4)$$

Using Eq. 3, the equilibrium constant K_{eq} can be expressed as:

$$K_{eq} = \frac{[S_i]^{eq}}{[S_o]^{eq}} = \exp\left(\frac{zF(\psi_o - \psi_i)}{RT}\right) \quad (5)$$

Note that, according to Eq. 5, for uncharged solutes at equilibrium and in the absence of any external forces: $K_{eq} = 1$ and $[S_i]^{eq} = [S_o]^{eq}$.

As discussed above, a general requirement for MT experiments is that separate NMR signals are observed for the solute (or solvent) inside and outside the cell. When a quantitative ('fully relaxed') one-dimensional (1D) pulse-and-acquire NMR spectrum is recorded (pulse sequence of Fig. 2a), the integrals of the two peaks are directly proportional to the mole quantities of the substrate in each compartment. This allows experimental determination of the relative amounts of the solute in the two compartments:

$$\frac{S_i^{eq}}{S_o^{eq}} = \frac{I_i^{eq}}{I_o^{eq}} \quad (6)$$

where S_i^{eq} and S_o^{eq} are the equilibrium mole quantities of the solute in the two compartments; I_i^{eq} and I_o^{eq} are the NMR peak integrals of the two signals, corresponding to the intracellular and extracellular population of the solute at equilibrium, respectively.

Therefore, using a conventional 1D pulse-and-acquire NMR spectrum, the equilibrium constant K_{eq} can be calculated as:

$$K_{eq} = \frac{[S_i]^{eq}}{[S_o]^{eq}} = \frac{S_i^{eq}}{S_o^{eq}} \frac{V_o}{V_i} = \frac{I_i^{eq}}{I_o^{eq}} \frac{V_o}{V_i} \quad (7)$$

where V_i and V_o are the respective volumes of the compartments inside and outside the cells, that are accessible for solute distribution; they can be calculated by measuring the value of the fractional volume of the sample, occupied by red blood cells (RBCs) (called haematocrit, Ht) via the following expressions:

$$V_o = V_{tot}(1-Ht) \quad (8)$$

$$V_i = V_{tot} f_{aq} Ht \quad (9)$$

$$V_o + V_i = V \quad (10)$$

where V_{tot} is the total physical volume of the sample, $f_{aq} = 0.717$ is the fraction of the total volume of an RBC that is aqueous (Raftos et al. 1990; Savitz et al. 1964) and V is the total aqueous volume of the sample, as introduced in Eqs. 1 and 2.

The overall concentration of the equilibrated substrate $[S]^{eq}$, which is known from the sample preparation procedure, can be expressed as:

$$[S]^{eq} = \frac{S^{eq}}{V} = \frac{V_i[S_i]^{eq} + V_o[S_o]^{eq}}{V} \quad (11)$$

where S^{eq} is the total mole quantity of the solute in the sample. Having determined the value of K_{eq} from Eq. 7, we can calculate the equilibrium concentration of the solute inside and outside the cells via Eq. 11:

$$[S_o]^{eq} = \frac{[S]^{eq} V}{K_{eq} V_i + V_o} = \frac{S^{eq}}{K_{eq} V_i + V_o} \quad (12)$$

$$[S_i]^{eq} = \frac{[S]^{eq} V}{V_i + V_o/K_{eq}} = \frac{S^{eq}}{V_i + V_o/K_{eq}} \quad (13)$$

Fig. 2 NMR pulse sequences used in magnetization-transfer experiments. The black rectangles represent high-power 90° radio-frequency (RF) pulses; the acquisition of the free induction decay is shown as a decaying oscillation at the end of the pulse sequence. The mixing delay is denoted by t_m . **a** Conventional 1D pulse-and-acquire. **b** Saturation-transfer experiment: the selective saturating irradiation, denoted by the open rectangle, can be achieved using a DANTE pulse train (Morris and Freeman 1978). **c** Inversion-transfer experiment: the rounded open rectangle represents a selective inversion (180°) RF pulse. **d** 1D EXSY: when the carrier frequency is at one of the

exchanging resonances and the free-precession delay δ is set to $\frac{1}{2\Delta\nu}$, where $\Delta\nu$ is the difference in the chemical shifts of the two magnetizations (in Hz), a selective inversion is achieved, as shown in Fig. 3 (Robinson et al. 1985); the recommended phase cycling: third pulse: $x, y, -x, -y$; receiver: $x, y, -x, -y$. **e** 2D EXSY: in comparison with the 1D EXSY, the first free-precession delay is now substituted by the evolution time t_1 , which is incremented in the usual 2D-NMR fashion. It is advisable to suppress the axial peaks by a two-step phase cycle: first pulse: $x, -x$; receiver: $x, -x$ (Claridge 2009)

As noted above, $K_{eq} = 1$ for passive (energy-independent) transport of an uncharged solute, and Eq. 13 leads to the following equality in that case: $[S_o]^{eq} = [S_i]^{eq} = [S]^{eq}$.

Assuming that the volumes of the two compartments do not change during the experiment, the flux of a solute at equilibrium can be expressed in terms of the mole quantities of the species in the two compartments by making the following substitutions in Eq. 4:

$$k_{oi} = \frac{k'_{oi} V}{V_o} \quad (14)$$

$$k_{io} = \frac{k'_{io} V}{V_i} \quad (15)$$

$$J^{eq} = k_{oi} S_o^{eq} = k_{io} S_i^{eq} \quad (16)$$

where J^{eq} is the flux (in mol s^{-1}) of the solute between the two compartments under equilibrium; k_{oi} and k_{io} are the *extrinsic* rate constants for influx and efflux, respectively (in s^{-1}). These two constants can be expressed as:

$$k_{oi} = \frac{1}{\tau_o} \quad (17)$$

$$k_{io} = \frac{1}{\tau_i} \quad (18)$$

where τ_o and τ_i are the mean residence times of a solute molecule in the extra- and intracellular compartments, respectively. For a specified total concentration of the membrane-permeable solute S , τ_o and τ_i are functions of the volume of the respective compartment, from which the solute is being transported. Since the transport experiments are mostly conducted under isotonic conditions, where the average volume of an RBC is constant, it is convenient to specify the transport rate constant as that of efflux, k_{io} , because it does not depend on the Ht value. On the other hand, the influx rate constant k_{oi} does depend on Ht and is calculated as follows:

$$k_{oi} = k_{io} \frac{S_i^{eq}}{S_o^{eq}} = k_{io} \frac{V_i [S_i]^{eq}}{V_o [S_o]^{eq}} = k_{io} \frac{K_{eq} f_{aq} Ht}{1 - Ht} \quad (19)$$

Alternatively, the exchange rates can be expressed in terms of the permeability coefficient P (classically expressed in units of cm s^{-1}), which is also not dependent on the value of Ht :

$$P = \frac{k_{io} V_i}{A_{tot}} = \frac{k_{oi} V_o}{A_{tot}} \quad (20)$$

where A_{tot} is the total surface area of the cells in the sample. Strictly speaking, however, the permeability coefficient should be only applicable to the transport of non-electrolytes via a non-saturable diffusion process (Kirk 1990).

NMR magnetization-transfer experiments

How can we detect the unidirectional fluxes of a solute at equilibrium when its distribution between the compartments remains constant? Historically, radioactive-tracer exchange was the most successful method for probing such systems, e.g. Morales et al. (1962). This technique requires a suitable radioactive form of the solute of interest and separation of the cells from their bathing solution; it is therefore applicable for relatively slow fluxes (slower than ~ 1 min time scale). NMR MT techniques overcome most of the limitations and challenges, associated with the radioactive-tracer exchange methods (Hills and Belton 1989), but, as introduced above, the time scale of their applicability is determined by the longitudinal relaxation time (T_1) of the spins, and this is usually of the order of a few seconds.

In all MT experiments, the nuclear spin system is perturbed from its thermal-equilibrium state by applying a combination of RF pulses, after which a new state of magnetization is established, and from which the system eventually returns to its original equilibrium state. These events lead to the changes in peak intensities in the spectra, which are determined by rates of exchange via the membrane-transport process and nuclear magnetic relaxation.

A mathematical description of the MT experiments is given by the Bloch–McConnell (B-M) equations. These describe magnetization evolution via the classical Bloch equations (Bloch 1946) that are elaborated upon to include exchange between sites with two different NMR characteristics (McConnell 1958). For a species exchanging between two compartments (outside and inside a cell as shown in Fig. 1), dependence of the longitudinal nuclear magnetizations of the extracellular (M_o) and intracellular (M_i) spin populations on time, t , are given by:

$$\frac{dM_o(t)}{dt} = -R_o^o [M_o(t) - M_o^{eq}] - k_{oi} M_o(t) + k_{io} M_i(t) \quad (21)$$

$$\frac{dM_i(t)}{dt} = -R_i^i [M_i(t) - M_i^{eq}] - k_{io} M_i(t) + k_{oi} M_o(t) \quad (22)$$

where R_o^o and R_i^i are the longitudinal relaxation rate constants, characterizing the return of the magnetizations towards their respective thermal-equilibrium values, M_o^{eq} and M_i^{eq} ; k_{oi} and k_{io} are the rate constants for the influx and efflux of the solute, respectively. Note that k_{oi} and k_{io} are the *extrinsic* rate constants (as in Eqs. 14–20), because they are multiplied by the NMR magnetizations from each compartment, which are in turn proportional to the mole amounts of solute in each compartment, rather than the concentrations. Usage of the intrinsic rate constants would be clumsier, since it would require factoring in the compartment volumes into these equations. R_o^o and R_i^i refer to the relaxation rate constants, pertinent to the spin populations in the absence of the transmembrane exchange. They can be expressed as $R_o^o = \frac{1}{T_1^o}$ and $R_i^i = \frac{1}{T_1^i}$,

where T_1^o and T_1^i are the respective longitudinal relaxation time constants.

As discussed above, at equilibrium, the unidirectional solute fluxes counter-balance each other. Since magnetizations M_i and M_o are directly proportional to mole quantities of the solute in the two compartments (S_i and S_o , respectively), from Eq. 16, it follows that:

$$k_{io}M_i^{\text{eq}} = k_{oi}M_o^{\text{eq}} \quad (23)$$

Combining Eqs. 21 and 22 with Eq. 23 allows the expression of the B-M equations in terms of deviations of the magnetizations from their equilibrium values:

$$\frac{d[M_o(t) - M_o^{\text{eq}}]}{dt} = -(R_1^o + k_{oi})[M_o(t) - M_o^{\text{eq}}] + k_{io}[M_i(t) - M_i^{\text{eq}}] \quad (24)$$

$$\frac{d[M_i(t) - M_i^{\text{eq}}]}{dt} = -(R_1^i + k_{io})[M_i(t) - M_i^{\text{eq}}] + k_{oi}[M_o(t) - M_o^{\text{eq}}] \quad (25)$$

Eqs. 24 and 25 can then be written down concisely in matrix form:

$$\frac{d\mathbf{M}}{dt} = \mathbf{R} \cdot \mathbf{M} \quad (26)$$

where a dot product is taken between time-dependent vector $\mathbf{M}$ that describes the evolution of the two magnetizations: $\mathbf{M} =$

$\begin{bmatrix} M_o(t) - M_o^{\text{eq}} \\ M_i(t) - M_i^{\text{eq}} \end{bmatrix}$ and time-independent matrix $\mathbf{R}$ that contains

the relaxation and rate constants: $\mathbf{R} = \begin{bmatrix} -R_1^o - k_{oi} & k_{io} \\ k_{oi} & -R_1^i - k_{io} \end{bmatrix}$.

The formal analytical solution of Eq. 26 is:

$$\mathbf{M}(t) = \exp(\mathbf{R}t) \cdot \mathbf{M}(0) \quad (27)$$

Eq. 27 allows us to estimate the kinetic parameters of the two-site exchange system, provided the appropriate NMR measurements are carried out. However, the precise nature of the required measurements varies for the different types of MT experiments; and the analytical solution of the B-M equations can be substantially simplified in some cases. Accordingly, some of the most commonly used types of MT experiments are described next.

Saturation transfer

The saturation-transfer (ST) experiment was pioneered by Forsén and Hoffman (1963) using continuous-wave NMR spectroscopy. In the ST experiment, selective RF irradiation

is applied at the chemical shift of one of the exchanging spin populations, before sampling the magnetizations with a hard RF pulse, as shown in the pulse sequence of Fig. 2b. The RF irradiation should be of sufficient power and duration for it to ‘saturate’ the targeted magnetization to zero; at the same time, it should not be so strong (or unselective) as to directly affect the equilibrium magnetization of the other spin population.

Due to the exchange of the species between the two sites, the saturated magnetization will ‘flow’ into the un-irradiated site. This saturation transfer competes with the relaxation of the magnetization towards the equilibrium state, thus leading to an overall steady state of the magnetization of the spin system. For example, if the intracellular spin population is chosen to be saturated, the signal of the extracellular resonance will be reduced to some finite value, M_o^{sat} . Thus, in the steady state under saturation, we obtain: $M_i(t) = 0$, $M_o(t) = M_o^{\text{sat}}$ and $\frac{dM_o(t)}{dt} = 0$. Substituting these expressions into Eq. 21 leads to:

$$R_1^o[M_o^{\text{eq}} - M_o^{\text{sat}}] = k_{oi}M_o^{\text{sat}} \quad (28)$$

$$R_1^{o,\text{sat}}\Delta M = k_{oi}M_o^{\text{eq}} \quad (29)$$

where $\Delta M = M_o^{\text{eq}} - M_o^{\text{sat}}$ and $R_1^{o,\text{sat}} = R_1^o + k_{oi}$ is the ‘effective’ relaxation rate constant, measured in the presence of the saturating irradiation (Brown 1980).

If $R_1^{o,\text{sat}}$ is estimated in a separate process, only two NMR spectra are required to be measured in the ST experiment: one in the presence and the other in the absence of the RF irradiation. This allows the determination of the values of M_o^{sat} and M_o^{eq} from the corresponding peak integrals and, therefore, estimation of k_{oi} as:

$$k_{oi} = \frac{R_1^{o,\text{sat}}\Delta M}{M_o^{\text{eq}}} \quad (30)$$

By analogy, k_{io} is obtained by saturating the extracellular population and measuring values of M_i^{sat} , M_i^{eq} and $R_1^{i,\text{sat}}$.

In practice, the selective saturating irradiation can be achieved using a DANTE pulse train (Morris and Freeman 1978). The main limitation of the ST method is that the two resonances must be sufficiently well resolved in the NMR spectrum to achieve the required saturation selectivity and avoid saturation ‘spill-over’ to the other population (Gabr et al. 2008). Therefore, control experiments are usually required, whereby the RF irradiation is applied at the same frequency offset from the un-saturated resonance, but to the other side of the spectrum. However, the situation might further be complicated by an asymmetric RF irradiation profile, and this should be overcome by using well-chosen control experiments with cell lysates, e.g. Potts et al. (1989).

Another problem, associated with the saturating RF irradiation, is that it may lead to sample heating, thus systematically

affecting the estimated values of the kinetic parameters. Therefore, in the ST experiment, the effective sample temperature should be estimated by using an independent method that takes into account the RF heating. For example, it can be done by using an ‘NMR thermometer’ consisting of a capillary with ethylene glycol that gives two NMR resonances, the separation of which is temperature sensitive (Bubb et al. 1988).

Considering the complications associated with selective RF irradiation, the other types of MT experiments are usually more reliable. Nevertheless, the ST method remains valuable because of its relative simplicity and an ability to provide clear evidence of exchange before the more time consuming estimate of $R_1^{0,\text{sat}}$ is made to actually quantify the exchange-rate constants.

Inversion transfer

The inversion-transfer (IT) experiment is conceptually similar to the ST experiment: as one of the exchanging resonances is perturbed, the effect of that perturbation is observed on the other (Dahlquist et al. 1975). However, instead of being selectively saturated, the magnetization (resonance) of one of the exchanging populations is inverted. For example, if a perfect selective-inversion RF pulse is applied at the resonance frequency of the intracellular spin population, we get the following starting conditions: $M_i(0) = -M_i^{\text{eq}}$, $M_o(0) = M_o^{\text{eq}}$. Therefore, the IT method employs perturbation of the original magnetization that is twice as large as in the ST experiment, thus improving the statistical quality of the data (Alger and Prestegard 1977). However, the selective inversion is rarely perfect and the starting magnetizations $M_i(0)$ and $M_o(0)$ should be measured in a separate process.

During the recovery of the inverted resonance to its thermal equilibrium, the longitudinal magnetization of the un-inverted resonance is affected by the exchange process, and both magnetizations can be read out by a hard 90° RF pulse upon waiting a chosen value of the mixing delay (pulse sequence of Fig. 2c). In the simplest form of the quantitative IT experiment, the magnetizations of the two resonances are sampled straight after the selective pulse (effectively zero mixing time) and at a finite value of the mixing time t_m , which produces strong perturbation of the un-inverted resonance. In order to determine all four unknowns of the matrix $\mathbf{R}$, this procedure has to be done by using inversion of one of the two resonances first, followed by the other (Bellon et al. 1987). Eq. 27 can be solved by constructing $\mathbf{M}(0)$ and $\mathbf{M}(t_m)$ as square 2×2 matrices from the numerical values of the measured

peak intensities, obtained by using zero and non-zero mixing times, respectively:

$$\mathbf{M}(0) = \begin{bmatrix} M_o^A(0) - M_o^{\text{eq}} & M_o^B(0) - M_o^{\text{eq}} \\ M_i^A(0) - M_i^{\text{eq}} & M_i^B(0) - M_i^{\text{eq}} \end{bmatrix} \quad (31)$$

$$\mathbf{M}(t_m) = \begin{bmatrix} M_o^A(t_m) - M_o^{\text{eq}} & M_o^B(t_m) - M_o^{\text{eq}} \\ M_i^A(t_m) - M_i^{\text{eq}} & M_i^B(t_m) - M_i^{\text{eq}} \end{bmatrix} \quad (32)$$

where superscripts A and B refer to the two separate experiments, where either one or another resonance is inverted. All elements of matrix $\mathbf{R}$ are then calculated from Eq. 27 using matrix algebra:

$$\mathbf{R} = \frac{\ln[\mathbf{M}(t_m) \cdot \mathbf{M}^{-1}(0)]}{t_m} \quad (33)$$

where $\mathbf{M}^{-1}(0)$ is the matrix, inverse to $\mathbf{M}(0)$. The natural logarithm of the dot product $\mathbf{M}(t_m) \cdot \mathbf{M}^{-1}(0)$ in Eq. 33 can be calculated by the diagonalization procedure via its eigensystem:

$$\ln[\mathbf{M}(t_m) \cdot \mathbf{M}^{-1}(0)] = \mathbf{U} \cdot \ln(\mathbf{\Lambda}) \cdot \mathbf{U}^{-1} \quad (34)$$

where $\mathbf{U}$ is the transformation matrix, composed of the eigenvectors of matrix $\mathbf{M}(t_m) \cdot \mathbf{M}^{-1}(0)$ as its columns; $\mathbf{\Lambda}$ is the diagonal matrix, containing the corresponding eigenvalues; and $\mathbf{U}^{-1}$ is the inverse of matrix $\mathbf{U}$. By definition, $\mathbf{U}^{-1}$ and $\mathbf{U}$ transform matrix $\mathbf{M}(t_m) \cdot \mathbf{M}^{-1}(0)$ into $\mathbf{\Lambda}$ (Perrin and Gipe 1984):

$$\mathbf{U}^{-1} \cdot \mathbf{M}(t_m) \cdot \mathbf{M}^{-1}(0) \cdot \mathbf{U} = \mathbf{\Lambda} \quad (35)$$

In a more advanced form of the IT experiment, the temporal evolution of the two magnetizations upon the selective inversion is recorded. The most convenient way to analyze the obtained recovery curves is by direct fitting of the B-M model onto the experimental data. This is done by a nonlinear least-squares iterative procedure such as the Levenberg–Marquardt algorithm (Moré 1978). After initial estimates/guesses of the relaxation and kinetic rate constants are put into the model, the B-M equations are numerically integrated ‘on the fly’, thus obviating the need for their analytical solution. Thus, the ‘best-fit’ parameter values for the relaxation and rate constants are obtained, together with estimates of their statistical uncertainties (standard deviation/standard error).

It is possible to reduce the number of the fitted parameters from a given dataset by separately measuring the values of the relaxation rate constants in the absence of exchange (Mariappan and Rabenstein 1992). Nevertheless, with modern software packages like *Mathematica* that we now routinely use (Mulquiney and Kuchel 2003), it is usually simpler to measure an overdetermined set of experimental points and

simultaneously fit all parameters of the model. The initial and equilibrium values of the two magnetizations can also be specified as fitted parameters.

For better precision of the determination of the rate constants, this variant of the IT experiment should be done by using inversion of one resonance first, followed by the other. This allows measurement of four magnetization time-courses, which are all used together in one global fitting procedure (Led and Gesmar 1982). When all the experimental conditions are optimized, the standard error of the estimates of the rate constants using a high-field NMR spectrometer is generally only a few percent (Bain and Duns 1997).

In practice, the selective inversion can be achieved by using a soft 180° RF pulse (Alger and Prestegard 1977; Bellon et al. 1987), as shown in Fig. 2c; however, greater selectivity can be achieved with so-called ‘ δ -ordered inversion’, which consists of two hard 90° pulses, separated by a delay δ , as shown in Fig. 2d (Robinson et al. 1985). Using the latter technique, a complete selective inversion is achieved when the frequency of the RF transmitter is set on one of the resonances, and $\delta = \frac{1}{2\Delta\nu}$, where $\Delta\nu$ is the difference in chemical shift between the two exchanging resonances (in Hz). The vector-model representation, illustrating how the selective inversion is achieved in this case, is shown in Fig. 3.

When the δ -ordered IT experiment is repeated several times using the same mixing time t_m , but different values of δ , it is referred to as ‘one-dimensional exchange spectroscopy’ (1D EXSY) (Engler et al. 1988); its name comes from its conceptual similarity to the two-dimensional counterpart (2D EXSY; see below). In 1D EXSY, the analysis of the data is readily performed using Eq. 33. However, when constructing matrices $\mathbf{M}(0)$ and $\mathbf{M}(t_m)$ as per Eqs. 31–32, superscripts A and B should now refer to the two separate experiments using two different values of δ . In order to obtain standard-error estimates, a number of experiments using a range of δ values needs to be performed. The pertinent analysis of such a set of data is referred to as ‘overdetermined’ 1D EXSY and is described in detail by Bulliman et al. (1989). Usually, acquisition of sets of spectra with 4–6 values of δ and a mixing time

of zero and a finite value are required for robust error estimation, as exemplified in Fig. 4.

Another approach to recording and analyzing 1D EXSY data, based on Eq. 33, entails linear least-squares fitting (Perrin and Engler 1990). It involves repeating the 1D EXSY measurements using the pulse sequence of Fig. 2d with varying the mixing time t_m , calculating the matrix $\ln[\mathbf{M}(t_m) \cdot \mathbf{M}^{-1}(0)]$ using Eqs. 31–32 and 34 for each value of t_m , and plotting the elements of this matrix, corresponding to k_{oi} or k_{io} , against t_m . According to Eq. 33, the slope of the straight line fitted to this plot yields the estimates of the values of the corresponding rate constants (Perrin and Engler 1990). This method is also applicable to the analysis of 2D EXSY data; however, in this case, $\mathbf{M}(t_m)$ and $\mathbf{M}(0)$ are constructed as square 2×2 matrices, consisting of the corresponding diagonal and cross-peak intensities (see below).

Two-dimensional exchange spectroscopy

Two-dimensional exchange spectroscopy (2D EXSY) employs the same RF pulse sequence as the more widely-known nuclear Overhauser effect (NOE) spectroscopy (2D NOESY), shown in Fig. 2e (Jeener et al. 1979). 2D EXSY provides a two-dimensional map, where the cross-peaks between the NMR signals reveal the presence of an exchange process (Perrin and Dwyer 1990) (or NOE peaks due to cross-relaxation (Macura and Ernst 1980)).

Quantitative treatment of 2D EXSY experiments requires extraction of the volumes of the two-dimensional diagonal and cross-peaks in the spectra. Measurements of peak volumes is often reliably carried out in the system software of the NMR spectrometer, such as Bruker’s TopSpinTM. However, a more advanced method, which involves fitting mixed Gaussian/Lorentzian lineshapes in a software package such as *Mathematica* (Wolfram 2003), or *Matlab* (MathWorks 2005), to the obtained peaks can also be used. Such procedures provide readily repeatable automation of the process and enable handling of moderate levels of peak overlap, effectively working as a spectral-peak deconvolution method. Once the

Fig. 3 Vector-model illustration of the ‘ δ -ordered inversion’ procedure, which is used in the 1D EXSY pulse sequence shown in Fig. 2d. **a** The two magnetization vectors are aligned along the z -axis (direction of the external magnetic field) at the beginning of the pulse sequence. **b** Both vectors are flipped onto the y -axis of the rotating frame of reference after the first 90°_x pulse. **c** Evolution of the vectors in the transverse plane during the free-precession delay δ ; the black vector does not move

because the carrier frequency is set at its chemical shift. **d** After delay $\delta = \frac{1}{2\Delta\nu}$, where $\Delta\nu$ is the difference in the chemical shifts of the two magnetizations (in Hz), the vectors are pointing in opposite directions along the y -axis. **e** The magnetization of one of the vectors is selectively inverted after the application of the second 90°_x pulse. Adapted with permission from Robinson et al. (1985)

Fig. 4 ‘Overdetermined’ set of one-dimensional ^{31}P (161.5 MHz) 1D EXSY NMR spectra of 40 mM hypophosphite in an RBC suspension at 37 °C. **a** The ‘fully relaxed’ equilibrium spectrum: the high and low frequency peaks are from the extra-(o) and intracellular (i) species, respectively. **b–g** The upper spectra were acquired using the 1D EXSY pulse sequence of Fig. 2d with zero mixing time ($t_m = 0$) and δ equal to $3/16\Delta\nu$, $4/16\Delta\nu$... $8/16\Delta\nu$ s, respectively; the corresponding lower spectra were acquired using the same parameters, except $t_m = 1$ s. Adapted with permission from Price and Kuchel (1990)

intensities (volumes) of the corresponding peaks are extracted, there are several methods that can be used in the subsequent analysis.

The earliest approach to analyzing 2D EXSY data was the initial-rate approximation method (Kumar et al. 1981). This method involves carrying out several 2D EXSY experiments using different values of the mixing time t_m and plotting the cross-to-diagonal peak-volume ratio against t_m . A straight-line equation is then fitted to the data and the slope of the best-fit line provides an estimate of the exchange-rate constant. The disadvantage of this method is that it is only accurate for short mixing times.

Alternatively, the analysis can be performed by the so-called ‘back-transformation’ analysis (Bremer et al. 1984; Perrin and Gipe 1984). This is based on the matrix-form solution of Eq. 27. In the simplest form of this experiment, matrices $\mathbf{M}(0)$ and $\mathbf{M}(t)$ are measured experimentally by conducting two 2D EXSY experiments: one using zero mixing time, and the second one with a finite value of t_m . Eq. 33 then allows determination of matrix $\mathbf{R}$, which contains all the information about the longitudinal relaxation and exchange-rate constants. According to the standard convention, the main diagonal in 2D NMR spectra goes from the lower-left corner to the upper-right one, while in matrix algebra, the main (leading) diagonal goes from the upper-left corner to the lower-right one. Therefore, we must take extra care when constructing matrices $\mathbf{M}$ and $\mathbf{R}$. For example, when $\mathbf{R}$ is given as $\begin{bmatrix} -R_1^o - k_{oi} & k_{io} \\ k_{oi} & -R_1^i - k_{io} \end{bmatrix}$, $\mathbf{M}$ should have the form: $\begin{bmatrix} I_{oo} & I_{io} \\ I_{oi} & I_{ii} \end{bmatrix}$, where I_{oo} and I_{ii} are the intensities of the diagonal peaks, corresponding to the extra- and intracellular populations, respectively; I_{io} is the intensity of the cross-peak for

efflux (intracellular chemical shift in the t_1 dimension, extra-cellular chemical shift in the t_2 dimension); and I_{oi} is the cross-peak intensity for influx (extracellular chemical shift in the t_1 dimension, intracellular chemical shift in the t_2 dimension).

The ability of the back-transformation method to provide statistically robust quantification of exchange-rate constants relies on a judicious choice of the mixing time. The optimal mixing time that maximizes the intensity of the cross peaks in the 2D EXSY experiments is calculated as (Perrin 1989):

$$t_m = \frac{1}{\bar{R} + \bar{k}} \quad (36)$$

where $\bar{R}$ and $\bar{k}$ are the mean values of the relaxation and exchange-rate constants, respectively. Using this optimal value for mixing time optimizes the precision of the estimates of the rate constant from both 1D and 2D EXSY spectra (Kuchel 1990).

Estimation of standard deviations in the back-transformation analysis has also been specifically addressed (Kuchel et al. 1988). However, sample-to-sample variations are usually higher than these statistical errors, so for proper error estimation it is advisable to repeat the experiment with samples that are prepared in an identical way and then to evaluate the inter-sample variations, e.g. O’Connell et al. (1994).

Back-transformation analysis of the 2D EXSY experiment can be further simplified if a simple quantitative 1D pulse-and-acquire experiment is also recorded. In this case, it is not necessary to measure a 2D EXSY spectrum with zero mixing time. Instead, matrix $\mathbf{M}(0)$ is substituted by a diagonal matrix that is composed of the corresponding intensities of the two peaks in the 1D spectrum, $\mathbf{S}(0)$ (Kuchel et al. 1988):

$$\mathbf{M}(0) = \alpha \mathbf{S}(0) \quad (37)$$

where the scaling factor α is usually unknown, yet its inclusion in the equations only affects the values of the diagonal entries of the matrix $\mathbf{R}$ and not the all-important off-diagonal elements. In other words, knowledge of the value of α is not required for the determination of the exchange-rate constants of interest (Kuchel et al. 1988).

For more precise quantification of the exchange-rate constants, several 2D EXSY spectra should be acquired using a range of mixing times. Then, the measured peak volumes can be used in a nonlinear regression fit of the B-M equations to the data. Such a method is more robust than the back-transformation procedure and allows estimates of the standard errors. However, recording several 2D EXSY spectra with different mixing times can be time consuming. Even if the spectral signal-to-noise ratio is high, acquisition of one 2D EXSY spectrum might take ~1 h in order to provide sufficient resolution in the indirect chemical shift dimension. This arises because complete recovery of longitudinal magnetization to its original unperturbed state is essential for quantitative analysis. Therefore, a

recovery delay of at least $5 \times T_1^{\max}$ is recommended between successive free induction decays (FIDs), where $T_1^{\max}$ is the longest of the relaxation time constants of all spin populations present in the exchanging system.

As in any quantitative NMR analysis, the precision of the measurement of peak volumes in 2D EXSY spectra needs to be maximized, so judicious choices of the FID-processing window functions and spectral baseline corrections are required. The experiment is preferably done using the hypercomplex States (Ohuchi et al. 1987; States et al. 1982) or TPPI (Marion and Wüthrich 1983) methods to achieve pure-absorption spectra. This is done routinely on modern spectrometers and allows discriminating between genuine exchange peaks and those due to cross-relaxation (Johnston et al. 1986). Other ‘false’ peaks in the 2D EXSY spectra might arise due to the presence of J -couplings in the spin system; this issue was specifically discussed by Macura et al. (1981). Axial peaks can be easily suppressed by a two-step phase cycle in the pulse sequence of Fig. 2e: first pulse: $x, -x$; receiver: $x, -x$ (Claridge 2009).

When the experimental setup is optimized, the precision of the 1D and 2D methods for estimating exchange-rate constants are comparable (Abel et al. 1986). However, 1D experiments usually take less experiment time than their 2D counterparts. Hence, when sample stability is under question, the 1D methods are preferable. Moreover, 1D variants are especially suitable when there are only a few NMR signals in the spectrum with no substantial peak overlap. On the other hand, 2D methods are required for studying samples that contain several poorly resolved resonances since the second chemical shift dimension generally reduces the spectral overlap (e.g. Fig. 6). Besides, the 2D spectrum gives direct visual evidence (useful for illustrations) of inter-site exchange via, often obvious, cross-peaks, as exemplified in Fig. 7.

Deeper analysis of transmembrane-transport kinetics

In the quantification of membrane-transport kinetics using NMR (or, in fact, any other technique), care must be taken to interpret correctly the results in terms of a model that is consistent across many datasets obtained under diverse experimental conditions.

When it is known that a particular component of the membrane, such as a carrier protein, is involved in the transport process, then solute flux can be ‘saturated’ at high substrate concentrations, reaching its maximum velocity ($V_{\max}$), as for a classical Michaelis–Menten enzyme (Stein 1986). In this case, the values of k_{io} and k_{oi} become dependent on the overall substrate concentration $[S]$. For such a system, it is useful to estimate the value of $V_{\max}$ and the Michaelis constant K_m (substrate concentration at half maximal velocity), the latter

being interpreted as the dissociation constant of the enzyme–substrate complex. The ratio of $V_{\max}/K_m$ is a useful indication of the catalytic activity of the transporter on a particular substrate and, in the context of membrane transport, can be interpreted as the overall ‘membrane permeability’ of a solute (Stein and Litman 2015). Therefore, a complete Michaelis–Menten kinetic analysis of such a system is performed by repeating measurements of exchange-rate constants k_{io} and k_{oi} for a range of substrate concentrations (which should cover values of $[S]$ well above and below the K_m). Then, fitting of the Michaelis–Menten model to the experimental data is performed for the estimation of the kinetic parameters.

In general, if the estimated values of the apparent rate constants k_{io} and k_{oi} do not depend on the overall concentration of the substrate (which means that the exchange rate varies linearly with the substrate concentration), the mechanism of the transport is surmised to be via passive diffusion through the phospholipid bilayer. However, it is possible that a saturable system is in fact in operation, but the tested substrate concentration range was not sufficiently broad to reveal at least partial saturation of the transport protein(s). Therefore, before assuming that the mechanism is in fact unassisted diffusion, it is also recommended to check whether the transport can be reduced by a range of known inhibitors of common transport proteins. Alternatively, an investigation of how the transport rate is affected by a change in temperature allows estimation of the activation energy of the process via the Arrhenius equation. The obtained activation energy will comment on the underlying mechanism of transport: passive diffusion is much less dependent on temperature and has a low activation energy when compared to protein-mediated transport.

Additionally, at least in the case of the simplest reversible enzyme-catalyzed reaction, it is possible to determine the rate constants of each of the kinetic steps in the mechanism from the equilibrium-exchange NMR MT studies, if the experiment is performed at several different temperatures (Kuchel 1987). However, as discussed above, equilibrium-exchange MT experiments only yield estimates of the *apparent* first-order rate constants for the overall efflux or influx of the substrate, which makes them unsuitable for determining the exact mechanism of enzyme operation, order of reaction, or values of unitary rate constant(s) of complex membrane-transport processes.

The Michaelis–Menten model

In its simplest form, the equilibrium-exchange kinetics of protein-mediated solute transport can be expressed as:

$$v^{\text{eq}}([S_i]) = \frac{V_{\max}^{\text{eq}}[S_i]}{K_m^{\text{eq}} + [S_i]} \quad (38)$$

where v^{eq} is the equilibrium-exchange velocity of the membrane-transport reaction, *viz.*, the equilibrium flux (properly normalized). The intracellular substrate concentration $[S_i]$ should be used in Eq. 38 in order to obtain values of $V_{\text{max}}^{\text{eq}}$ and K_m^{eq} that are independent of the experimental Ht value. $V_{\text{max}}^{\text{eq}}$ is the maximum value of the transport velocity, reached under ‘saturated’ equilibrium-exchange conditions when $[S_i] \gg K_m^{\text{eq}}$. The Michaelis constant K_m^{eq} is the concentration of the substrate S that yields half of the maximum transport velocity under equilibrium exchange. Thus, $v^{\text{eq}}([S_i] = K_m^{\text{eq}}) = \frac{V_{\text{max}}^{\text{eq}}}{2}$.

The equilibrium-exchange velocity of the membrane-transport process can be expressed as the equilibrium substrate flux J^{eq} , normalized to the total number of cells in the sample, n_{RBC} :

$$v^{\text{eq}} = \frac{J^{\text{eq}}}{n_{\text{RBC}}} = \frac{k_{\text{io}} S_i^{\text{eq}}}{n_{\text{RBC}}} \quad (39)$$

$$n_{\text{RBC}} = \frac{V_{\text{cell}}}{V_{\text{RBC}}} = \frac{V_i}{f_{\text{aq}} V_{\text{RBC}}} \quad (40)$$

where V_{cell} is the total sample volume, occupied by the cells; and V_{RBC} is the average volume of an RBC ($V_{\text{RBC}} = 86$ fL in isotonic solution (Dacie and Lewis 1975)).

Substituting Eqs. 39–40 into Eq. 38, after some algebraic rearrangements, gives:

$$k_{\text{io}} f_{\text{aq}} V_{\text{RBC}} = \frac{V_{\text{max}}^{\text{eq}}}{K_m^{\text{eq}} + [S_i]} \quad (41)$$

As discussed above, the MT experiments can be used to obtain estimates of the apparent rate constants k_{io} at several different concentrations $[S_i]$. Then, fitting Eq. 41 to the experimental dependence of k_{io} on $[S_i]$ allows estimation of the Michaelis–Menten parameters $V_{\text{max}}^{\text{eq}}$ and K_m^{eq} , as exemplified in Fig. 5.

Fig. 5 Michaelis–Menten plot demonstrating the dependence of the fluoride efflux rate from RBCs on the intracellular fluoride concentration, under equilibrium-exchange conditions. The solid line is the fit of the Michaelis–Menten model that allowed estimation of the K_m and V_{max} values at 27 ± 3 mM and 18.0 ± 0.5 fmol cell⁻¹ s⁻¹, respectively. Reproduced with permission from Chapman and Kuchel (1990)

Examples of equilibrium-exchange membrane-transport studies in RBCs

Table 1 presents a summary of the published NMR MT studies that were used to quantify equilibrium-exchange kinetics of solutes in RBCs. These studies are briefly described in the following.

Dimethyl methylphosphonate

The earliest quantitative estimate of the rapid (sub-second) transmembrane exchange rate of a solute in RBCs was carried out on the non-electrolyte dimethyl methylphosphonate (DMMP) using ST ³¹P NMR (Kirk and Kuchel 1986). DMMP displays a ‘split-peak effect’ in an RBC suspension, which is manifested as separate ³¹P NMR signals from populations inside and outside the cells; this beneficial property was initially used to monitor changes in cell volume (Kirk and Kuchel 1985). The intracellular ³¹P signal is shifted to lower frequency due to differences in the magnetic susceptibility and the extent of hydrogen bonding of the phosphoryl oxygen with water (Kirk and Kuchel 1988b). It was concluded that this compound enters RBCs via unassisted dissolution in the membrane bilayer, since its exchange rate ($k_{\text{io}} = 0.3$ s⁻¹; $P = 9.47 \pm 1.01 \times 10^{-5}$ cm s⁻¹ at 25 °C) was not affected by several well-known inhibitors of membrane-transport proteins, and it was not changed as the substrate concentration was increased up to 600 mM (Potts et al. 1989).

Hypophosphite

As in the case of DMMP, ³¹P NMR spectra of hypophosphite (H_2PO_2^-) display the split-peak effect (Labotka and Omachi 1987). This allowed its use as a probe of membrane potential (Kirk et al. 1988) and to measure its transmembrane exchange rate using 1D EXSY experiments (Price and Kuchel 1990). A typical ‘overdetermined set’ of the obtained 1D EXSY spectra is shown in Fig. 4. The transmembrane exchange of hypophosphite was shown to be inhibited by 4,4'-dinitrostilbene-2,2'-disulfonic acid (DNDS), a known inhibitor of capnophorin (‘band 3’ protein) (Cabantchik and Rothstein 1974). Therefore, in order to characterize the membrane-transport kinetics of H_2PO_2^- properly, the experiments were carried out using a range of substrate concentrations from 20 to 240 mM. This led to the following estimates of the Michaelis–Menten kinetic parameters of capnophorin for equilibrium exchange of H_2PO_2^- : $K_m = 75 \pm 16$ mM and $V_{\text{max}} = 1.6 \pm 0.2$ fmol cell⁻¹ s⁻¹ at 37 °C (Price and Kuchel 1990).

Table 1 Summary of the conducted quantitative measurements of equilibrium-exchange membrane-transport kinetics of various compounds in human RBCs using magnetization-transfer NMR techniques

Compound	Mechanism	NMR probe nucleus	MT method	Reference
Dimethyl methylphosphonate	Passive diffusion	^{31}P	ST	(Kirk and Kuchel 1986; Potts et al. 1989)
Hypophospite	Capnophorin	^{31}P	1D EXSY	(Price and Kuchel 1990)
Trifluoroacetamide	Passive diffusion	^{19}F	IT	(London 1994)
Fluoride ion	Capnophorin	^{19}F	1D EXSY	(Chapman and Kuchel 1990)
Difluorophosphate	Capnophorin	^{19}F	1D EXSY	(Kuchel et al. 1992)
FDG-3	GLUT1	^{19}F	ST	(Potts et al. 1990)
FDG-3	GLUT1	^{19}F	1D EXSY	(Potts and Kuchel 1992)
FDG-2, FDG-3, FDG-4, FDG-6	GLUT1	^{19}F	1D EXSY	(O'Connell et al. 1994)
FDG-223344	GLUT1	^{19}F	2D EXSY	(Kim et al. 1998)
FDG-234, FDA-234	GLUT1	^{19}F	2D EXSY	(Bresciani et al. 2010)
Ammonia	RhAG	$^{15}\text{N}/^{14}\text{N}$	ST	(Labotka et al. 1995)
Fluoroethylamine	RhAG	^{19}F	1D EXSY	(Szekely et al. 2006)
Formate	Capnophorin	^{13}C	ST	(Kuchel et al. 1992)
Bicarbonate	Capnophorin	^{13}C	DST	(Kuchel et al. 1987a)
[1- ^{13}C]D-glucose	GLUT1	^{13}C	DST	(Kuchel et al. 1987b)
HDO	Aquaporin and passive diffusion	^2H	2D EXSY	(Kuchel and Naumann 2008)

Trifluoroacetate and trifluoroacetamide

^{19}F NMR spectra of fluorinated compounds in general also display the split-peak effect in RBC suspensions; this was first reported for trifluoroacetate (TFA) and trifluoroacetamide (TFM) by London and Gabel (1989). The advantage of ^{19}F as a probe nucleus is its high NMR sensitivity and virtual absence of background signals in biological samples. The physical basis of this effect is similar to that of the phosphoryl compounds; however, the intracellular shift of ^{19}F is in the opposite direction (to higher frequency) to that of ^{31}P . This distinction arises because the F atom participates directly in forming hydrogen bonds whereas it is the phosphoryl oxygen (and hence only indirectly ^{31}P) in the case of ^{31}P compounds (Xu et al. 1991). This underscores the fact that the split-peak effect is primarily not because of the magnetic-susceptibility differences across the cell membrane but due to chemical interactions like hydrogen bonding that differ between the inside and outside of cells.

Rapid transmembrane exchange of TFM was studied using the IT experiment (London 1994). Under the conditions used, the apparent efflux rate constant k_{io} was estimated to be 12.5 s^{-1} ; and the exchange of TFM was not competitive with urea and not inhibited by phloretin (inhibitor of the glucose transporting GLUT1 protein). Therefore, it was concluded that the likely mechanism of its transport was by passive diffusion through the phospholipid bilayer (London 1994).

Fluoride ion

Using ^{19}F NMR spectroscopy, the rate of exchange of fluoride ion (F^-) in RBCs was measured using overdetermined 1D EXSY analysis by Chapman and Kuchel (1990). The transmembrane transport of F^- was concluded to be via capnophorin due to its inhibition by DNDS. The measurements of fluoride efflux rate constants at 37°C using a range of its intracellular concentrations (11 to 136 mM) yielded the Michaelis–Menten plot shown in Fig. 5. Fitting of the Michaelis–Menten model to the experimental data produced best-fit parameter estimates of $K_{\text{m}} = 27 \pm 3 \text{ mM}$ and $V_{\text{max}} = 18.0 \pm 0.5 \text{ fmol cell}^{-1} \text{ s}^{-1}$ (Chapman and Kuchel 1990).

Difluorophosphate

Difluorophosphate (DFP) displays the split-peak effect in both ^{19}F and ^{31}P NMR spectra (Xu et al. 1991). ^{19}F is the preferred nucleus for MT experiments, due to its higher inherent sensitivity. The rate of transmembrane exchange of DFP was quantified using the overdetermined 1D EXSY ^{19}F NMR: K_{m} and V_{max} were estimated to be $50 \pm 10 \text{ mM}$ and $3.8 \pm 2.0 \text{ fmol cell}^{-1} \text{ s}^{-1}$ under equilibrium-exchange conditions at 37°C (Kuchel et al. 1992). The exchange of DFP across the membrane was also inhibited by DNDS, implying its transport via capnophorin (Kuchel et al. 1992).

Fluorinated analogues of D-glucose

One of the favourable cases for MT analysis is the transmembrane exchange of the fluorinated analogues of D-glucose (Glc). Introduction of fluorine atoms into the Glc scaffold slows down its catabolism by cells and provides a convenient handle for MT experiments, based on the ^{19}F split-peak effect. Individual ^{19}F resonances are also routinely observed for the α - and β -anomers of these sugars, which makes the NMR MT approach especially suitable for investigating the differences between the catalytic activity of the glucose transporter GLUT1 on the two anomeric forms (O'Connell et al. 1994; Potts and Kuchel 1992). In turn, the data analysis is slightly more complicated due to competition between the α - and β -anomers of the sugar for transport via GLUT1; this requires appropriate modifications in the Michaelis–Menten analysis (Potts and Kuchel 1992).

The transmembrane exchange of 3-fluoro-3-deoxy-D-glucose (FDG-3) was measured using ST ^{19}F NMR (Potts et al. 1990) and the overdetermined 1D EXSY method (Potts and Kuchel 1992). The latter analysis produced more robust data: the transmembrane exchange of FDG-3 was measured for a range of substrate concentrations (3.4–113 mM), which established that the ratio of Michaelis constants for α - to β -anomers was 0.75 ± 0.07 (34 °C) and 0.83 ± 0.07 (37 °C). The transport was shown to be via GLUT1, by blocking it with the known inhibitors of the transporter: phloretin and cytochalasin B (Bloch 1973; Griffin et al. 1982).

Using quantitative 1D EXSY measurements, O'Connell et al. (1994) concluded that 2-fluoro-2-deoxy-D-glucose (FDG-2) and FDG-3 are transported at a similar rate to Glc, while the transport of 4-fluoro-4-deoxy-D-glucose (FDG-4) and 6-fluoro-6-deoxy-D-glucose (FDG-6) is at $\sim 1/2$ that rate. The transport of FDG-2 and FDG-3 was also significantly inhibited by the adenosine antagonist BW-A1433U (Gabel et al. 1997).

Kim et al. (1998) quantified the transmembrane exchange of 2,2,3,3,4,4-hexafluoro-2,3,4-trideoxy-D-glucose (FDG-223344). Spectral overlap between the extra- and intracellular signals due to the extensive ^{19}F - ^{19}F coupling network necessitated 2D EXSY analysis. Estimates of $k_{\text{io}} = 22.2 \text{ s}^{-1}$ (α -anomer) and 7.6 s^{-1} (β) were obtained for transport of FDG-223344 at 37 °C at 17.6 mM of substrate, thus its exchange was measured to be ~ 10 times faster than for Glc (Kim et al. 1998). The exchange rate was also reduced by a factor of three upon incubation of the cells with phloretin (Kim et al. 1998). When the experiments were repeated at 25 °C, the exchange peaks disappeared from the 2D EXSY spectrum. This suggests that the hexafluorinated sugar is transported via GLUT1 and not by passive transmembrane diffusion, because of the high apparent activation energy of the exchange process.

In a more recent study, Bresciani et al. (2010) measured transmembrane exchange of 2,3,4-trifluoro-2,3,4-trideoxy-D-glucose

(FDG-234) and 2,3,4-trifluoro-2,3,4-trideoxy-D-altrose (FDA-234) using 2D EXSY ^{19}F NMR. ^1H -decoupled ^{19}F 1D pulse-and-acquire and 2D EXSY ^{19}F NMR spectra of FDG-234 are shown in Fig. 6. The transport was assumed to be via GLUT1 and both of these fluorinated sugars were shown to be transported more slowly than FDG-3, but FDG-234 had higher apparent efflux rate constants [$k_{\text{io}} = 0.97 \pm 0.21$ (α); $k_{\text{io}} = 0.22 \pm 0.07$ (β)] than FDA-234 [$k_{\text{io}} = 0.33 \pm 0.07$ (α); $k_{\text{io}} = 0.40 \pm 0.05$ (β)] at 37 °C and 10 mM substrate concentrations (Bresciani et al. 2010). It is worth noting that Kim et al. (1998) and Bresciani et al. (2010) used 5 mM ascorbate in the washing media of the cells, which might potentially compete with the fluorosugars for transport via GLUT1 (Rumsey et al. 1997).

Ammonia

Ammonia (NH_3) exchanges across the RBC membrane via the Rhesus associated glycoprotein (RhAG) (Ripoche et al. 2004). Its exchange rate was measured using ST experiments

Fig. 6 ^{19}F (470.4 MHz) NMR spectra of a fluorine-labelled analogue of glucose. **a** 1D pulse-and-acquire; and **b** 2D EXSY ($t_m = 0.5 \text{ s}$) of 2,3,4-trifluoro-2,3,4-trideoxy-D-glucose (FDG-234) in 123 mM NaCl, 15 mM Tris/HEPES, 5 mM ascorbate, recorded at 37 °C. The EXSY experiment was conducted using 10 mM FDG-234 in the presence of RBCs (H_t 0.5). All six resonances, visible in (a) were split into two components: a broader peak, shifted to higher frequency, which corresponds to the intracellular resonance; and a sharper peak, corresponding to the extracellular resonance. The cross-peaks, visible in the 2D EXSY spectrum, indicate the exchange between the intra- and extracellular pools. Reproduced with permission from Bresciani et al. (2010)

by Labotka et al. (1995). The intra- and extracellular ^{14}N and ^{15}N NMR signals were resolved by the addition of the paramagnetic shift reagent dysprosium tripolyphosphate (Gupta and Gupta 1982). The apparent influx rate of ammonia was seen to be directly proportional to its concentration ($0.39 \pm 0.012 \text{ fmol cell}^{-1} \text{ s}^{-1} \text{ mM}^{-1}$ at pH 7.0) and it was not saturable in the range of concentrations used (Labotka et al. 1995), implying a high K_m value.

Fluoroethylamine

Another compound that is rapidly exchanged via RhAG is fluoroethylamine (FE) (Szekely et al. 2006). Its transport rate was measured using overdetermined 1D EXSY, and the k_{io} was estimated to be $3.39 \pm 0.02 \text{ s}^{-1}$ for 4.1 mM concentration of the intracellular FE at 37°C (Szekely et al. 2006). The exchange rate of FE varied linearly with the concentration of substrate, and the transport was not saturated even at the highest concentration of intracellular FE used (33.5 mM). Due to an upper limit on the experimental concentration of the substrate, imposed by the requirement of isotonicity of the medium with RBCs, the Michaelis–Menten parameters could not be estimated in this case. Binding of Rhesus antigen complex antibody to RBCs resulted in $\sim 29\%$ reduction in the exchange rate of FE (Szekely et al. 2006), thus underscoring RhAG to be a major molecular route for entry of FE into RBCs.

^{13}C -labelled compounds

In ^{13}C NMR of cell suspensions, the intra- and extracellular peaks for most compounds are not sufficiently resolved, which usually precludes using conventional MT techniques with this nucleus as a probe. Nevertheless, the ^{13}C NMR spectrum of formate in an RBC suspension contains two (albeit not fully resolved) resonances. This partial overlap between the intra- and extracellular peaks implies that the spill-over effects in the ST experiment are unavoidable. Hence, only a semi-quantitative estimate of the efflux rate for this compound was made: $\sim 3.2 \text{ fmol cell}^{-1} \text{ s}^{-1}$ (Kuchel et al. 1992). This value was nevertheless useful to demonstrate that the transport is inhibited by DNDS, thus showing that the transport of formate is via capnophorin (Kuchel et al. 1992).

Since for most compounds intra- and extracellular ^{13}C NMR resonances are not resolved at all, measuring their transmembrane exchange rates requires a modification of the classical MT analysis. Thus, a new method, ‘differential saturation transfer’ (DST), was developed by Kuchel et al. (1987a); this approach is based on employing another chemical reaction that has differential rates inside and outside the cell. Namely, quantification of the transport rate of $\text{H}^{13}\text{CO}_3^-$ across the RBC membrane relied on the more easily detectable exchange

between $\text{H}^{13}\text{CO}_3^-$ and $^{13}\text{CO}_2$, which is catalyzed inside RBCs by carbonic anhydrase (Kuchel et al. 1987a). Nevertheless, the analysis of such an experiment is more demanding, because of the requirement for precise characterization of all involved kinetic processes, and the reader is referred to the original publication for details (Kuchel et al. 1987a).

Fig. 7 ^2H (61.422 MHz) 2D EXSY spectra of HDO, recorded at 15°C in the presence of RBCs in a gelatin gel, stretched to twice its original length. **a** Oblique view of a spectrum acquired using $t_m = 1 \text{ ms}$. The two most prominent resonances were assigned to the extracellular HDO while the central resonance was assigned to HDO inside the RBCs. The two remaining peaks are the cross-peaks between the two extracellular spin-populations. **b** Oblique view of a spectrum acquired using $t_m = 75 \text{ ms}$. The spectrum was scaled to give the largest diagonal signal with the same amplitude as in (a); the additional four cross-peaks indicate the transmembrane exchange of HDO. The insets show the corresponding contour plots. Adapted with permission from Kuchel and Naumann (2008)

Another process, suitable for the DST analysis, is the equilibrium-exchange transport of [$1\text{-}^{13}\text{C}$]D-glucose, for which the ^{13}C NMR signals of α - and β -anomers are well resolved (Kuchel et al. 1987b). By adding external mutarotase, the rate of the anomerization process was accelerated in the extracellular compartment, but not inside the cells. For a total glucose concentration of 25.5 mM, the efflux rate constants for the α - and β -anomers at 40 °C were measured to be $1.20 \pm 0.40 \text{ s}^{-1}$ and $0.71 \pm 0.30 \text{ s}^{-1}$, respectively (Kuchel et al. 1987b).

HDO

Another special case of the MT analysis was conducted to measure transmembrane exchange of HDO via aquaporin and passive diffusion in RBCs, which are incorporated into a stretched gelatin gel. The intra- and extracellular ^2H NMR signals of HDO became resolved due to the residual quadrupolar splitting of the ^2H resonance in a partially-aligned gel environment (Kuchel and Naumann 2008). In this specific case, the ST experiment must be used with caution due to the overlap of the intracellular signal with the double-quantum transition of the extracellular resonance (Eliav et al. 2009). Therefore, with such a sample, 2D EXSY spectra (Fig. 7), analyzed using the back-transformation method, delivered more statistically robust results than the ST approach, and the estimate of the efflux rate constant at 15 °C was $31.5 \pm 2.2 \text{ s}^{-1}$, while at 25 °C it was $\sim 50 \text{ s}^{-1}$ (Kuchel and Naumann 2008).

Conclusions

NMR MT experiments allow a convenient alternative to the classical radioactive-tracer exchange experiments for the characterization of rapid sub-second transmembrane-exchange kinetics. In the present review, we have summarized the procedures that are involved in the acquisition and analysis of such experiments using human erythrocytes as an example. Such experiments, however, are applicable to other type of cells and even to in vivo studies, as long as separate NMR signals from the exchanging populations of the solute under investigation are seen either due to the intrinsic properties of an NMR-active nucleus, or with addition of external shift reagents.

Future directions

Perhaps, the largest value of MT experiments would be for in vivo studies. In fact, the so-called CEST (chemical exchange saturation transfer) method is conceptually similar to the experiments described here and is now widely used in magnetic resonance imaging. Equilibrium-exchange enzyme

kinetics can be fruitfully complemented by another type of analysis, such as *zero-cis*, *zero-trans*, *infinite-cis*, *infinite-trans* experiments (Stein 1986). These types of transport kinetics are more difficult to quantify for fast reactions, because of the inherent time constraints on obtaining NMR spectra with high signal-to-noise ratios. A modern approach, which allows significant enhancement of the NMR signals, called dissolution dynamic nuclear polarization (Ardenkjær-Larsen et al. 2003), can potentially obviate the low-sensitivity problem and allow monitoring fast non-equilibrium reactions with NMR (Pagès and Kuchel 2013).

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Compliance with ethical standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human erythrocytes were in accordance with the ethical standards of the University of Sydney Human Ethics Committee. Informed consent was obtained from all individual participants included in the study.

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