

Spatially Resolved STD-NMR Applied to the Study of Solute Transport in Biphasic Systems. Application to Protein-Ligand Interactions

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Fluid biphasic systems are one of the most interesting dynamic systems in chemistry and biochemistry. In NMR spectroscopy, the study of the solute dynamics across fluid biphasic systems requires the introduction of dedicated NMR methods, due to their intrinsic heterogeneity. Diffusion and spatially resolved NMR techniques represent a useful approach for dealing with the study of solutes in biphasic systems and have been applied lately with success. Nevertheless, other potential applications of NMR spectroscopy for biphasic systems remain to be explored. In this proof of concept communication, we specifically aimed to investigate whether solute exchange between two immiscible phases can be followed by NMR experiments involving transfer of magnetization. To that aim, we have used Spatially Resolved Saturation Transfer Difference NMR (SR-STD NMR) experiments to analyze solute exchange by transfer of saturation from one phase to the other in a biphasic system and have explored which are the underlying mechanisms leading to the transfer of magnetization between phases and the limits of the approach. We hereby demonstrate that SR-STD NMR is feasible and that it might be implemented in pharmacological screening for binders of biological receptors or in the study of chemical and biochemical reactions occurring at interfaces.

Keywords: SR-STD NMR, Biphasic system, CEST, ¹H-NMR, Interface.

The study of reactions at interfaces along with solute diffusion or transport through fluid biphasic systems is of major interest both in chemistry and biochemistry. In chemistry, interface reactions have opened the way to novel methodologies of synthesis of important products impossible to obtain via monophasic approaches [1]. In biochemistry, interface reactions are at the heart of very important mechanisms for life itself, spanning from enzymatic reactions (*e.g.* lipases), shuttle systems, and transport to diffusion of solutes across the membranes.

Some latest NMR applications have successfully explored phenomena related to Brownian molecular diffusion or to distribution of solutes between two phases. In fact, DOSY [2] and spatially resolved NMR techniques [3] are all well suited for the study of solutes diffusing through fluid interfaces. Lately, a hybrid methodology, known as Chemical Exchange Saturation Transfer (CEST), has been applied with success in the field of Magnetic Resonance Imaging (MRI) to enhance the contrast between phases [4,5]. In the CEST approach, either exchangeable protons or molecules are selectively saturated. The transfer of this saturation is detected indirectly by monitoring the bulk water signal, which decreases as a consequence of the exchange with saturated protons. The difference between the saturated and not saturated spectra plotted as a function of the saturation frequency is called the CEST spectrum [4]. For the CEST experiment to be informative, the system under investigation should involve nuclei under slow to intermediate chemical exchange and the molecular species in analysis should have separated resonances. Typically, in a slow to intermediate exchange regime the following is true:

$$k_{ex} \leq \Delta\omega \text{ [eq.1]}$$

where k_{ex} is the chemical exchange rate constant, and $\Delta\omega$ is the frequency difference between the exchanging nuclei (in $\text{rad}\cdot\text{s}^{-1}$) [6]. Only if such condition is satisfied the saturation will be transferred by chemical exchange. We have recently demonstrated that a CEST-like NMR approach (SSTD NMR) can be successfully applied to characterise internal rotations in small molecules [7].

Translating the principles underlying the CEST experiment to the transfer of magnetization between two immiscible phases, we firstly probed the feasibility of the approach on a simple biphasic system. Hence, we carried out the study of the immiscible fluid biphasic system constituted by CDCl_3 as organic phase and 10% D_2O in H_2O as aqueous phase ($\text{CDCl}_3/\text{H}_2\text{O}:10\% \text{D}_2\text{O}$). The system was prepared in an NMR tube as described in the Experimental section and shown in Fig. 2a. From the ¹H NMR spectrum of the biphasic system (Fig. 1), we can easily identify the H_2O peak at 4.7 ppm in the water phase and the $\text{HDO}/\text{H}_2\text{O}$ peak at 1.53 ppm in the CDCl_3 phase. The exchange between HDO and H_2O in the water phase and the following transfer through the interface (transport) to the CDCl_3 phase can be described by the model in Fig. 2b.

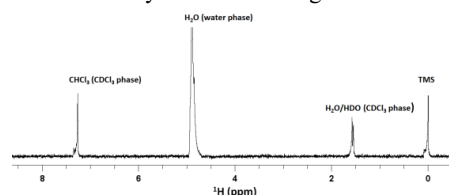


Figure 1: ¹H NMR spectra of the biphasic system $\text{CDCl}_3/\text{H}_2\text{O}:10\% \text{D}_2\text{O}$. The H_2O peak in the aqueous phase at 4.7 ppm and the $\text{H}_2\text{O}/\text{HDO}$ peak in CDCl_3 at 1.53 ppm are easily identifiable. The shape of the peaks is not due to poor homogeneity correction on the spectrometer (shimming) but to the intrinsic inhomogeneity in magnetic susceptibility of the biphasic system.

In this model, the hydrogen isotope exchange (H-D) between H_2O and HDO species occurs in the H_2O phase and is to be considered in fast exchange regime (only one peak is observed in $\text{H}_2\text{O}:\text{D}_2\text{O}$ solutions) [8]. In contrast, the interface transfer (transport) of the $\text{HDO}/\text{H}_2\text{O}$ to the CDCl_3 phase is slower and can be considered the rate limiting step of the entire system (Fig. 2b). In this case, the CEST requirement ($k_{ex} \leq \Delta\omega$) is fulfilled by the interface transfer rate of $\text{HDO}/\text{H}_2\text{O}$ to the CDCl_3 phase. We have to emphasize that in this system the chemical species in exchange with each other ($\text{HDO}/\text{H}_2\text{O}$ in water phase and $\text{HDO}/\text{H}_2\text{O}$ in CDCl_3 phase) are not only in two different chemical environments but, are found in two different immiscible and physically separated phases (Fig. 2 a,b).

In order to experimentally demonstrate the transfer of magnetization from one phase to the other following the CEST approach, we decided to resort to Saturation Transfer Difference (STD) NMR experiments, which, to the best of our knowledge have not been previously applied to this type of system [9].

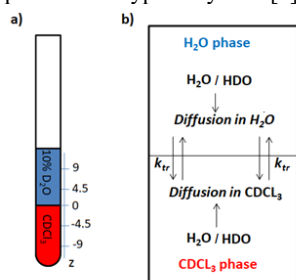


Figure 2: a) Scheme of the biphasic $\text{CDCl}_3/\text{H}_2\text{O}$ system in a 5 mm NMR sample tube. The origin of z-coordinates is considered at the center of the 18 mm active coil window. b) Proposed mechanism for the transfer of saturation from the H_2O species in water phase to the $\text{HDO}/\text{H}_2\text{O}$ species found in CDCl_3 phase. The water species in the aqueous (top) phase are directly saturated by the saturating on-resonance train of selective pulses, and saturation is transferred by diffusion and transport to the organic phase, where it accumulates and is detected.

As biphasic systems are intrinsically inhomogeneous, a new Spatially Resolved STD NMR (SR-STD NMR) pulse sequence was specifically written (Fig. 3).

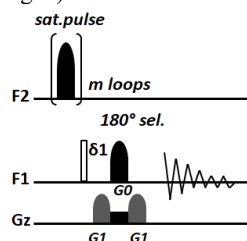


Figure 3: Spatially resolved Saturation Transfer Difference NMR (SR-STD NMR) pulse sequence. The saturation pulse was a *Gauss1.1000* shaped pulse while the 180° selective pulse was a *Reburp.1000*. $G1$ gradient shape was *SMSQ10.100* while the rectangular gradient $G0$ is applied for the whole duration of the selective 180° pulse; for this reason, care should be taken. All the pulses and gradients have to be calculated accordingly as usual for the NMR spectrometer in use.

The pulse sequence resembles the original STD NMR experiment, in which a 180° selective pulse flanked by two shaped gradients and one rectangular gradient (simultaneous to the 180° pulse) are added. With this modification, the intrinsic inhomogeneities of the biphasic systems are avoided and it is possible to read the output of the experiment in the desired phase. From the 180° selective pulse bandwidth ($\Delta\omega_p$) and its carrier frequency (Ω) it is possible to calculate the thickness and position of the reading slice in the desired phase using the following equations [10, 11]:

$$z = \Omega / (\gamma G_0) \quad [\text{eq.2}]$$

$$\Delta z = \Delta\omega_p / (\gamma G_0) \quad [\text{eq.3}]$$

The experiments were acquired by positioning the on-resonance saturation pulse at 4.7 ppm (H_2O resonance in the aqueous phase) and the off-resonance pulse at 40 ppm. The saturation time was increased from 1.5 to 18 s. Both, reference and saturated spectra were acquired reading in a single slice at -5.5 mm from the origin by positioning the 180° selective pulse carrier frequency in the CDCl_3 phase. As a convention, the origin of the z axis is considered the center of the length (L) of the active volume coil ($L=18$ mm, Fig. 2a).

The feasibility of the proposed approach was demonstrated by the observation of saturation transfer from the H_2O phase to the CDCl_3 phase, detected as a decrease of the $\text{HDO}/\text{H}_2\text{O}$ peak intensity at

1.53 ppm. Furthermore, a build-up of saturation as a function of the saturation time was obtained, indicating efficient accumulation of saturated water molecules in the organic phase. Fig. 4a shows the intensity of the saturation transferred to the CDCl_3 phase as a function of the saturation time. The saturation transfer to the chloroform phase increases as the saturation time increases, reaching a plateau around 12% after 6 s.

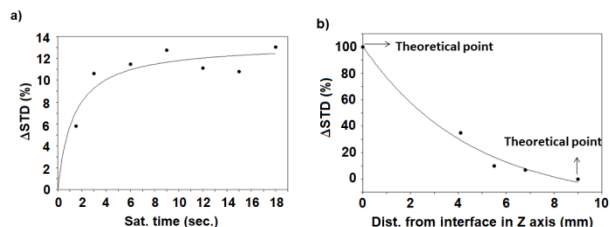


Figure 4: a) Saturation transfer from the aqueous to the organic phase detected by monitoring the $\text{HDO}/\text{H}_2\text{O}$ peak intensity (calculated as $I_0 - I_{\text{sat}}/I_0$) in the CDCl_3 phase. b) SR-STD NMR experiments reading at different distances from the interface, with a constant saturation time of 9 s. The transferred saturation decreases moving away from the interface in agreement with the general mechanism depicted in Figure 2b. The theoretical lower point at -9 mm and its symmetric counterpart at +9 mm are impractical points for the measurement purposes due to r.f. inhomogeneity.

We further investigated the system performing the SR-STD NMR experiments with a fixed saturation time (9 s) while moving the position of the carrier frequency of the 180° selective pulse on the CDCl_3 phase, to read at different distances (-4.1, -5.5, and -6.8 mm) from the interface (Fig. 4b). In agreement with our model (Fig. 2b), the level of transferred saturation increased in the organic phase from the bottom towards the interface. Indeed, the saturation at the interface can be considered as 100% due to the fact that the HDO and H_2O resonance frequencies are indistinguishable in this region. Moving towards the bottom of the active coil the saturation decreases. This decrease of the transferred saturation is dependent on the mechanisms of interface transfer (k_{tr}), Brownian diffusion (D) and self-relaxation (R_1) of the HDO protons. Taken together, these results show unequivocally that observation of interface transport via transfer of saturation between the two phases of this model of an aqueous/organic biphasic system is possible.

Although the above study was on a simple model, it is a solid proof of principle supporting that our approach could be applied and transferred to other systems. To this aim, we explored the use of a similar experimental setup to study the case of a ligand-receptor system. STD NMR experiments have been successfully used in the past to study protein-ligand systems, providing structural information on biomolecular complexes [12-17]. In that sense, our novel experimental setup could be of interest when a protein-ligand interaction cannot be studied due to either a strong inhomogeneity of the sample, or to a low aqueous solubility of the ligand. In this latter case, the ligand would accumulate efficiently in an organic phase, while the protein would be present in the aqueous one.

The ligand-receptor system selected for this experiment was Naproxen and Bovine Serum Albumin (BSA) (Fig. 5). For protein-ligand systems we need to consider that ligand molecules are bigger than the water molecules, hence diffusing through the phases with a slower interface transport, slower Brownian diffusion in both phases, and more efficient self-relaxation. To avoid these pitfalls, we resorted to: 1) increase the interface surface by incorporating a 2% Agarose gel in the water phase and 2) move the position of the reading slice nearer to the interface between the agarose/aqueous phase and the other one (from 1.1 to 2.5 mm from the interface). In this case, the phase to which the saturation is transferred to is positioned on the upper half of the active coil volume, while the 2% Agarose gel containing the BSA protein is positioned for practical

convenience in the bottom half of the active coil volume (Fig. 5). Two different setups, involving two different types of upper phase in contact with the 2% Agarose gel phase, were considered: first, an aqueous D₂O phase (Fig. 5a) and, second, an organic CDCl₃ phase (Fig. 5b). This choice was made to evaluate the impact of the interface transport on the transfer of saturation.

The SR-STD NMR experiments were carried out following the routine protocol for STD NMR used for ligand-receptor interactions [18]. In the first case, the upper phase (D₂O) containing Naproxen (20 mM) was added to the top of the 2% Agarose gel in D₂O containing BSA (60 μM) in a 5 mm diameter NMR sample tube. Then, Naproxen was allowed to diffuse in the Agarose gel phase. The on-resonance saturation pulse was positioned at 0.7 ppm (aliphatic residues of BSA), the off-resonance saturation pulse was at 40 ppm, and the reading 180° selective pulse was positioned at 1.1 mm from the origin (on the upper part of the active coil).

The feasibility of the proposed approach for protein-ligand interactions was demonstrated by the observation of interface magnetization transfer by the detection of a decrease of the Naproxen aromatic peaks in the region from 7.0 to 8.0 ppm. In this case, the interface transport step is considered absent between the Agarose:D₂O phase (bottom) and the D₂O phase (top) (Fig. 5a) as D₂O is the solvent in both phases. Thus, the transfer of saturation from the Agarose:D₂O phase to the D₂O phase depends on the dissociation constant (K_D) of the BSA/Naproxen complex, the diffusion constant (D) of Naproxen and its protons self-relaxation rate (R_1). Accordingly, in the model of the saturation transfer between the two phases in Fig. 5a the interface transfer step is absent due to free diffusion of Naproxen in both phases.

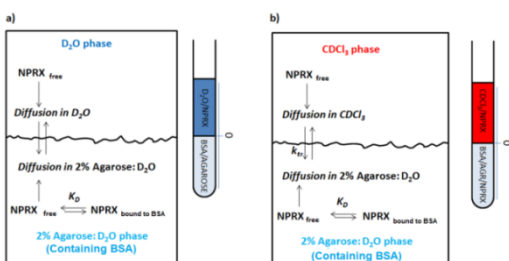


Figure 5: Schematic of the proposed mechanism for the saturation transfer between phases in the Naproxen:BSA:D₂O:Agarose system. In **a)** the upper phase is D₂O while in **b)** the upper phase is CDCl₃. (For the sake of clarity on the right of each proposed mechanism are shown cartoon representations of the NMR tube with the biphasic systems)

The outcome of the experiment is shown in Fig. 6a. As in the case of the biphasic CDCl₃/H₂O:10%D₂O system, for the Naproxen:BSA:D₂O:Agarose system the results indicated an efficient ligand accumulation in the second phase, after saturation transfer from its interaction with the receptor protein. Fig. 6a shows that the transfer of saturation increases with the increase of saturation time, reaching the plateau after about 4 s. In this case, the level of saturation observed is higher (50 %) than in the model system studied before, as the reading slice was positioned closer (at 1.1 mm and not at -5.5 mm) from the interface.

The last series of experiments on the Naproxen:BSA system was then performed using an organic solvent (CDCl₃) as the upper phase. The model for the transfer of saturation on this system is shown in Fig. 5b. In contrast to the previous system with D₂O as the top phase, in this system an interface transfer step has to be considered. This is usually the rate limiting step of the system and might impact negatively the whole saturation transfer efficiency, preventing the detection of the Naproxen-BSA interaction via saturation observation on the organic phase.

The output of the experiment is shown in Fig. 7a. Again, but now in an aqueous:organic biphasic system, the feasibility of this approach

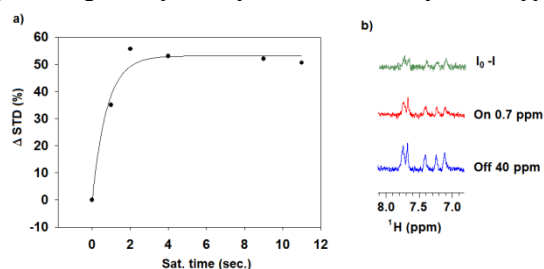


Figure 6: **a)** Saturation transfer detected monitoring the aromatic peaks of Naproxen for the 2% Agarose:D₂O:BSA lower phase and D₂O as upper phase. **b)** Example of the spectra obtained using the SR-STD NMR pulse sequence confirming the transfer of saturation to the Naproxen found in the upper phase (see general mechanism in Figure 5a). The measurement was performed at 1.1 mm from the interface.

for protein-ligand interactions was demonstrated by the observation of interface saturation transfer via the detection of saturated signals of Naproxen in the organic phase. As in the previous cases, the ligand saturation was also efficiently accumulated on the second phase (CDCl₃), as it increased with saturation time, reaching the plateau after 4 s (Fig. 7a). In this case, the level of saturation transfer was lower, as expected, due to the interface transport step constituting a bottleneck of the transfer process, compared to the experiment with D₂O as the upper phase. Still, surprisingly, this reduction amounted only to 10%. The overall small reduction is due to the increase of the interface surface area using the gel that might compensate for a lower velocity of interface transfer of Naproxen. In fact, data acquired without the presence of the agarose gel in the lower aqueous phase showed no transfer of saturation between the phases (data not shown).

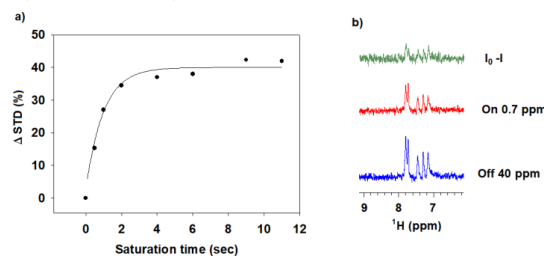


Figure 7: **a)** Saturation transfer detected monitoring the aromatic peaks of Naproxen for the 2% Agarose:D₂O:BSA lower phase and CDCl₃ as upper phase. **b)** Example of the spectra obtained using the SR-STD NMR pulse sequence confirming the transfer of saturation to the Naproxen found in the upper phase (see general mechanism in Figure 5b). The measurement was performed at 1.1 mm from the interface.

Finally, to confirm that the saturation transfer from one phase to the other has its origin in the transfer of saturation from BSA to Naproxen, control experiments without BSA were analyzed under identical conditions (Fig. S1 Supplementary Data). None of them showed saturation transfer between the phases, confirming that the saturation generated on the protein is transferred to Naproxen during the binding process, and it is further transported to the top phase of the sample.

In conclusion, using model systems, we have shown promising and conclusive results supporting the applicability of the Spatially Resolved STD NMR (SR-STD NMR) approach to the analysis of solute transport on fluid biphasic systems. We foresee that this novel approach can become a powerful tool for the *in vitro* studies of complex chemical or biochemical systems that involve transport between hydrophilic and lipophilic phases. Several applications may be developed using a similar setup either for screening or mechanistic studies. Most importantly, protein-ligand interactions in which the small binder shows moderate solubility in water may also be studied through the detection of the saturation in an immiscible

organic phase, where the solubility is higher and the signal accumulates more efficiently.

Experimental

Naproxen, D₂O, CDCl₃ and Bovine Serum Albumin (BSA) were purchased from Sigma-Aldrich.

CDCl₃/ H₂O:10% D₂O biphasic system: The biphasic system was obtained by initially adding the CDCl₃ phase directly in the 5 mm NMR tube reaching exactly the center of the active coil volume. Shortly after that the 10% D₂O water solution was added slowly on the top. The sample was then transferred to a 500 MHz Avance I NMR Bruker machine for the SR-STD measurements.

2% Agarose:D₂O:BSA/ D₂O:Naproxen biphasic system: The 2% Agarose gel in D₂O was obtained by heating the sample up to 80° C in order to obtain a clear solution. After this the solution was cooled to 38° C and maintained in a water bath for the time needed to add the BSA (Bovine Serum Albumin) and mixed gently. Quickly after that, the “sol” phase was transferred in the 5 mm NMR tube covering exactly the half of the active coil volume (see scheme in Fig. 5a insert) and was let to make its transition into the “gel” phase. At the top of the gel a solution of 20 mM of Naproxen was then added which was allowed to slowly diffuse into the gel phase.

2% Agarose:D₂O:BSA/ CDCl₃:Naproxen biphasic system: For this system the D₂O phase of the previously described 2% Agarose:D₂O:BSA/D₂O:Naproxen system was discarded and fresh Naproxen (7.0 mM) in CDCl₃ was added on the top of the gel phase.

NMR parameters and SR-STD pulse sequence: The SR-STD NMR experiment derives from the original STD experiment and maintains the same experimental setup. For the CDCl₃/H₂O:10% D₂O system the on-resonance saturation pulse was positioned on the

water peak resonance at 4.7 ppm while the off-resonance saturation pulse was positioned at 40 ppm. The spectra were obtained by positioning the selective 180° pulse at the CDCl₃ phase (at -5.5 mm from the interface) calculating the distance using equation 2 while the saturation time was increased from 1.5 to 18 s with a D1 of 18 s.

For the 2% Agarose:D₂O:BSA/D₂O:Naproxen and 2% Agarose:D₂O:BSA/CDCl₃:Naproxen the same experimental setup as before was followed. The on-resonance saturation pulse was positioned on at 0.7 ppm (aliphatic residue side chains of BSA) while the off-resonance saturation pulse was positioned at 40 ppm. The saturation time was increased from 0.5 to 12 s with a D1 of 12 s. Further, in this case, the selective 180° pulse was positioned on the upper part of the active coil at 1.1 mm and at 2.5 mm from the interface. 64 scans were acquired for all the SR-STD NMR experiments while the temperature of the samples was maintained at 298 K.

Supplementary data: Control SR-STD NMR spectra to demonstrate the mechanism of interface saturation transfer are included in the electronic document “Supplementary Data”.

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