Uniform water-mediated saturation transfer: a sensitivity-improved alternative to WaterLOGSY

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Abstract. In the study of small molecule ligands and candidate macromolecular targets, water spins in long-lived association with macromolecules (proteins or nanoparticles) constitute a remarkable source of magnetization that can be exploited to reveal ligand-target binding. In this work we show how the selective saturation of water spins complemented with adiabatic off-resonance spin-locks can remove the NOE contribution of bulk water in the final difference spectrum, leading to uniformly enhanced signals that reveal weak ligand-target interactions.

1. Introduction.

The WaterLOGSY experiment introduced by Dalvit [1] has rapidly established itself as one of the standard methods to probe interactions between small molecules (*i.e.* candidate drugs) and macromolecules (typically proteins). Unlike the Saturation Transfer Difference (STD) experiment [2], which uses the macromolecule spins as the source of magnetization, WaterLOGSY aims at transferring magnetization between water molecules and small molecules in solution via the Nuclear Overhauser Effect (NOE). The rationale behind WaterLOGSY is that there actually exist two populations of water molecules in solution, namely water molecules in the bulk and water molecules in long-lived association with the macromolecular receptor. Because of the different dynamics in the relevant dipolar interactions for these two populations, WaterLOGSY experiments eventually generate NOE enhancements of different polarization depending on whether the small molecules tend to associate with the macromolecules or not.

While Dalvit indicated two possible alternatives between transient NOE (driven by inversion of the water resonance) and steady-state NOE (driven by saturation of the water resonance), the former is by far the most used version of the two experiments. Indeed, when a train of selective (generally 180° Gaussian) pulses is used to saturate the water spins, the two approaches produce very similar results: the transient approach however has a simpler implementation (NOE-ePHOGSY [3]) compared to the steady-state counterpart, which suffers from the disadvantage that a reference spectrum must be recorded separately.

In the attempt to increase the sensitivity of "NMR chemosensing" experiments [4], our group has recently shown that saturating, rather than inverting, the populations of water spins leads to significant sensitivity enhancements when specific experimental conditions are met [5]. Notably, if the power of the selective

pulses is raised, the saturation band becomes much broader and selectivity on water spins is lost. In this situation we demonstrated that, when the saturation power is carefully calibrated, it is possible to drive the longitudinal magnetization of unbound analytes back towards the same value as in the reference spectrum, making their resonances disappear from the difference spectrum. This approach has been originally referred to as WaterSTD.

The use of high-power (HP) RF pulses in WaterSTD promotes two effects: first, the signals of non-interacting species are greatly reduced in the final spectrum and second, the signals of interacting species become more intense. Both these effects are mostly related to the cancellation of the positive NOE experienced by all the fractions of free species in solution. Note that, under fast chemical exchange, in WaterSTD and WaterLOGSY experiments the free and bound populations of interacting analytes contribute to the same signals with opposite signs, because the NOE they experience originates from two different dynamic regimes.

Along with the aforementioned advantages in sensitivity gain, rising the power of the saturating pulses brings however a major disadvantage: namely, spins that resonate close to the offset are much more affected by the saturating RF field than those resonating far from the offset, a situation which may ultimately lead to false positives (*i.e.* seemingly interacting species) in the final difference spectrum.

In this work we propose a new strategy that retains the WaterSTD advantages in terms of sensitivity while minimizing the occurrence of false positives. This is achieved through to a combination of soft and adiabatic spin-lock pulses that respectively saturate the water resonance in a selective way and slightly perturb a large portion of the NMR spectrum in a homogeneous way.

2. Experimental

The advantages of the proposed method are demonstrated on a mixture containing the main components of the commercial drug “Deltacortene” (Italy) or “Deltasone” (USA), whose tablets formulation typically contain 5 to 25 mg prednisone (**1**) as the active principle and lactose (**2**), stearic acid, corn starch and microcrystalline cellulose as excipients. Prednisone is known to interact with human serum albumin (HSA) [6] while lactose does not, making the two molecules good candidates for WaterLOGSY and WaterSTD experiments. In addition, prednisone has a low solubility in water (about 0.2 mM at 25°C [7]), which hampers its detection in aqueous media. Accordingly, a phosphate buffered solution (pH 7.0) containing saturated prednisone (about 5 times the solubility in water) and 1.26 mM lactose in H_2O/D_2O = 90/10 was prepared and filtered on 0.22 μm Millex-GV filters to remove fine precipitate. The resulting solution was subsequently complemented with HSA (20 μM), and a portion of the same solution without added HSA was saved for reference purposes. A sample with only prednisone in D_2O was also prepared with the same procedure.

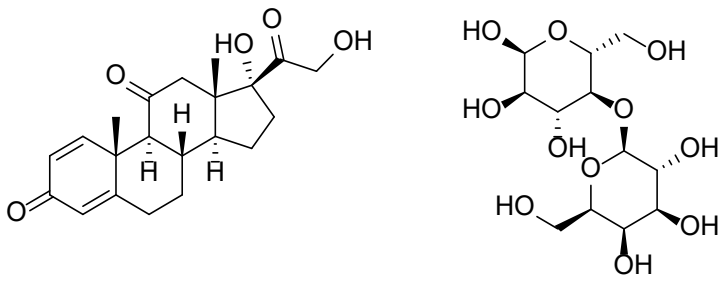

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Chart 1. Sketch of prednisone (**1**) and lactose (**2**) used in this work.

All the NMR spectra were recorded at 25 °C on a Bruker AVANCE III spectrometer operating at 500.13 MHz ^{1}H Larmor frequency and equipped with a 5 mm *z*-gradient broad-band inverse noncryogenic probe.

The pulse sequence used for classic WaterLOGSY experiments was the NOE-ePHOGSY available in the Topspin 3.6.2 library, yet with the solvent suppression scheme modified into a double pulsed field gradient (DPFG) perfect-echo (PE, Figure 1a) [8,9]. The relaxation delay was set to 3 s, and the mixing time (2 s) was tested among different values to provide an optimum signal enhancement.

The pulse sequence used for WAter Saturation Transfer spectroscopY (WASTY hereafter) was designed to acquire multiple spectra interleaved between on- and off-resonance saturation of water, achieved with a train of Gaussian-shaped pulses of 50 ms duration each. Solvent suppression was incorporated before acquisition in the form of a DPFG-PE with W5 clusters as the refocusing element (Figure 1b). Note that, when the power of the Gaussian pulses is sufficiently low (*i.e.* calibrated to deliver a 180° rotation), only the resonances close to the RF carrier are affected and a selective saturation profile is achieved (see par. 3.1). We will refer to this setup as "low-power (LP-) WASTY". As opposite, when the power of the Gaussian pulses is calibrated to deliver rotation angles much larger than 180°, a non-selective saturation profile results due to the increase of the effective RF B_1 field at larger offsets. Accordingly, we will refer to this setup as "high-power (HP-) WASTY". In both LP- and HP-WASTY experiments the relaxation delay was set to 3 s and the total saturation time was set to 2 s.

The novel approach proposed in this work relies on a modified WASTY experiment that will be referred to as "uniform (uni-) WASTY". The corresponding pulse sequence (Figure 1c) was designed with the same approach of LP- and HP-WASTY, replacing the Gaussian pulses with pairs of combined 50 ms Gaussian-AM-WURST-20 (amplitude modulated WURST-20 [10,11]) pulses whose aim is to provide both a sharp saturation on-resonance with water and alternating adiabatic off-resonance spin-lock fields [12] that uniformly affect all the ^{1}H resonances in a typical 5 kHz range (10 ppm at 500 MHz). In particular, two pairs of Gaussian-AM-WURST-20 pulses were synthesized using the WaveMaker software (Bruker). The first pair consists of two consecutive pulses (SAT1ON and SAT2ON) constructed as follows: SAT1ON was obtained by combining a 50

ms Gaussian pulse (truncated at 1% and set on resonance with the water signal) with a 50 ms adiabatic off-resonance spin-lock with WURST-20 amplitude profile (placed at +9.2 ppm from the water signal). SAT2ON was constructed in the same way as SAT1ON, but with the AM-WURST-20 spin-lock placed at -9.2 ppm from the water signal. The second pair (SAT1OFF- SAT2OFF) was constructed in the same way, but with all the pulses (both Gaussian and AM-WURST-20) shifted to -80 ppm to generate a reference spectrum where no resonances are saturated. Again, the relaxation delay was set to 3 s and the total irradiation time for the SAT1-SAT2 train was set to 2 s.

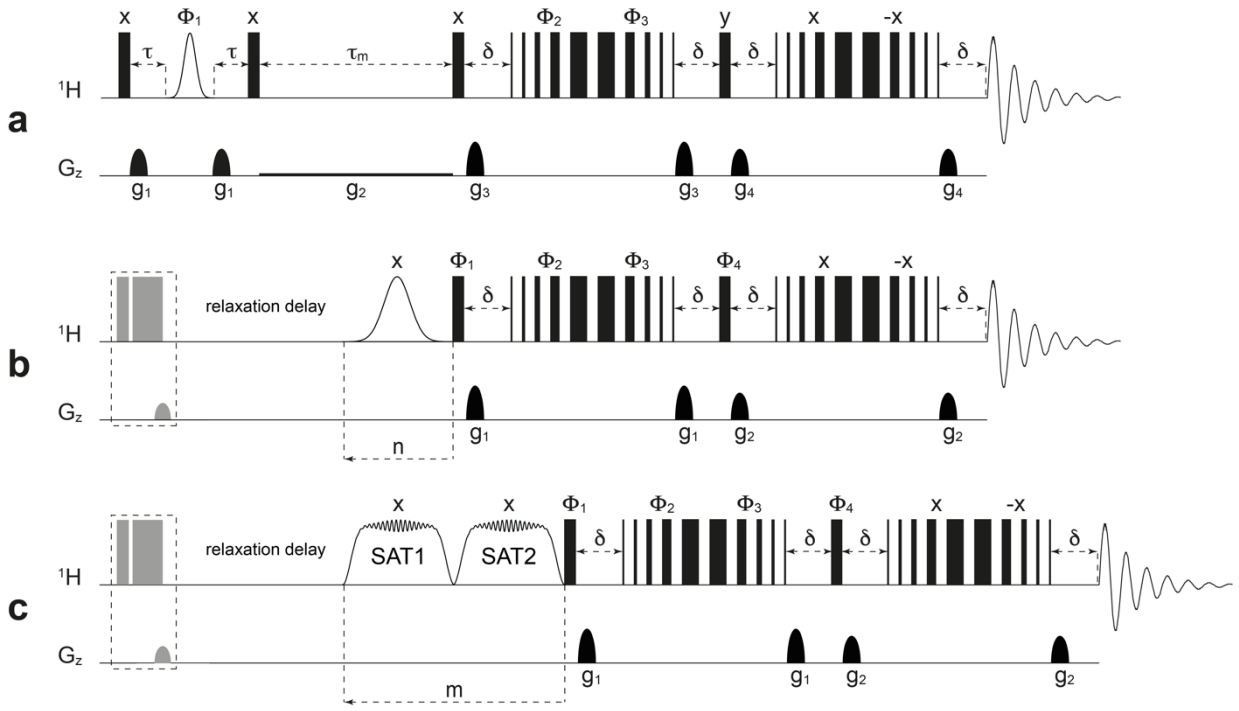

Figure 1. Pulse sequences used in this work. a) WaterLOGSY with W5 DPFG-PE. Φ_1 = x, y, -x, -y; Φ_2 = x, x, y, y, -x, -x, -y, -y; $\Phi_3 = \Phi_2 + 180°$. b) low-power or high-power WASTY with W5 DPFG-PE. The RF carrier of the saturating pulse train is alternated between on- and off-resonance with respect to the signal of water to generate an interleaved pseudo-2D dataset. Φ_1 = x, -x; Φ_2 = x, x, y, y, -x, -x, -y, -y; $\Phi_3 = \Phi_2 + 180°$; $\Phi_4 = \Phi_1 + 90°$. c) uni-WASTY with W5 DPFG-PE. The saturating pulses SAT1 and SAT2 are obtained by combining Gaussian and AM-WURST-20 pulses with the WaveMaker software (see text for details). Two pairs [SAT1ON-SAT2ON] and [SAT1OFF-SAT2OFF] are alternated to generate an interleaved pseudo-2D dataset. Phase cycling is identical to that of pulse sequence b. WASTY pulse sequences also include an optional spoil block (in grey) before relaxation delay. The cumulative duration of the saturation is accounted for by the loop counters *n* and *m*. If required, the time intervals δ can be extended to achieve T_2 filtration of broad signals.

3. Results and discussion.

Figure 2 compares the ^{1}H NMR spectrum of prednisone alone (trace a) and together with lactose and HSA (trace b). Not surprisingly, the signals of prednisone become broader in the presence of HSA because of its interaction with the protein. This aspect must be carefully considered when T_2 filtration is used to remove

unwanted signals of HSA, since the same filter would also lead to a significant loss of prednisone signals. Furthermore, a preliminary WaterLOGSY spectrum was acquired on the sample without added HSA to rule out possible aggregation of prednisone in the adopted conditions (see supporting information).

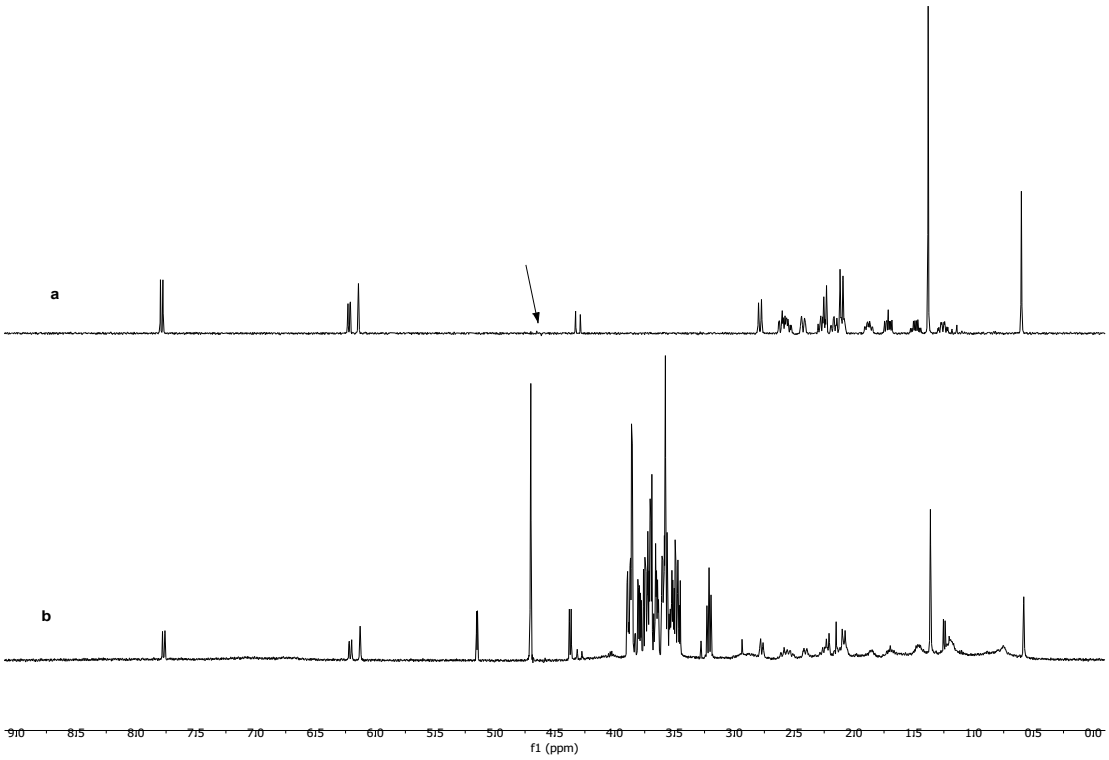

Figure 2: ^{1}H DPFG-PE spectra with W5 refocusing elements of a) prednisone in D_2O with 10% aqueous phosphate buffer (36 mM, pH 7.0); b) prednisone, lactose and 20 μM HSA in H_2O/D_2O = 90/10 with phosphate buffer (36 mM, pH 7.0). The arrow indicates a resonance from prednisone which lies at about 35 Hz from water signal and is therefore cancelled by the W5 clusters.

Figure 3 shows the ^{1}H NMR spectra obtained with standard WaterLOGSY experiments and with uni-WASTY experiments (both without and with T_2 filtration) on the sample containing prednisone, lactose and HSA. In this respect, we have chosen to keep the T_2 filtration time at no more than 15 ms (δ = 3.75 ms) not to reduce the signals of prednisone by an excessive amount.

A comparison between traces a and c (no T_2 filtration) or b and d (15 ms T_2 filtration) highlights that the signals of prednisone are much more evident in uni-WASTY experiments than in conventional WaterLOGSY experiments of equal duration. The performance of uni-WASTY is particularly relevant on the aliphatic resonances of prednisone (0.5 to 3 ppm), where the complex hyperfine structures together with the line broadening induced by the presence of HSA leads to a critical S/N ratio on several signals. Indeed, WaterLOGSY even struggles to reveal the singlets belonging to the two methyl groups.

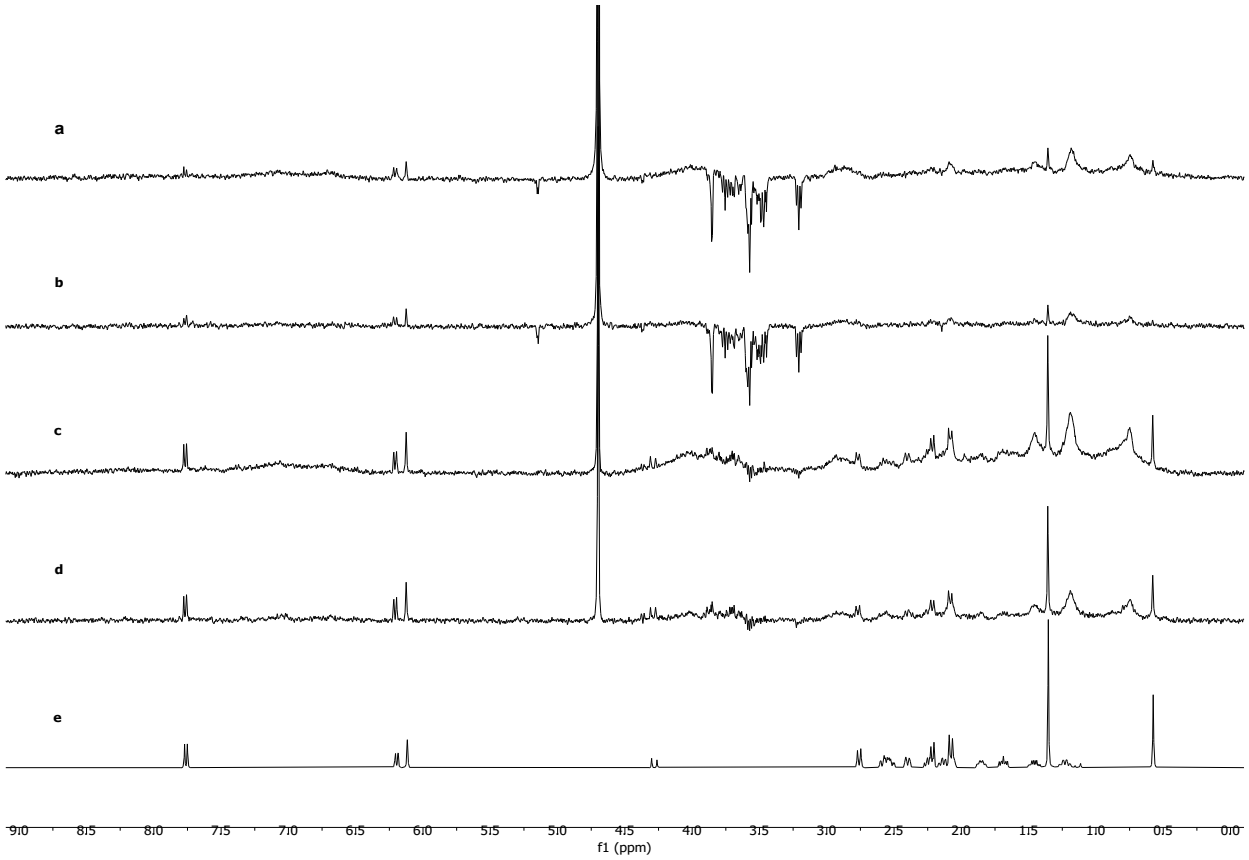

Figure 3. a) 180°-phased WaterLOGSY (non-interacting species down, interacting species up) obtained with the pulse sequence in Figure 1a with 2 s mixing time, 128 scans, δ = 0.2 ms; b) same as a), but with the time interval δ extended to 3.75 ms to achieve a total 15 ms T_2 filtration. c) ^{1}H spectrum obtained from a uni-WASTY experiment with 2 s saturation time, 64+64 scans, δ = 0.2 ms. d) same as c), but with the time interval δ extended to 3.75 ms to achieve a total 15 ms T_2 filtration. Spectra a-d were obtained from the same sample of prednisone, lactose and 20 μM HSA in H_2O/D_2O = 90/10 with phosphate buffer (36 mM, pH 7.0). The duration of each experiment is about 13 min. e) reference spectrum of prednisone in 36 mM phosphate buffer, processed with a 1.5 Hz line broadening.

3.1 Saturation profiles

To better clarify the results outlined in the previous section, we have investigated the performances of the saturation schemes of HP-WASTY, LP-WASTY, and uni-WASTY on a sample of doped water (see figure caption for details). It is clear from the traces presented in Figure 4 how rising the power of the Gaussian pulses results in a broad saturation profile, while a sharp saturation profile is observed under low-power conditions obtained with a train of calibrated 180° Gaussian pulses (blue trace). Conversely, the combination of low-power Gaussian pulse and adiabatic off-resonance AM-WURST-20 spin-locks used in uni-WASTY provides a saturation profile featuring a narrow dip on resonance and a weak homogeneous broadband saturation (black trace). Indeed, such saturation induced by the AM-WURST-20 spin-lock is tuneable depending on the instrument and the sample: in the case under exam, we have found that a $\gamma B_{1,max}$ value of 1280 Hz is just right to cancel the signals of non-interacting sucrose in the difference spectrum. Interestingly, simulations of the

saturation profiles have revealed that the effect of the adiabatic off-resonance AM-WURST-20 spin-locks is rather insensitive to the relaxation times of the saturated spins (see supporting information).

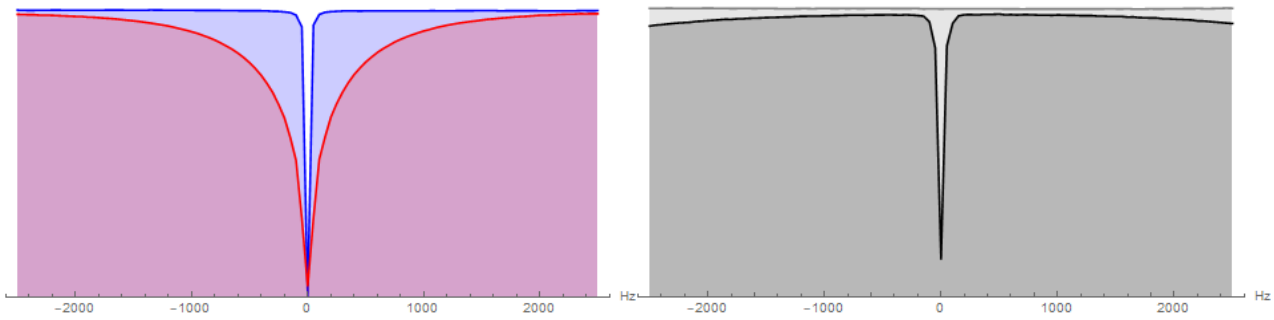

Figure 4.: Left panel: experimental saturation profiles emerging after a train of 40 Gaussian pulses (each of 50 ms duration and 1% truncation) set at low power (blue, $\gamma B_{1,max}$ = 24.3 Hz) and high power (red, $\gamma B_{1,max}$ = 750 Hz). Right panel: experimental saturation profiles (black) emerging after a train of 40 combined Gaussian-AM-WURST-20 spin-lock pulses each of 50 ms duration (see pulse sequence c in Figure 1 for details). The grey trace represents the reference magnetization emerging from the same pulse sequence, with the saturating pulses set at zero power. The profiles were obtained by varying the transmitter offset by 50 Hz within a 5 kHz band and observing the water signal in a H_2O/D_2O = 10/90 mixture doped with 0.3 mM $GdCl_3$ ($T_{1,water}$ = 0.210 s, $T_{2,water}$ = 0.194 s, T = 25 °C).

The evolution of the magnetization under a train of SAT1ON-SAT2ON pulse pairs has been also investigated by means of SpinDynamica calculations, with emphasis on the effect of the high/low-field AM-WURST pairs. To this aim, a probe spin has been set to resonate at +1000 Hz from the offset, where the effect of the Gaussian pulse is negligible. The two AM-WURST-20 spin-locks ($\gamma B_{1,max}$ = 1280 Hz) have been centred at ±4600 Hz from the offset (±9.2 ppm at 500 MHz). The results presented in Figure 5 show how these off-resonance spin-locks induce an overall slight saturation (about 5%) of the magnetization of the probe spin (orange trace).

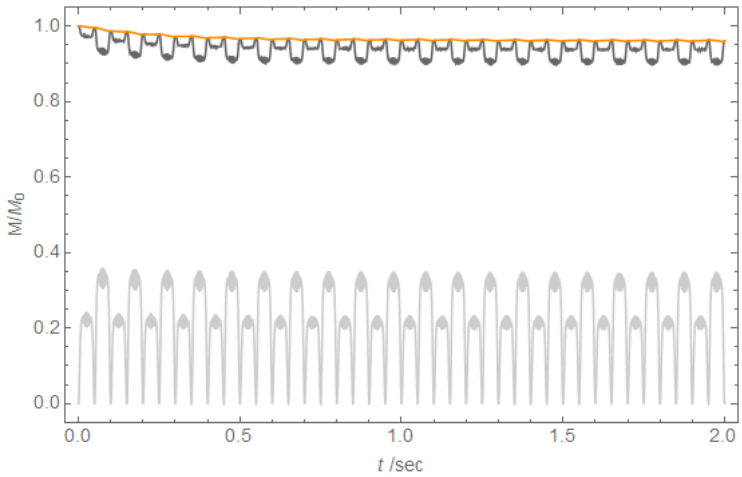

Figure 5.: Effect of a train of SAT1ON-SAT2ON pairs (see text for details) on the magnetization of a probe spin resonating at +1000 Hz from the offset. The simulation was run under SpinDynamica 3.6 with phenomenological relaxation parameters T_1 = 0.210 s, T_2 = 0.194 s for the probe spin. Dark grey: M_z, light grey: M_{xy}, orange: M_{tot}.

It is worth to point out that the two high- and low-field spin-locks are meant to ensure a uniform and symmetric saturation profile, a strategy also found in JS-ROESY and EASY ROESY experiments [13]. However, the magnetization in uni-WASTY is slightly displaced back and forth by the AM-WURST-20 spin-locks in such way that a partial saturation results, whereas adiabatic spin-locks in EASY ROESY are set to draw the magnetization to significantly larger tip-angles by low- and high-field B_1 fields.

Figure 6 finally compares the performances of HP-WASTY (a), LP-WASTY (b), and uni-WASTY (c) on the sample of prednisone, lactose and HSA. Consistent with the results outlined in Figure 4, the HP-WASTY spectrum displays false positive signals of lactose due to the broad saturation profile delivered by the train of high-power Gaussians. As opposite, the LP-WASTY spectrum shows the same features of a WaterLOGSY spectrum (180°-phased). Finally, for the sake of comparison, panel c reports the same spectrum found in Figure 3c: it is evident that uni-WASTY provides a spectrum very similar to that obtained from HP-WASTY, but with no false-positive signals.

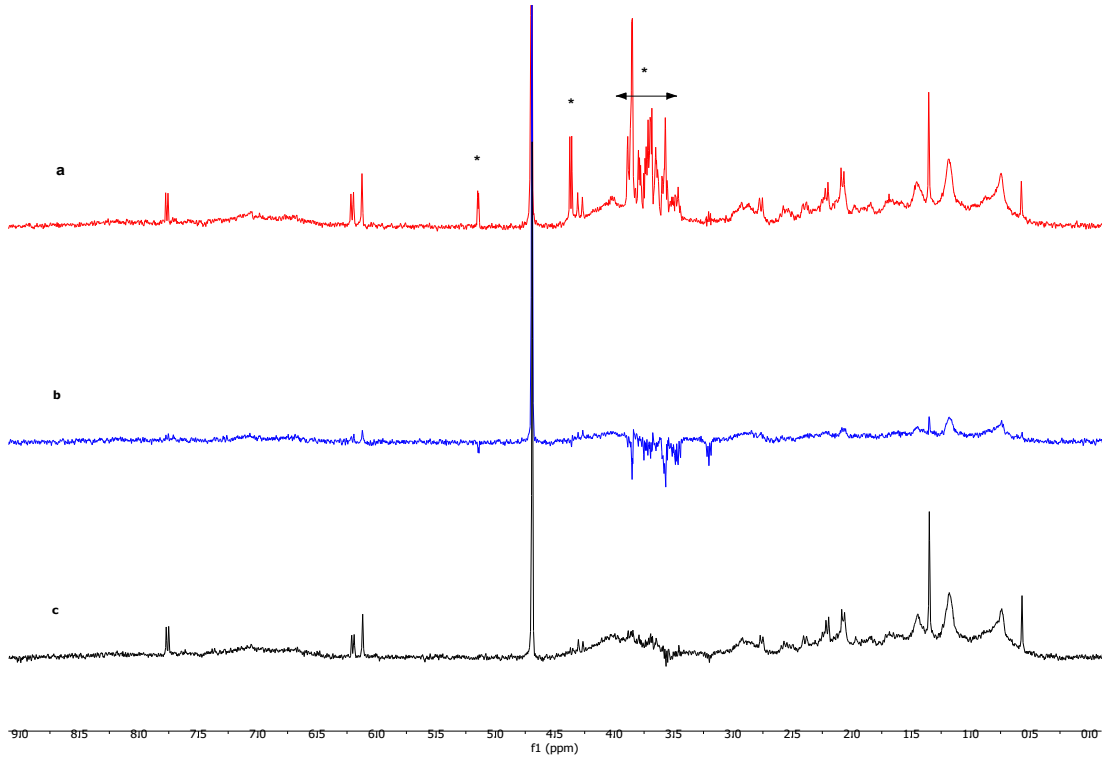

Figure 6. a) HP-WASTY; the asterisks denote false-positive signals that result from over-saturation of the spectral region around the offset (red curve in Figure 4). b) LP-WASTY spectrum. c) uni-WASTY spectrum. All spectra are phased to display non-interacting species down and interacting species up (as in a 180°-phased WaterLOGSY) and have been collected without T_2 filtration on the sample of prednisone, lactose and 20 μM HSA in H_2O/D_2O = 90/10 with phosphate buffer (36 mM, pH 7.0).

4. Conclusions.

In this work we have significantly improved previous results obtained with high-power saturation in WaterSTD experiments. The proposed experiment, which we have dubbed uni-WASTY, relies on the combined effect of a selective on-resonance saturation of water spins and adiabatic off-resonance spin-locks that uniformly saturate all the ^{1}H resonances in a typical 5 kHz range. In this way, possible false positive signals from over-saturation of protons resonating close to the water signal are avoided.

We expect this approach to be useful in the screening of drugs with weak affinity for the receptors, or when the ligand-binding interactions do not provide a clear polarization of the signals in WaterLOGSY experiments. In addition, the same technique finds a straightforward application in the domain of NMR chemosensing, where suitably designed nanoparticles (among other possible receptors) allow a selective magnetization transfer only to specific classes of molecules, thus enhancing the signals of some analytes over others.

It is foreseeable that, when the strategy discussed in this work is implemented on spectrometers with high magnetic fields and state-of-the -art cryogenic probes, the dramatic sensitivity gains would open the pathway to the analysis of mixtures in the micromolar range. Along this line, NMR chemosensing protocols for the detection of catecholamines in urines are currently being investigated in our labs for the prognosis evaluation of neuroblastoma.

Declaration of Competing Interest.

The authors declare no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References.

[1] C. Dalvit, P. Pevarello, M. Tato, M. Veronesi, A. Vulpetti, M. Sundström, Identification of compounds with binding affinity to proteins via magnetization transfer from bulk water, J. Biomol. NMR. 18 (2000) 65–68. doi:10.1023/A:1008354229396.

[2] M. Mayer, B. Meyer, Characterization of ligand binding by saturation transfer difference NMR spectroscopy, Angew. Chemie - Int. Ed. 38 (1999) 1784–1788. doi:10.1002/(SICI)1521-3773(19990614)38:12<1784::AID-ANIE1784>3.0.CO;2-Q.

[3] C. Dalvit, U. Hommel, Sensitivity-Improved Detection of Protein Hydration and Its Extension to the Assignment of Fast-Exchanging Resonances, J. Magn. Reson. Ser. B. 109 (1995) 334–338.

doi:10.1006/jmrb.1995.9984.

[4] F. De Biasi, F. Mancin, F. Rastrelli, Nanoparticle-assisted NMR spectroscopy: A chemosensing perspective, Prog. Nucl. Magn. Reson. Spectrosc. 117 (2020) 70–88. doi:10.1016/j.pnmrs.2019.12.001.

[5] F. De Biasi, D. Rosa-Gastaldo, X. Sun, F. Mancin, F. Rastrelli, Nanoparticle-assisted NMR spectroscopy: Enhanced detection of analytes by water mediated saturation transfer., J. Am. Chem. Soc. (2019) jacs.8b13225. doi:10.1021/jacs.8b13225.

[6] G. Colmenarejo, In silico prediction of drug-binding strengths to human serum albumin, Med. Res. Rev. 23 (2003) 275–301. doi:10.1002/med.10039.

[7] L. Zhou, L. Yang, S. Tilton, J. Wang, Development of a high throughput equilibrium solubility assay using miniaturized shake-flask method in early drug discovery, J. Pharm. Sci. 96 (2007) 3052–3071. doi:10.1002/jps.20913.

[8] K. Takegoshi, K. Ogura, K. Hikichi, A perfect spin echo in a weakly homonuclear J-coupled two spin-system, J. Magn. Reson. 84 (1989) 611–615. doi:10.1016/0022-2364(89)90127-3.

[9] J.A. Aguilar, M. Nilsson, G. Bodenhausen, G.A. Morris, Spin echo NMR spectra without J modulation, Chem. Commun. 48 (2012) 811–813. doi:10.1039/C1CC16699A.

[10] E. Kupče, R. Freeman, Strange Effects of Pulse Shaping in Water Presaturation Experiments, J. Magn. Reson. 146 (2000) 240–244. doi:10.1006/jmre.2000.2151.

[11] Ē. Kupče, Applications of Adiabatic Pulses in Biomolecular Nuclear Magnetic Resonance, in: U.S. T. L. James, V. Dötsch (Ed.), Methods Enzymol., Acad. Press, San Diego, 2001: pp. 82–111. doi:10.1016/S0076-6879(02)38216-8.

[12] H. Desvaux, P. Berthault, N. Birlirakis, M. Goldman, M. Piotto, Improved Versions of Off-Resonance ROESY, J. Magn. Reson. Ser. A. 113 (1995) 47–52. doi:10.1006/jmra.1995.1054.

[13] C.M. Thiele, K. Petzold, J. Schleucher, EASY ROESY: Reliable Cross-Peak Integration in Adiabatic Symmetrized ROESY, Chem. - A Eur. J. 15 (2009) 585–588. doi:10.1002/chem.200802027.