PATHOS - Deliverable D1.6
Report on AZE for homonuclear mixing and recoupling

Abstract

Multidimensional Exchange, TOtal Correlation and Nuclear Overhauser Effect SpectroscopY (EXSY, TOCSY and NOESY respectively) are at the foundation of homonuclear mixing and recoupling in magnetic resonance experiments. NOESY and TOCSY in particular play numerous fundamental roles in structural and dynamic applications based on NMR in the organic chemistry, pharmaceutical and biomolecular sciences. Low efficiency is an intrinsic downside of all these methods, and this is particularly true for systems involving fast relaxing or rapidly exchanging. These in turn include labile protons of the kind that are ubiquitous in polysaccharides, disordered proteins, nucleic acids, etc. The fast, effective relaxation then imparted on these protons complicates their observation, and restricts magnetization transfer processes involving them. As part of PATHOS we have begun to rely on Anti-Zeno Effects (AZEs) to dramatically increase the efficiency of these methods –not by percents or 10s of percents, but by factors of 2-6! As initial step in this we have recently introduced Looped PROjected SpectroscopY (L-PROSY), an approach that actually uses AZEs to enhance NOESY and TOCSY cross-peaks for amide, hydroxyl and amine groups in biomolecules. This deliverable demonstrates that much larger sensitivity gains per unit time can be achieved if using a looped inversion or a saturation procedure, where NMR NOESY or TOCSY correlations are achieved by irradiating *a priori* selected frequencies according to the Hadamard encoding recipe. Then, provided that the spectrum is generally sparse and that the different frequency-selective "channels" (peaks) along the indirect dimension are known, experiments that are much faster (fewer scans) and more sensitive (per scan) than either conventional or even L-PROSY counterparts, can result. In the resulting AZE effect is mediated by a looped magnetization-transfer (MT) process, whereby labile protons of different systems are selectively addressed via a train of inversion or a long saturation pulse, and then repeatedly subjected to either NOE, exchange or broadband isotropic mixing with other protons in the molecule. Ensuing NMR detection, followed if needed by Watergate-based water suppression module, provide an enhanced homonuclear mixing/recoupling. These gains can be achieved at any fields, but gain a lot from ultrahigh field situations where peaks are better separated (important for the Hadamard encoding) and where T_1s are long (important for enhancing the MT aspects of the correlations). This is here corroborated by experiments performed in Weizmann's brand new 1 GHz NMR spectrometer. The effectiveness of the ensuing three-way polarization transfer interplay between water, labile and non-labile protons is illustrated here by experimental data for numerous systems, all of which reveal cross-peaks that are barely detectable in conventional 2D NMR counterparts with 100s % enhancements, in acquisitions that take only a fraction of these conventional counterparts. The efficiency of these new modules, their application to additional systems –including glycans and paramagnetically labeled proteins, as well as extensions to higher dimensionalities, are also discussed and presented.

Introduction

With the developments of 2D homonuclear correlation experiments,[1,2] NMR spectroscopy became an integral part of research efforts to elucidate the structure and dynamics of organic, pharmaceutical and biological molecules.[3,4] These correlations can be mediated by chemical exchange or by Nuclear Overhauser Enhancements (NOEs),[5,6] and are probed by monitoring how off-equilibrium polarization from one spin reservoir travels to another via dipolar interactions or chemical kinetics.[7–12,15–17] Transfer of magnetization among members of a J-coupled spin network can also be achieved by TOtal Corelation SpectroscopY (TOCSY),[13,14] leads to a complementary set of information based on bonded connectivities. Despite being widely performed these 2D NMR experiments, and particularly NOESY, suffer from a relatively low efficiency leading to small cross-peaks and requiring in turn extensive signal averaging for their characterization. Retrieval of such cross-peaks becomes even more difficult when NOESY and TOCSY start competing against chemical exchanges with the solvent. Hydroxyl protons in saccharides, amino groups in proteins and nucleic acids, amides in disordered proteins, and imino protons in RNA/DNA are prototypical examples of these challenging systems, as when placed in water they will undergo a rapid chemical exchange with the solvent that can dramatically reduce the efficiency of their intramolecular polarization transfers. We have recently introduced Looped PRObjective SpectroscopY (L-PROSY),[18] an approach that was shown to alleviate these problems in proteins by means of Anti-Zeno Effects (AZEs). Indeed, instead of applying only a single mixing period for facilitating homonuclear transfers to happen and reach maximum amplitude, L-PROSY "freezes" these transfers after they begin to act with their (fastest) initial rate, resets the labile protons' states to their initial conditions by exploiting their exchanges with the massive solvent reservoir, and repeats this process multiple times.[19,20] The ensuing 'L-PROSY encoding' acts then as a sort of conveyor, causing the NOE/TOCSY cross-peaks to grow with the much more favorable rates characterizing their initial buildups, before performing the signal detection. By selectively addressing only the targeted protons and avoiding water perturbation L-PROSY exploits some elements of the SOFAST NMR experiment;[21,22] at the same time, by its repeated action, it also reminds of certain CEST-like polarization transfers.[23-26] Despite the sensitivity gains imparted by the AZE procedure these experiments are still long, requiring traditional incrementation of evolution periods to build-up second dimension as in conventional experiments. Even though these looped experiments could also be paired with NUS sampling[27–29], the possibility also arises to achieve even faster and more complete ways to exploit these polarization transfer improvements, especially given the generally sparse distribution of the labile protons in molecules. The present study explores this by introducing a new way to implement magnetization transfers (MTs) while exploiting AZEs based on a Hadamard encoding[30,31] of the targeted labile peaks. It is shown that when combined with multiple selective inversions or with a continuous saturation procedure, this provides the highest enhancements per unit time we have seen to NOESY, TOCSY and EXSY experiments involving labile and fast relaxing protons.

Results and discussion

Frequency-domain Hadamard spectroscopy[30,32] offers the possibility to speed up a large number of experiments, while enjoying the benefits of multiplexing. Within the framework of time-domain NMR experiments Hadamard replaces the conventional t_1 incremented evolution delay, with a "comb" of frequency-selective RF pulses that directly address peaks in the F_1 frequency domain. Prior knowledge of the one-dimensional spectrum to be addressed and a need to deal with sparse spectra with clearly addressable peaks, are thus main limitations of Hadamard spectroscopy. These however, will be readily fulfillable conditions for most of the high field, labile proton scenarios to be considered in this study. So far, Hadamard excitation and decoding in NMR has been based on imparting 180˚ phase shifts on the targeted peaks, followed by a suitable addition/subtraction reconstruction of the indirect dimension according to the Hadamard excitation matrix. In order to bring the aforementioned AZE-based advantages to bear into this Hadamard spectroscopy scheme, excitations were replaced by selective saturations or looped inversion pulses addressing fast-exchanging labile protons. This led to the experimental scheme depicted in Figure 1a, where what we denominate for simplicity as a Magnetization Transfer (MT) block encodes as "on" or "off" the labile protons according to a Hadamard matrix, without perturbing either the solvent nor the peaks that will eventually receive polarization from the labile sites. The fact that the large water spin reservoir is not perturbed provides constant repolarization of the labile protons during the encoding process,

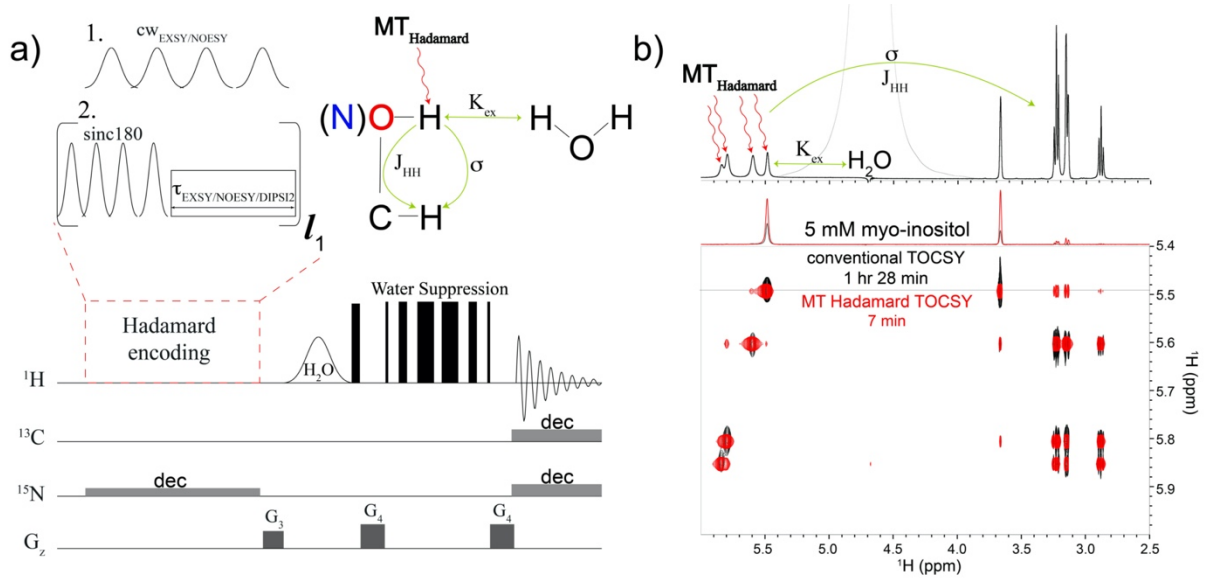

Figure 1. a) MT Hadamard pulse sequence illustrating that two types of perturbation procedures can be utilized for efficient magnetization transfer according to Hadamard encoding– selective saturations or looped inversions followed by a delay for NOESY/EXSY transfer or DIPSI2 to achieve isotropic mixing for TOCSY (selective saturations can be used only for the NOESY/EXSY transfers; repeated inversions work for all). During these long MT processes a three-way polarization transfer is effectively established, where water constantly repolarizes labile protons enabling prolonged magnetization transfer to non-labile protons. An AZE-like effect is thus achieved. Dec refers to GARP4 decoupling, that was used during Hadamard encoding and acquisition for the labeled samples. b) 1D spectrum of myo-inositol showing in gray the dominating water resonance and illustrating the homonuclear transfers occurring during the MT. Shown below is a conventional TOCSY spectrum of myo-inositol acquired with 48 ms DIPSI2 mixing, overlaid on a MT Hadamard TOCSY spectrum obtained using 12 loops of 24 ms DIPSI2 mixing. Notice the different acquisition times shown in the figure, as well as the substantial MT enhancement of the cross peaks. Spectra were acquired at 600 MHz Avance III, equipped with a Prodigy probe.

prolonging the MT operating through cross-relaxation, J-coupling[33] or chemical exchange. Figure 1b illustrates the ensuing gains in overlaid conventional and MT Hadamard encoded TOCSY spectra addressing the hydroxyl sites of myo-inositol, a prototypical saccharide.

Whereas in conventional TOCSY the chemical exchange that exchangeable protons undergo with the solvent averages out J-couplings and prevents transfer through full J-coupling network, in the MT Hadamard they enhance the correlation, and magnify cross-peaks several-fold while requiring an order-of-magnitude shorter acquisition time.

It makes sense to compare MT Hadamard performance to the L-PROSY experiment. Figure 2a illustrates 2D TOCSY NMR comparisons between conventional, L-PROSY and MT Hadamard methods on a 5 mM sucrose sample at 2 ºC. Both L-PROSY and Hadamard MT yielded large (≈2-4x) sensitivity enhancements compared to the conventional experiment, with the former being slightly larger but the latter lasting an order of magnitude less time. Figure 2b shows another comparison for a NOESY experiment acquired on 5 mM myo-Inositol; again, notice the resemblance between L-PROSY and MT Hadamard results– same SNR, but with the Hadamard encoding requiring a 28-fold shorter acquisition.

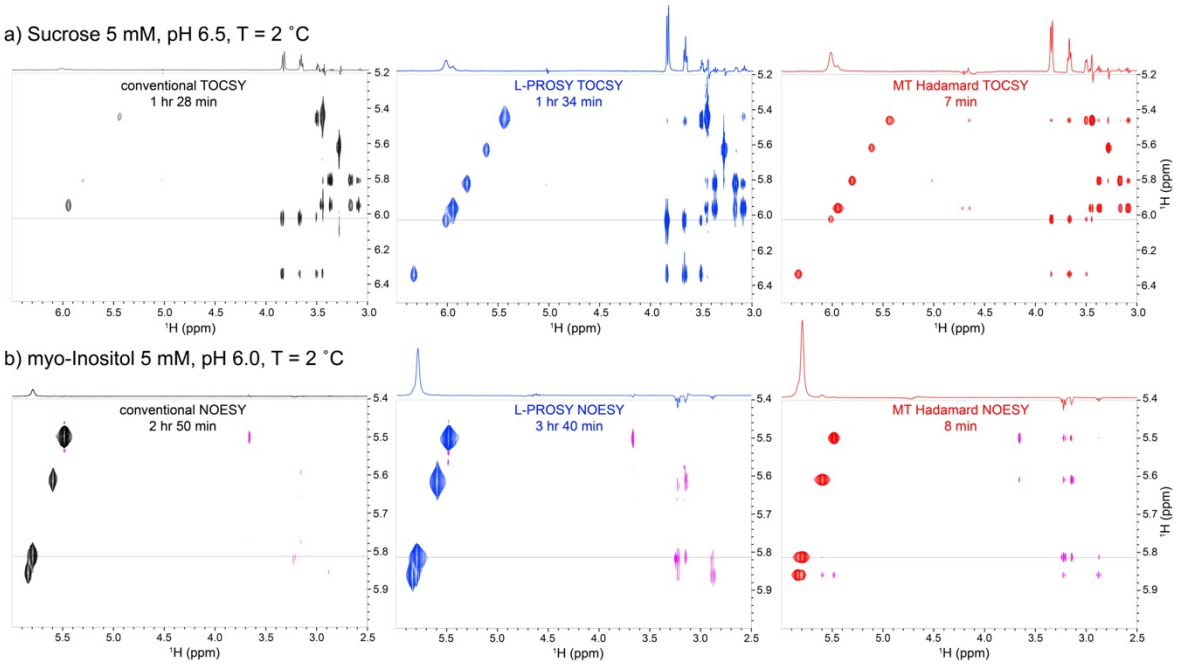

Figure 2. a) TOCSY spectra acquired on 5 mM sucrose using conventional (48 ms DIPSI2), L-PROSY (11x20 ms DIPSI2) and MT Hadamard TOCSY (12x20 ms DIPSI2) schemes. Notice the superior spectral quality in both the L-PROSY and MT Hadamard experiments vis-à-vis the conventional TOCSY acquisition. b) Similar comparison but for NOESY experiments on 5 mM myo-Inositol. The conventional NOESY experiment was acquired with 80 ms mixing, L-PROSY used 14 loops, 35 ms each; MT Hadamard was acquired using 800 ms CW saturation. All data were acquired on a 600 MHz Avance III Bruker equipped with Prodigy probe.

These AZE methods, but MT Hadamard NMR in particular, benefit significantly from operating at ultrahigh field. Under these conditions, superior resolution leads to better line separation, facilitating the Hadamard encoding. Moreover, the exchangeable protons signals are sharper due to larger frequency separation from the water resonance, and hence decreased exchange broadening. This is clearly depicted in Figure 3, for the hydroxyl and amide protons of the tetramer glycan sialic acid. In addition the T_1 relaxation of the non-labile protons receiving the labile protons' polarization and dictating the upper limit for the duration of MT encoding, lengthens at higher magnetic fields.[34–37] This increases the extent of the magnetization transfer during Hadamard encoding. Furthermore, at higher fields the same spectral resolution can be achieved for the labile protons while studying them at higher temperatures. This brings the multiple benefits of enabling studies nearer physiological

conditions (37 ˚C), of benefiting from faster exchange rates with the solvent that magnify the AZE enhancement, and of even longer T_1s as shorter tumbling times prolong T_1s in small molecules.[38]

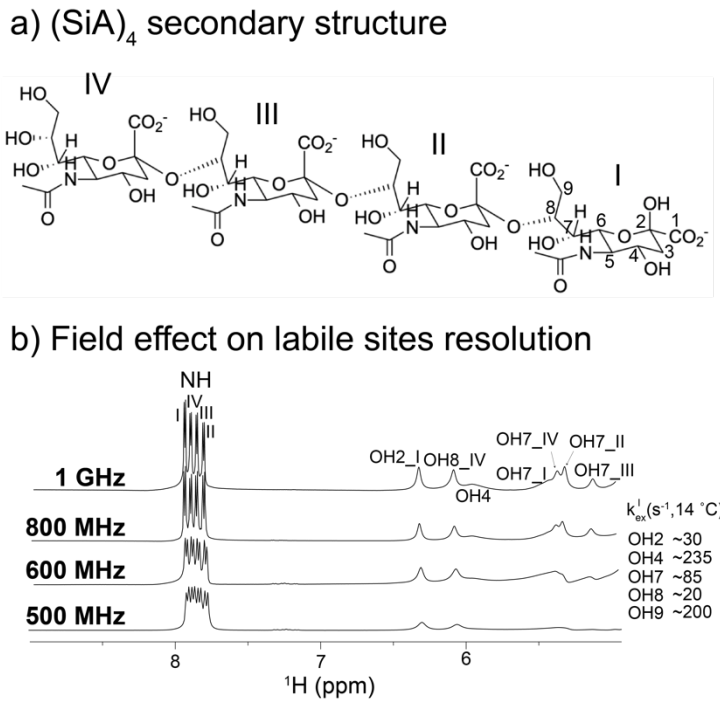

Figure 3. a) Secondary structure of $(SiA)_4$ depicting multiple hydroxyl and amide sites. b) Magnetic field effect on the linewidth of hydroxyl and amide protons. Notice how the chemical exchange broadens certain hydroxyl protons beyond detectability at 500 MHz, while at 1 GHz, most of the peaks can be resolved well. Something similar happens, to a smaller degree, with the NH protons. In both cases this is due to the transformation of an exchange process from the intermediate to the slow exchange regime, mediated not by a change in temperature but by the field-induced spreading of the exchanging sites.

Figure 4 compares conventional and MT Hadamard NOESY spectra acquired on the $(SiA)_4$ glycan at 5 ºC and at a 1 GHz frequency. While it is hard to discern any correlations involving the hydroxyl protons in the conventional experiment due to the very weak cross-peaks and pronounced T_1 noise, MT Hadamard provides quality NOESY data in a fraction of time needed for conventional experiment, that can be used for both structural assignments and hydrogen bonds detections.

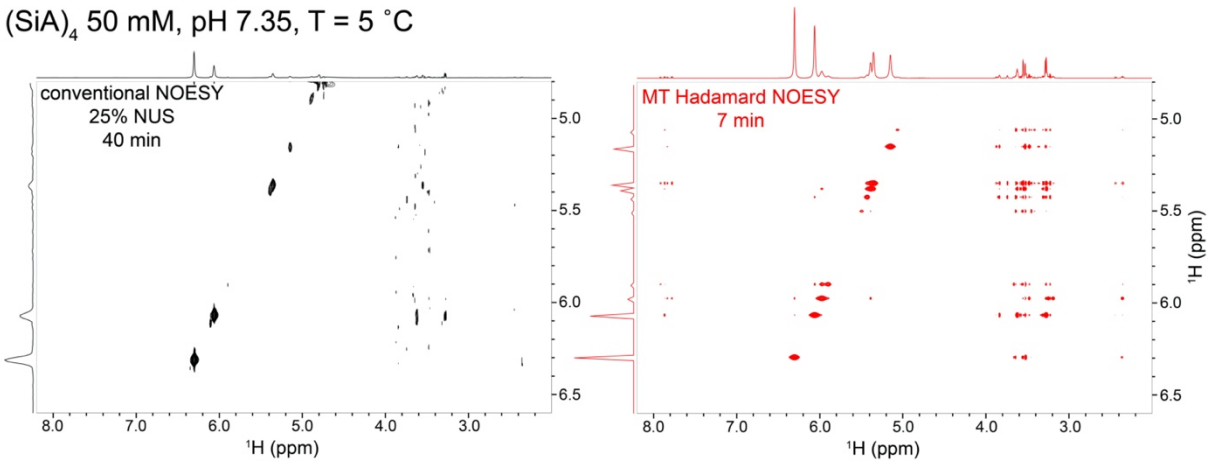

Figure 4. Comparison between a conventional 2D NOESY acquired using 25% non-uniform sampling for a minimal acquisition time with faithful spectral reconstruction, and an MT Hadamard experiment. Notice the much higher quality provided by the latter, coupled to the much shorter acquisition. All spectra were acquired at 1 GHz Avance Neo, equipped with TCI cryoprobe.

High magnetic fields turned out to be especially valuable for implementing this kind of correlations on the imino protons of nucleic acids. At 1 GHz the imino resonances, which can be broadened by chemical exchange with the solvent, are fairly sharp and fully resolved, opening the possibility for implementing MT Hadamard experiments. In particular, NOESY correlations involving the imino protons could be of great usefulness in elucidating RNA peak assignments, and thereby a wide variety of ensuing NMR analyses. Figure 5 shows again superior efficiency of MT Hadamard experiment over conventional NOESY for detecting cross peaks involving these imino resonances, utilizing the14mer hairpin RNA as prototypical example.

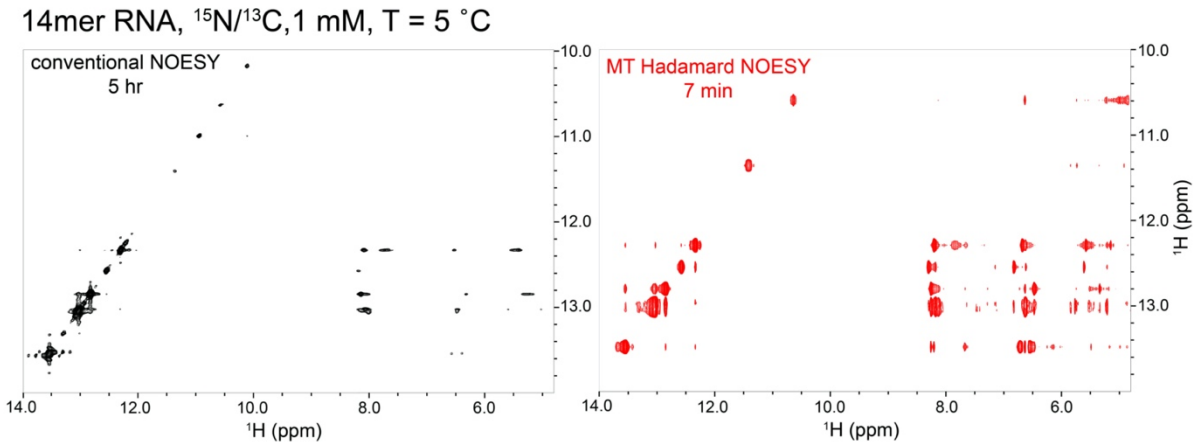

Figure 5. Comparison between conventional NOESY (100 ms mixing, 30 us delay for binomial water suppression – optimal for imino resonances) vs. MT Hadamard NOESY (14 loops 40ms mixing each) spectra. The conventional spectrum shows NOESY correlations with the imino resonances along F_1, and was flipped to match the MT Hadamard that actually encoded imino protons: otherwise, the conventional experiment would show no peaks originating from the imino altogether. For this ^{15}N-labeled sample, looped inversions and mixing periods were preferable to long saturations as otherwise the constant ^{15}N decoupling during the long saturation period can lead to sample heating. Large number of imino correlations with other imino, amino and sugar aliphatic protons can be observed in MT Hadamard, while many of them are undetectable in conventional experiment. Spectra are acquired at 1 GHz using a Bruker Avance Neo spectrometer equipped with a TCI cryoprobe.

At 1 GHz, most of the H_N signals in small and medium-sized proteins are fully resolved enabling MT Hadamard NOESY experiments on these polypeptides as well. Figure 6 illustrates the benefits resulting from this with a comparison between conventional and MT Hadamard NOESY experiments recorded for a) LA5, a 40-residue protein and b) Ubiquitin, a 76-residue protein. As in the case of the RNA (Fig. 5), the MT Hadamard utilized a looped encoding instead of a single long saturation as both of these proteins were ^{15}N labeled, and looping facilitated the heteronuclear decoupling (sequence in Fig. 1a). Extracted 1D projections show 1:1 match between two spectra with noticeable sensitivity enhancements of ≈2x provided by the faster MT Hadamard scheme.

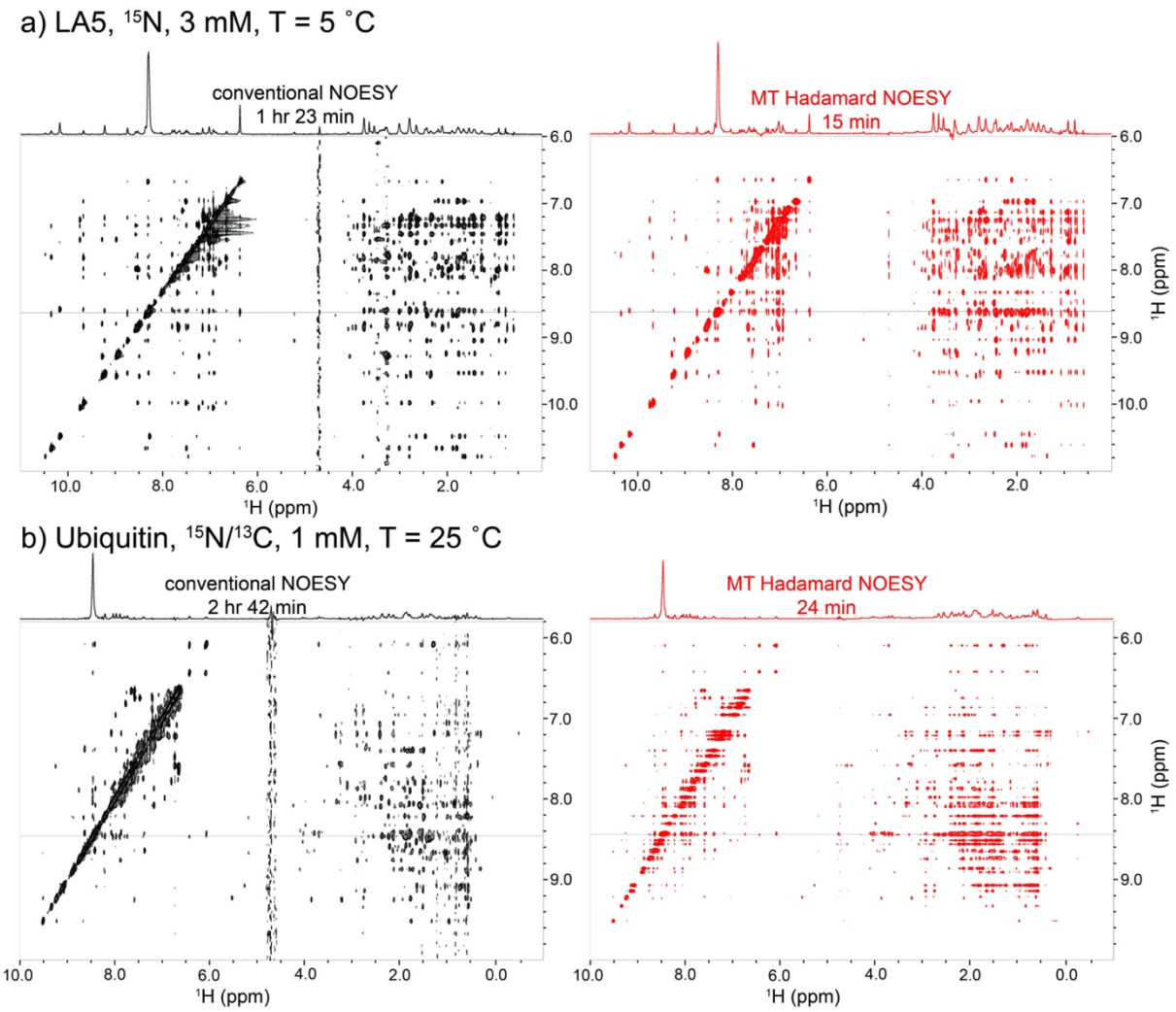

Figure 6. Conventional vs MT Hadamard NOESY spectra recorded for a) LA5 and b) Ubiquitin samples. Notice how the 23.5 T field is sufficient to resolve almost entirely the amide/amino resonances in these proteins (regions between 6.6 – 9.5 ppm) and enable fast, highly sensitive NOESY experiments by MT Hadamard. Conventional experiments were acquired with 300 ms and 250 ms mixing respectively, MT Hadamard employed 6x150 ms and 6x140 ms looped encoding. Spectra were acquired at 1 GHz using a Bruker Avance Neo console equipped with a TCI cryoprobe.

Another benefit of the MT Hadamard scheme compared to the conventional experiments, rests in their ability to selectively target resonances of interest. For instance, in the protein spectra shown in Figure 6 it is not straightforward to distinguish between the correlations stemming from amide and from side-chain amino protons. Hadamard encoding allos one to selectively address the latter, providing 2D correlations with improved resolution. Figure 7 shows such 2D correlations acquired for these sites at 1 GHz, in only 4 minutes for Ubiquitin and for the 14mer RNA, based on the MT Hadamard experiment. These experiments evidence the expected NOE-driven cross-peaks with the aliphatic protons for the protein case and with the imino protons of the bases for the nucleic acid case. Strong correlations, however, are also observed in both spectra among the amino protons themselves; we ascribe those to chemical exchange effects among these moieties, facilitated as well by the MT scheme.

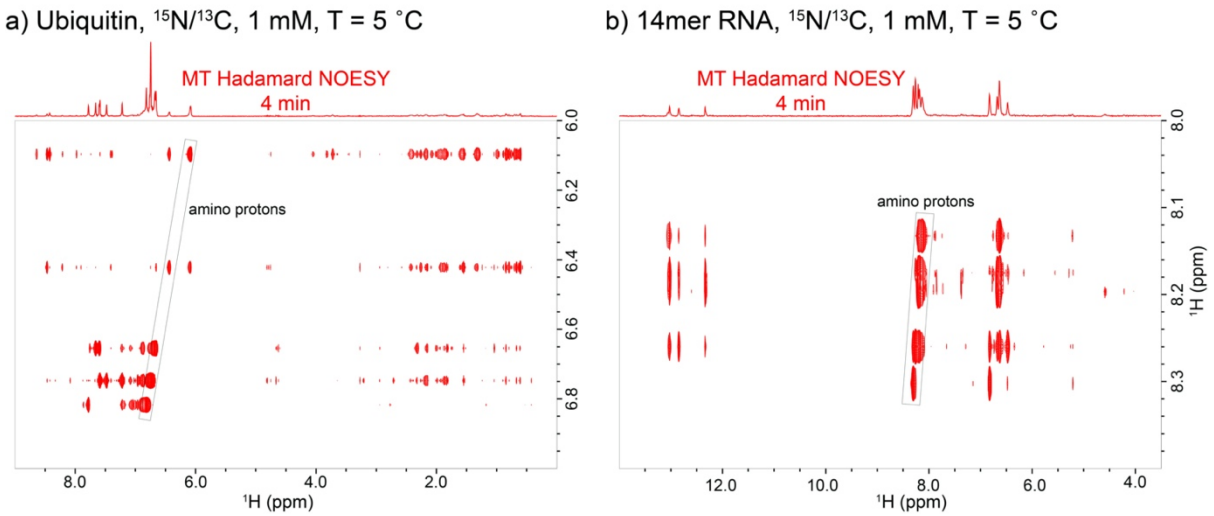

Figure 7. Amino protons targeted with MT Hadamard NOESY experiment in a) Ubiquitin and b) 14mer RNA. In a) spectrum is acquired with 12 loops and 80 ms per loop, while in b) saturation of 600 ms was used instead. Since only one pair of amino protons is targeted during Hadamard encoding, the strongest correlations correspond to exchange cross-peaks. Spectra were acquired at 1 GHz using a Bruker Avance Neo console equipped with a TCI cryoprobe.

In addition to nitrogen-bound labile protons, notoriously hard targets to correlate in protein and nucleic acid experiments concern the hydroxyl protons in the former sidechain groups and in the sugars of the latter molecules; these -OHs are often buried under other, sharper and more intense amino and amide resonances. On the other hand, hydroxyl protons usually undergo faster chemical exchanges with water than the latter. As illustrated with the small saccharide examples given in Figures 1-4, these qualifies them for potentially large cross-peak enhancements when targeted by the MT Hadamard scheme, that could provide a plethora of information about the protein and nucleic acid structure and folding. Figure 8a shows a version of the MT Hadamard pulse sequence that, by incorporating additional $^{15}N/^{13}C$-based filters aimed at suppressing the intense signals from proton bound to ^{15}N and ^{13}C that would otherwise complicate the OH's observation, allow 'hidden' hydroxyl hydrogens to be encoded. To ensure that only OH protons are targeted in these experiments, a series of variable-temperature (5-25 ºC) 1D, $^{15}N/^{13}C$ suppressed spectra were acquired; proton resonances that survived the N-H and C-H suppression became sharper at lower temperatures due to slower chemical exchanges with water, and were confirmed as likely candidates to arise from the labile hydroxyl protons. Figures 8b and 8c exemplify the cross-correlations arising from this experiment on a doubly $^{13}C/^{15}N$-labeled ubiquitin and on a 14mer RNA sample, respectively. Highlighted in these spectra are the hydroxyl hydrogens that are addressed in MT Hadamard. Particularly interesting are OH-OH inter-residue correlations detected in Ubiquitin, and the long-range correlations between the sugar's OH and the base protons resonating between 7 and 8 ppm in the 14mer RNA; both of these could contribute substantially to 3D structure determinations.

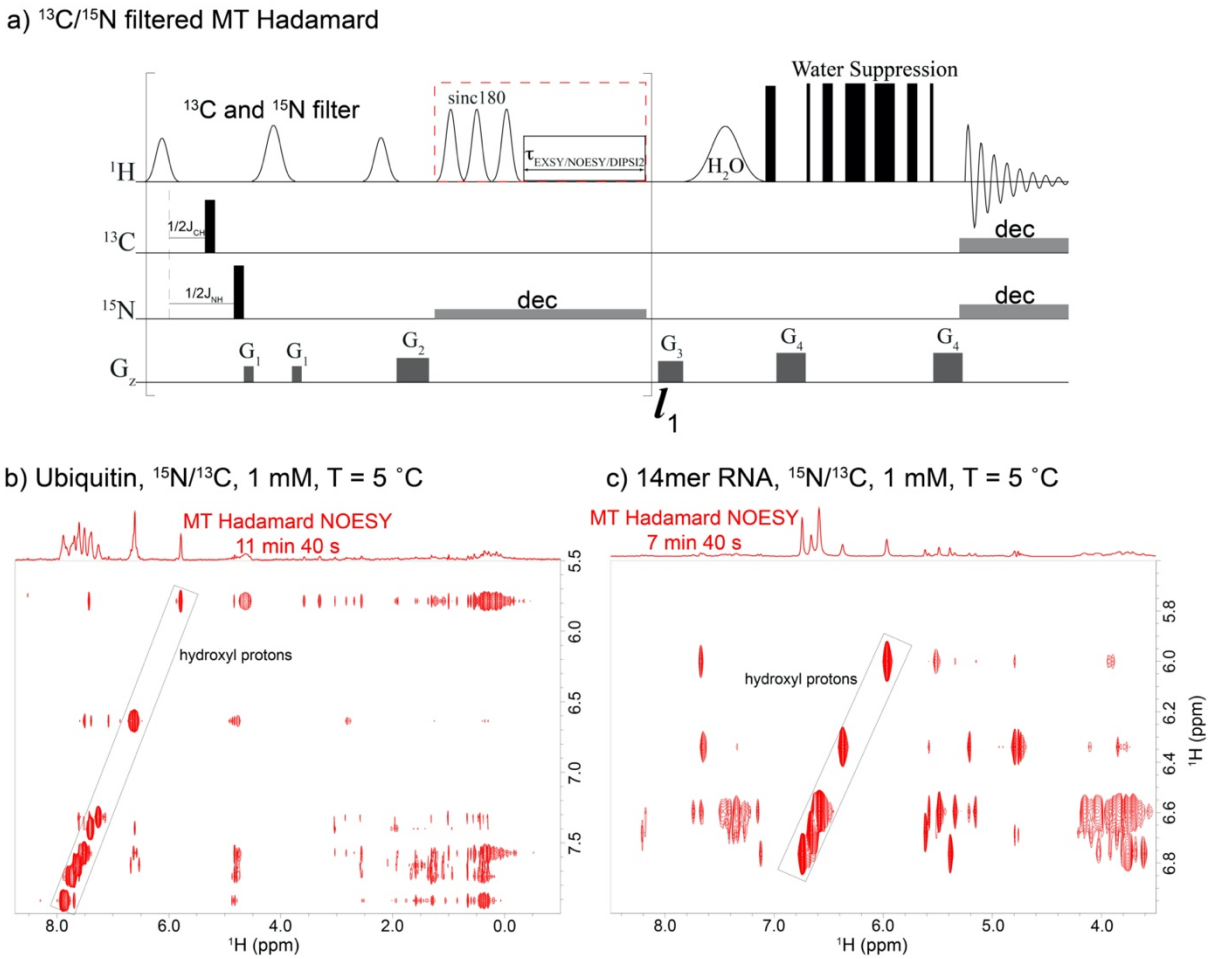

Figure 8. a) MT Hadamard pulse sequence with optional filter to suppress protons bound to ^{13}C and/or ^{15}N in labeled compounds in order to selectively address 'invisible' hydroxyl hydrogens. Filter has to be applied at the beginning of every MT Hadamard loop in order not to allow suppressed resonances to start recovering during mixing periods. Consequently, short mixing times has to be used per loop, which is possible due to fast chemical exchange with water. In filter, selective proton pulses are applied only in H_O-bearing regions not to suppress other protons that are receiving magnetization. Examples with b) Ubiqutin acquired using 10 loops and 50 ms per loop and c) 14mer RNA using 15 loops and 40 ms mixing time per loop. Spectra were acquired at 1 GHz using a Bruker Avance Neo console equipped with a TCI cryoprobe.

Conclusions

MT Hadamard is introduced here as an extension of AZE-based experiments where, instead of a t_1 time-domain encoding, selective irradiations are used to achieve significant sensitivity gains in EXSY, NOESY and TOCSY homonuclear correlation experiments involving fast-exchanging protons. For achieving these gains MT Hadamard exploited the flow of fresh water polarization resetting the states of the targeted sites, whose perturbation away from equilibrium via saturations or inversions could then be used to magnify polarization transfer processes that through either dipole-dipole relaxation or through J-coupling, spread between distinct molecular sites. Thus, while chemical exchange with the solvent deteriorates conventional homonuclear transfer experiments, the abundant, slowly-relaxing water resonance improves these processes by several-fold when switching to this new encoding scheme. Relaxation properties of the non-labile protons on the receiving end of these transfers will limit the extent of these gains, as the MT process will only be effective over their 'memory

times'. It follows that operating at higher fields, where T_1 relaxation times are usually longer, will improve the efficiency of these magnetization transfers. So will the line narrowing that labile ^{1}Hs will experience as a result of exchanges with the water –particularly when involving hydroxyl, amino or imino sites. Possible drawbacks of relying on ever longer MT processes concern potential sample heating, and an enhanced spin-diffusion among the non-labile sites in systems with high proton density. The former was not found to be a problem even when operating at 1 GHz, where dielectric losses could be substantial. The latter, however, could rob the MT experiments from certain specificity. We investigated a similar possibility for the case of L-PROSY, where it was found that this new method still provided a 1:1 correlation with conventional NOESY cross-peak intensities when concerning translation of cross-peak intensities into inter-nuclear spatial distances –even in the presence of a spin-diffusion sink pool. We assume that this correspondence will persist over a wide range of solvent exchange rates and correlation times, a matter that is still under investigation.

While this study focused on homonuclear transfers originating from labile ^{1}Hs being replenished by the solvent, the MT Hadamard concept could be exploited in additional NMR settings where fast-relaxing sites can be individually addressed. These include methyl groups in otherwise deuterated proteins, inter-molecular binding interactions including protein/drug binding processes,[39–41] as well as fast-relaxing sites in paramagnetic biomolecules. The 2D MT Hadamard concepts here introduced could also be included as part of correlations with heteronuclei in 3D spectral acquisitions.[30,42] Heteronuclear analogues of the homonuclear polarization transfer processes here discussed can also be proposed. Further discussions on all these cases will be presented in an upcoming report.

Experimental section

Sample preparation

Myo-Inositol was purchased from SigmaAldrich (Israel) and prepared as 5 mM solution at pH 6.0. 5mM sucrose solution was prepared using household sugar at pH 6.5. Natural abundance α2-8 $(SiA)_4$ was purchased from EY Laboratories Inc (San Mateo, CA). 25 mg of α2-8 sialic acid tetramer was dissolved in 400 μL (~50 mM) of 20 mM phosphate buffer at pH 6.5, containing 0.05 % NaN3 that yielded the final solution at pH of 7.35. 14mer haipin RNA was synthesized as described and a 1 mM solution was prepared at pH 7.2. Several proteins were analyzed over the course of the MT Hadamard tests. Ubiquitin was purchased from Asla Biotech and was dissolved in PBS (Dulbecco's Phosphate Buffer Saline at physiological pH, purchased from Biological Industries) at a concentration of 1 mM. LA5, the ligand bind- ing domain 5 of the low density lipoprotein receptor LDLR, was prepared at pH 7.4 and concentration 3 mM in 10 mM Tris buffer with 1 mM CaCl2. All samples were prepared containing NaN3 and H_2O/D_2O (90%:10%).

NMR experiments

NMR experiments were conducted using either a 14 T Bruker magnet equipped with an Avance III console and TCI Prodigy probe, or a 1GHz, 23.5 T Bruker Avance Neo equipped with a TCI cryoprobe. Hadamard experiments were carried out using 8-64 Hadamard encoding

channels depending on the number of peaks in the spectrum. 10 Hz nutation field was used for saturation, while 20-25 Hz bandwidth *sinc1* inversion pulses were used in looped inversion method. Number of loops and duration saturation were determined according to T_1 values of receiving protons. Optimal values for NOESY and TOCSY mixing times were used in conventional experiments. Conventional TOCSY experiments were acquired using dipsi2gpph19/dipsi2esgpph standard Bruker sequences using DIPSI2 isotropic mixing, while for NOESY experiments noesyfgpph19/noesyesgpph was used. In all cases, optimal mixing times and optimal WATERGATE delays for binomial water suppression according to the magnetic field were used. All spectra were processed in Bruker® TopSpin® 4.0.6. All spectra were apodized with QSINE or SINE window functions and while conventional spectra were zero-filled once, all Hadamard spectra were zero-filled to 256-1024 points.

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