Enhancing the Sensitivity of Solution-Phase Protein NMR by Optimized Injections of Hyperpolarized Water (see page XA)
On the Potential of Hyperpolarized Water in Biomolecular NMR Studies

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ABSTRACT: A main obstacle arising when using ex situ hyperpolarization to increase the sensitivity of biomolecular NMR is the fast relaxation that macromolecular spins undergo upon being transferred from the polarizer to the spectrometer, where their observation takes place. To cope with this limitation, the present study explores the use of hyperpolarized water as a means to enhance the sensitivity of nuclei in biomolecules. Methods to achieve proton polarizations in excess of 5% in water transferred into the NMR spectrometer were devised, as were methods enabling this polarization to last for up to 30 s. Upon dissolving amino acids and polypeptides sited at the spectrometer into such hyperpolarized water, a substantial enhancement of certain biomolecular amide and amine proton resonances was observed. This exchange-driven 1H enhancement was further passed on to side-chain and to backbone nitrogens, owing to spontaneous one-bond Overhauser processes. 15N signal enhancements >500 over 11.7 T thermal counterparts could thus be imparted in a kinetic process that enabled multiscan signal averaging. Besides potential bioanalytical uses, this approach opens interesting possibilities in the monitoring of dynamic biomolecular processes, including solvent accessibility and exchange process.

1. INTRODUCTION

Recent developments in high-field dynamic nuclear polarization (DNP), can greatly enhance the sensitivity of nuclear magnetic resonance (NMR) in solids and liquids. Most promising among these methods, particularly within the context of solution-phase NMR spectroscopy and imaging (MRI), is the dissolution DNP approach. Dissolution DNP improves NMR’s sensitivity by executing the nuclear hyperpolarization ex situ, on a custom polarizer where the targeted sample is comixed with a stable (often organic) radical and cooled into an amorphous frozen glass. After exposing such cryogenic system to suitable microwave radiation, the very high polarization of the electron spins (>90%) is efficiently transferred to the surrounding nuclei in bulk. This microwave-driven polarization transfer happens over minutes or hours at $T \leq 1.5$ K; the sample is subsequently returned to the liquid state by exposing it to hot vapors, and the resulting liquid is then flushed from the polarizer into the NMR/MRI probe/coil for a rapid inductive-based detection. This ex situ method can create nuclear polarizations in excess of 30%, and for the case of small molecules, its sudden-dissolution nature can preserve much of these earnings for subsequent liquid-phase NMR observations. Such sensitivity gains can be truly outstanding, akin to years of nonstop conventional signal averaging. Still, when considering the use of this setup for biomolecular applications, a serious limitation arises. This derives from the short relaxation times that characterize biomolecules, particularly in the very low (<0.1 T) magnetic fields that the dissolved sample has to negotiate between the polarizer and the spectrometer. Indeed, relaxation rates in excess of a kilohertz are typical of medium-sized biomolecules tumbling with nanosecond correlation times, implying that in the 1–3 s time scales that the dissolution DNP method requires for the sample to traverse through a low-field region, most of the hard-earned polarization gains will be lost. The sample hyperpolarization will be further depleted by the additional relaxation induced by the paramagnetic polarizing agent, which gets dissolved and transferred together with the targeted sample into the NMR spectrometer.

A number of alternatives have emerged over recent years to deal with this limitation. The most general among these solutions is arguably the proposal by Kockenberger et al., which employs a dual-magnet approach whereby the solid sample is transported from an upper DNP magnet into a lower NMR magnet, where samples are melted and observed. While also in this setup the sample transverses a low-field region in-between the magnets, it does so as a cryogenic pellet, opening an opportunity for preserving the hyperpolarization of even large biomolecules thanks to their cryogenic state. In a scheme that follows more closely the original ex situ DNP setup, Hilty and coworkers have recently described a dissolution device that maximizes sample transport speed while minimizing turbulence through a system of back-pressure regulation. Using this

Received: October 17, 2013
Revised: January 13, 2014
Published: January 13, 2014

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system and a modified Hypersense polarizer, a total sample dissolution-to-NMR delay of 1.2 s was achieved; short enough to endow the original ex situ approach with 300–3000× sensitivity gains for certain 13C sites in perdeuterated, unfolded polypeptides.20 Yet another interesting option recently demonstrated within the context of DNP-enhanced biomolecular NMR, focuses the hyperpolarization on perdeuterated 15N-labeled systems, which were allowed to slowly exchange their deuterons with protons of water acting as dissolution solvent.27 Because this H/D exchange process takes place once the sample has reached the high-field NMR magnet and probe, the 15N sensitivity enhancement is preserved and can be passed onward to protons.

The present study examines an alternative way to cope with these limitations, that uses hyperpolarized water as a means to enhance the sensitivity of biomolecular nuclei. We find that water protons could be spin-aligned rapidly in a cryogenic DNP made relatively long-lived, thanks to extended relaxation times and transfer to the NMR scanner. This enhancement could be helium gas applied over 1.5 s.1H dilution factors in these equilibrium 15N polarization could thus be recorded for both Overhauser-driven heteronuclear effects and a spontaneous magnetization enhancement.15N signal went a build-up behavior for this microwave-driven water polarization, as measured by the liquid state enhancement observed after a 492 nm wavelength using a 17.8 MΩ-cm H2O sample as blank.

Sample Preparation. For the exchangeable 1H NMR experiments (Figure 4), a concentrated sample of partially deuterated arginine was prepared by dissolving this amino acid at natural abundance and in powdered form (≥98% pure, Sigma Aldrich, St. Louis, MO) in 99.9% D2O (Sigma Aldrich), adjusting the pH to ~3 with concentrated HCl, and drying it by rotary evaporation. The procedure was repeated, and the remaining powder was dissolved in 3 mL of 99% D2O to a final concentration of 1 M. This arginine sample was inserted into the 10 mm NMR tube subsequently used in the hyperpolarized water injection experiments. For the water-derived 15N enhancement experiments of small molecules (Figures 5 and 6), sample volumes and concentrations included: 500 μL of 200 mM 15N-urea (Cambridge Isotopes, Cambridge, MA), 350 μL of 500 mM 15N-alanine (Cambridge Isotopes), and 700 μL of 2.1 M natural abundance arginine (Sigma Aldrich) at pH ~3. All samples were prepared in 99.9% D2O and analyzed in 10 mm NMR tubes. Finally, for the water-derived 15N enhancement experiments of biomolecules (Figure 7), modified aldehyde reductase (40 kDa) was cloned into pET28_TEVH and expressed in BL21 (DE3) bacteria using 4 L of M9 minimal media supplemented with 15N labeled ammonium chloride. The bacterial lysate was applied to a Ni column (HisPrep FF 16/10, GE Healthcare Biosciences, Uppsala, Sweden) and eluted with imidazole to yield a partially purified protein mix. The imidazole was removed by applying the protein mix to a preparative desalting column (HiPrep 26/10, GE Healthcare) equilibrated with phosphate-buffered saline (PBS). The protein was filtered, 0.02% NaN3 plus Trypsin was added to it, and the mixture was subsequently incubated overnight at 37 °C to digest the reductase. The ensuing polypeptide mix was then concentrated on a Centricon with a 10 kDa molecular weight cutoff (Millipore, Billerica, MA). The flow-through contained peptides with a Mw < 10 kDa that were subsequently removed from a Resource column (GE Healthcare) with 90% acetonitrile and 0.1% TFA. The resulting mixture of polypeptides was frozen and lyophilized to obtain a dry powder. An ~11 mg/mL solution was prepared by dissolving the powder in 97% D2O buffer (25 mM KH2PO4, 50 mM NaCl), and its pD was adjusted to ~10 with NaOH to ensure rapid hydrogen exchange.

NMR Spectroscopy. NMR experiments were conducted in an 11.7 T Magnex magnet (Abingdon, Oxfordshire, U.K.) run by a Varian iNova console (Palo Alto, CA) and equipped with a QNP Bruker (Karlsruhe, Germany) 10 mm probe. NMR experiments were triggered upon dissolution and injection of the hyperpolarized water sample into the NMR tubes waiting with their samples inside the magnet bore. All NMR data were processed using Matlab software (The MathWorks, Natick, MA) using an exponential decay as a line-broadening function, and when needed peaks were fitted as Lorentzians using Dmfit (The Comfit Consortium, Orleans, France).28 29

3. RESULTS

Hyperpolarizing Water. Dissolution DNP studies have shown that water samples containing 10–40 mM of a nitroxide radical mixed with the appropriate proportion of glassing agent can be efficiently polarized when irradiated by microwaves at T ≤ 1.5 K in high magnetic fields.18,29,30 Figure 1 illustrates the build-up behavior for this microwave-driven water polarization, as measured by the liquid state enhancement observed after
dissolving a sample polarized in a 3.35T Hypersense, with 3 mL D2O. This curve evidence a 10 ± 2 min characteristic buildup time for the solid-state polarization; in terms of the achievable postdissolution enhancement, such optimized hyperpolarization conditions led to signals decaying by ≈1000-fold as they reach thermal equilibrium in the 11.7 T NMR used in this study (Figure 1, inset).

Although very promising, such enhancement figures are deceptively high. Comparisons between a hyperpolarized and a thermal signal measure relative enhancements but ignore the 1H signal reduction due to the dilution of the hyperpolarized water with the glassing agent needed for an effective cryogenic DNP process, or the substantial dilution with nonpolarized solvent that the hyperpolarized sample undergoes upon melting and flushing it across the two magnets. To address the first of these concerns, we used glycerol as water’s coglassing agent. Glycerol was chosen over other possible cosolvents, given this compound’s relatively high concentration of exchangeable protons. These will be polarized as well by the solid DNP process and eventually contribute to the pool of exchangeable protons whose hyperpolarization one aims to transfer to the biomolecule. At a 3:2 water/glycerol v/v ratio, the ensuing sample polarized efficiently and still delivered ∼76% of the exchangeable protons expected from a pure water counterpart.

To address the second concern, we attempted to decrease the dilution factor by increasing the volume of hyperpolarized sample without a concomitant increase in the volume of the dissolution solvent. While the water’s dilution could be reduced by a factor of ∼10 in this fashion, this came at the cost of severely reducing the T1 of the hyperpolarized water. This penalty reflects the fact that all efforts aimed at reducing a pellet’s dilution will de facto increase the nitroxide’s postdissolution concentration; because this radical efficiently polarizes the protons but is also an effective water T1 relaxation agent, particularly in the low magnetic fields experienced by H2O during its transfer from the polarizer to the NMR magnet, the net hyperpolarization achievable from these reduced-volume solutions actually drops. To reduce water’s post-DNP dilution without decreasing the T1 of the hyperpolarized 1H, a number of alternatives were tested. The most

Figure 1. DNP-enhanced 1H signal buildup observed for water as a function of the polarization time under cryogenic conditions. The experimental points arise from independent dissolution experiments, where the water signal enhancement was compared with the thermal counterpart after returning to equilibrium. Samples consisted of 30 μL of H2O/glycerol 3:2 (v/v) hyperpolarized at 1.5 K and 94.1 GHz using 25 mM TEMPO as polarizing agent and were subsequently dissolved with 3 mL of D2O. Comparison of the resulting data to its thermal counterpart (inset) indicates a plateauing 1H polarization under these conditions of (3.9 ± 0.3)% and a buildup time constant of (10 ± 2) min. Alternative polarization and dissolution conditions (cf. Figure 2) can elevate the former figure beyond 5%.

Figure 2. Improving water’s hyperpolarized signal by codissolution with heptane. (a) Water signal evolution following hyperpolarization of a 150 μL 3:2 (v/v) mixture of H2O/glycerol with 25 mM TEMPO and dissolution in either 3 mL at ~35 °C D2O (top) or in a mixture of 1.5 mL of D2O and 3 mL of heptane with transport and measurement at ca. 50 °C (bottom). The relaxation time T1 of the water resonance is extended from 3.6 (top) to 18.2 s (bottom), and the absolute enhancement at t = 0 is increased by a factor of 4.5. (b) Comparison between the hyperpolarized 1D 1H NMR arising from the D2O/heptane dissolution 15 s after it has reached the NMR magnet and a thermal spectrum of the same sample. All spectra were obtained by acquiring 28 k complex data points using a small (~1°) flip-angle pulse excitation and a carrier frequency set to 2.9 ppm; time zero corresponds to the conclusion of the sample flushing from the DNP polarizer.
efficient among these ended up being the combined use of immiscible organic and aqueous solvents to melt and transfer the hyperpolarized water pellet. This method reduces the dilution factor because of the phase separation that the immiscible organic solvent will undergo after the sample is transferred, as it settles outside the NMR observation coil region. At the same time, a suitable organic phase can extract the organic copolarizing TEMPO radical over the course of the sample-transfer process, thereby decreasing the aqueous phase relaxivity. Heptane was found as a useful cosolvent for achieving these dual goals without introducing substantial susceptibility-derived distortions in the ensuing lineshapes. Typically, 150 μL of hyperpolarized water samples were thus dissolved and transferred with a 1.5/3 mL mix of water/heptane, leading to a net dilution factor of ~8. Further reductions in the aqueous phase dissolution volumes did not significantly reduce the hyperpolarized pellet’s dilution factor.

Figure 3. Calculations of the relative $^1$H magnetization enhancements of protons $H_e$ due to exchange with hyperpolarized water protons. Unless otherwise stated, the relaxation of water was kept constant at $T_1^{H_2O} = 10$ s, and the initial relative enhancement of water was $\langle H_2O \rangle_z(0)/\langle H_2O \rangle_z(Th) = 1000$. (a) Enhancement as a function of time since the hyperpolarized (HP) water injection for the different exchange rate ($k_{ex}$) values indicated in the bottom panel. Calculations are given for three different values of $T_1(H_e)$ (top, middle, and bottom panels); the dashed orange line in all panels represents the decay of the water polarization with time. (b) Enhancement achievable by $H_e$ as a function of $k_{ex}$ calculated assuming that: a single scan was measured by a 90° pulse at an optimal time after injection of hyperpolarized water (blue lines), that thermal signal averaging was performed over the course of 10 h with optimal conditions (i.e., with 90° pulses and recycle delays given by $k_{ex}$ and not solely by $T_1(H_e)$; red lines), or that multiple hyperpolarized scans were done on $H_e$ at optimum times assuming minimal TR of 100 ms (cyan lines; in this latter case, we display the sum of signals collected with 90° pulses divided by square root of the number of scans). Other parameters are the same as in panel a. (c) The effect of $T_1(H_2O)$ on the $H_e$ enhancement, shown for an optimized single scan acquisition (top), or for multiple scans seeking maximum SNR as a function of $k_{ex}$. $T_1(H_e) = 2$ s, and the $T_1(H_2O)$ is varied 5, 10, and 15 s (red, cyan, and green lines, respectively).
yet these lead to negligible enhancements, and their use was
thus discontinued. Table 1 in the Supporting Information gives
further quantitative data on how each of the processes
described in this paragraph assisted in achieving an enhanced
water hyperpolarization at the NMR probe position.

The outcome of these efforts is summarized by the
postdissolution traces in Figure 2. This compares results
obtained for a dissolution employing solely D$_2$O, with those
stemming from a joint D$_2$O/heptane dissolution mix
obtained for a dissolution employing solely D$_2$O, with those
postdissolution traces in Figure 2. This compares results
steps are clearly evidenced; unfortunately, so are the signi-
al longer-lasting enhancements a
incorporating heating of the transfer line. The stronger,
corresponding peaks. (b) Peak intensities arising from the experimental time course, together with
to the indicated relaxation times
characteristic decay
Figure 4.
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ficiently slow in the NMR time
scale to give distinct peaks in the ensuing
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spectrum, and at
the same time sufficiently fast to accommodate significant gains
for the above-mentioned hyperpolarized water
T
1
times. Because the ultimate goal is to exploit these exchange processes
in biomolecules with significantly shorter
T
1
values than those
of the hyperpolarized water, the investigated paradigm explored
the gains in polarization achieved by biomolecules that were
waiting in the NMR magnet/probe and exchanged their labile
protons with those of water that was suddenly injected
following dissolution DNP. This approach would have the
advantage that during the transfer process the polarization will
decay with the longer
T
1
of the water protons, and significant polarizations could be imparted even on species with short
proton
T
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values. To investigate under what conditions this
approach would be beneficial, basic calculations were
performed on the extent by which protons
H
ex
that are initially
thermally polarized will enhance their
z-magnetizations
H
ex
by chemical exchange with hyperpolarized water. Assuming that
the injected water hyperpolarization is much higher than its
thermal
Th counterpart (i.e., that
H
32
H
2
(Th) ≫
H
2
ex
(Th)) and that
H
1
H
2

and of approximately
10× obtained at 1.5 T by liquid state
continuous-flow DNP.

Sensitivity Enhancement of Exchangeable Protons in
Small Biomolecules. With these gains at hand, the use of
DNP-enhanced water protons toward the magnification of
NMR signals arising from labile biomolecular protons was
explored. To this end, we targeted protons possessing solvent
exchange rates
k
ex
that are sufficiently slow in the NMR time
dscale to give distinct peaks in the ensuing
H
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spectrum, and at
the same time sufficiently fast to accommodate significant gains
for the above-mentioned hyperpolarized water
T
1
times.

Figure 4. Transferring water hyperpolarization to the resonance of arginine’s exchangeable protons. (a) Progression of arginine’s
H
1
NMR spectrum upon sudden dissolution of hyperpolarized water into 3 mL of a 1 M arginine sample (pD ∼3) dissolved in D$_2$O and waiting in the 500 MHz
spectrometer used to collect the data. Each trace involved the acquisition of 4k complex points, arising from a small flip-angle (∼1°) excitation
(carrier at 7.3 ppm) with a 0.25 s TR. The different types of protons in the arginine sample (inset: molecular formula) are indicated above their corresponding peaks. (b) Peak intensities arising from the experimental time course, together with fits to eq 2 for each arginine site (solid lines), lead
to the indicated relaxation times
T
1
and exchange rates
k
ex.
These fits revealed an initial water polarization enhancement of (438 ± 3)× and a characteristic decay
T
1
(H
2
O) of 10.9 ± 0.1 s; the ensuing decay curve is presented in the Figure as green dots.

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follow from modified Bloch–McConnell equations and predict a time-dependent exchangeable proton magnetization:

\[
\langle H_{ex}(t) \rangle(t) \approx \langle H_{ex}(0) \rangle \cdot \frac{k_{ex}}{k_{ex} + \frac{1}{T_{1H}} - \frac{1}{T_{1NOE}}} \cdot e^{-\frac{t}{T_{1NOE}}} \left( 1 - \exp \left( -\left( k_{ex} + \frac{1}{T_{1H}} - \frac{1}{T_{1NOE}} \right) t \right) \right)
\]

where the \( T_1 \) denotes the spin–lattice relaxation delay of the species and \( k_{ex} \) is their mutual exchange rate.

Plots of this equation for a variety of conditions are given in Figure 3a. These show that the maximum magnetization achievable by the exchangeable protons will be relatively insensitive to \( T_1^{\text{H}_2\text{O}} \) or even to the water \( T_1 \), but will be sensitive to the \( T_1^{\text{H}_2\text{O}} - k_{ex} \) product. Importantly, not only can high levels of single-shot polarization be achieved in this manner for \( H_{ex} \) relative to the polarization arriving with the water protons, but by applying selective pulses on the exchangeable proton sites, the water polarization can be preserved and multiple scans with enhanced signal can be acquired from a single dissolution. For a \( \theta = 90^\circ \) selective pulsing taking place at a constant repetition time \( TR \), the polarization contributions to the exchangeable proton signals will be described by

\[
\langle H_{ex}(t, TR) \rangle \approx \langle H_{ex}(0) \rangle \cdot \frac{k_{ex}}{k_{ex} + \frac{1}{T_{1H}} - \frac{1}{T_{1NOE}}} \cdot e^{-\frac{t}{T_{1NOE}}} \left( 1 - \exp \left( -\left( k_{ex} + \frac{1}{T_{1H}} - \frac{1}{T_{1NOE}} \right) TR \right) \right)
\]

As shown in Figure 3b for a variety of instances, substantial sensitivity increases can then be obtained for the exchangeable protons. This can also be important if attempting to acquire multidimensional spectra or to follow a dynamic process. It follows as well from the last expression that although an increase in the water \( T_1 \) leads to only a slight increase in the initial \( H_{ex} \) magnetization, the achievable polarization enhancement of the exchangeable sites in a multiscan experiment can be significantly increased by prolonging \( T_1^{\text{H}_2\text{O}} \) (Figure 3c).

With these expectations as background, Figure 4 illustrates the gains that this procedure afforded when applied to a small molecule possessing multiple exchanging sites. This compound exhibits different \( \text{H}_2\text{O} \leftrightarrow \text{HN}^- \) exchange rates \( k_{ex} \) for the nonequivalent \( \text{NH} \) and \( \text{NH}_2 \) groups in the molecule, with strong \( \text{pH} \) and temperature dependencies. The polarization buildup is thus different for each group but is in all cases significant. A train of acquisitions following a water-based dissolution DNP experiment allows one to obtain insight into the rates of hydrogen exchange of these sites with the solvent (Figure 4a,b).

**Heteronuclei Signal Enhancement.** Interestingly, not only can exchangeable protons be polarized but also heteronuclei directly bound to such exchangeable protons are spontaneously polarized by injection of hyperpolarized water. This is illustrated in Figure 5a, which demonstrates how polarization from DNP-enhanced water \( ^1\text{H} \) migrates to urea’s \( ^{15}\text{N} \) without the need for any \( ^1\text{H} \) pulsing. A train of low flip angle pulses on the \( ^{15}\text{N} \) channel evidences the slow buildup of urea’s \( ^{15}\text{N} \) polarization, reaching a maximum at \( \sim 40 \text{ s} \). The decay of this polarization is also slow, reflecting a \( T_1^{\text{N}} \) that for urea in a partially deuterated solution like the one arising in this case is on the order of minutes. A number of factors are involved in this buildup/decay function, including the rate of amide/water \( ^1\text{H} \) exchange \( k_{ex} \), the rate of \( ^1\text{H}^{\rightarrow^{15}\text{N}} \) cross-relaxation \( k_{\text{NOE}} \), and the rate of polarization decay given by the \( ^1\text{H} \) \( T_1 \) values of the water and urea sites as well as by the \( ^{15}\text{N} \)’s own \( T_1 \). Three-site exchange simulations (Supporting Information) show that the magnitude of the \( ^{15}\text{N} \) enhancement will depend in a complex fashion on these multiple factors. Still, fits of the experimental data based on this model reveal that the heteronuclear Overhauser transfer \( k_{\text{NOE}} \) is the rate-determining step of this \( ^1\text{H} \) \( T_{\text{water}} \) \( \frac{k_{ex}}{k_{\text{NOE}}} ^1\text{H}_{\text{ex}} \) \( ^{15}\text{N} \) polarization transfer process. With this knowledge at hand, one can propose a
Figure 6. Enhancement vis-à-vis thermal counterparts of the 15N signals of 15N-alanine (a) and of natural abundance arginine (b,c) by polarization transfers from hyperpolarized water. The optimal delay in each case was extracted from simulations of the kind given in Figure 5. Hyperpolarized 15N NMR spectra in panels a and b were detected in single-scan experiments using a 90° 15N pulse, applied 43 and 18 s after the injection of the water, respectively. Thermal acquisitions took ca. (a) 2 and (b,c) 14 h, respectively. (c) Sum of the first eight scans collected after injection of hyperpolarized water to an arginine sample, over a total time of 24 s. An effective average enhancement ≥500X is observed. All measurements were done at ∼50 °C under conditions akin to those in Figure 5. Notice that the NH of arginine has significantly lower enhancement than the other two peaks due to its slow kex at pH ~3.

A simpler two-site model whereby the observable 15N magnetization only arises from a 1H reservoir made available by the DNP experiment. Because this is left unperturbed apart from its relaxation back to equilibrium, the 15N polarization's time evolution can be described by:

\[
\frac{d}{dt} \langle N_z(t) \rangle = \left[ \frac{1}{T_1} - \frac{1}{T_{1e}^N} \right] \langle H_z(t) \rangle \langle N_z(t) \rangle
\]

where \( k_{1H\rightarrow15N} \) summarizes the average effects of the H2O→15N process and \( T_{1e}^N \) is a decay time factoring both the natural 1H and the depleting effects of the pulses used to interrogate the signal.

The enveloping line in Figure 5 shows a fit of this simplified model to traces arising from this kind of experiment, leading to an effective rate \( k_{1H\rightarrow15N} = 0.29 ± 0.02 \) s\(^{-1}\) and times \( T_{1e}^N \approx 89.2 \) s and \( T_{1H} \approx 16 \) s. By setting \( d/dt[\langle N_z(t) \rangle]_{t=0} \) to zero, this model also lets us find the approximate time leading to the maximal 15N enhancement: \( t_{max} = 34 \) s. For an initial degree of maximal 1H polarization injected in the reservoir, the solution of eq 3 also predicts a maximum achievable 15N polarization that from the parameters fitted in Figure 5 should be ~344X, close to the experimentally observed value of 320X (Figure 5, inset).

Figure 6 illustrates an application of this strategy to the enhancement of 15N sites in alanine and arginine. For alanine, a similar analysis as the one just described suggests a maximal 15N sensitivity enhancement ca. 40 s after sample injection, although with a polarization enhancement of ~180X. A similar experiment on a 2.1 M D2O solution of natural abundance arginine at pD ∼3 shows a maximum enhancement at ~20 s, with the 15NH3 and 15NH2 sites showing ~360X and ~280X levels of enhancement, respectively. Much lower enhancements (~50X) are observed for the NH site due to its slower rate of hydrogen exchange. It is noteworthy that because the hydrogen exchange with water for the former two arginine sites is fairly rapid, it is not necessary to wait a relatively long \( T_{1H} \) delay to obtain the optimum enhancement: multiple scans collected at times ~\( (k_{ex})^{-1} \) lead to significantly enhanced signals that can be averaged over several repeated scans (Figure 6c). A similar approach could prove to be useful in the acquisition of Hadamard-encoded or sparsely sampled 2D NMR spectra.

To investigate whether these initial observations can be extended to larger biomolecules, we applied the heteronuclear transfer experiment on a lysate of 15N-labeled aldehyde reductase. Trypsin-based lysis reduced the original 40 kDa Mw of this well-folded protein into an array of peptides of mostly Mw ~3 kDa, with some 5% reaching up to the 10 kDa molecular weight cutoff used. It can be assumed that the peptides in this mixture do not contain residual structure and that their chains are fully extended. These conditions should favor a rapid exchange of their amine and amide NHs with the hyperpolarized water protons. To examine what kind of effective 15N signal enhancement this could lead to, we collected a series of 15N NMR spectra using 90° excitation pulses following the injection of hyperpolarized water. These results are shown in Figure 7a and confirm a sensitivity enhancement of both backbone amide and side-chain amine resonances. The build-up of these signals is relatively rapid, as expected for the high kex rates characterizing these unfolded peptides. The apparent decay of the signal enhancement by contrast, 20 ± 1 s, is much slower than the overall relaxation of the backbone amides, whose global \( T_{1H} \) is 1.8 ± 0.8 s. This relatively slow decay reflects the \( T_{1H} \) of the hyperpolarized water protons, which supports the 15N repolarization process between consecutive 15N scans. These long lifetimes allow one to achieve an 15N enhancement beyond what would be possible with a single acquisition; comparing a sum of scans collected over a 25 s period (Figure 7b, upper trace) against a thermal equilibrium 15N spectrum (Figure 7b, middle trace) indicates that most peaks in the amide backbone region can be enhanced in this multiscan fashion by >500X. A similar enhancement characterizes 15N sites in the NH3 region as well as arginine’s guanidine 15N sites in the lysate. The only amide nitrogens that do not appear enhanced are those belonging to proline groups, owing to their lack of exchangeable protons.

4. DISCUSSION AND CONCLUSIONS

Bringing the benefits of DNP to bear onto the study of biomolecules in solution is an important challenge in contemporary NMR. The present work investigated a way of bypassing the \( T_{1H} \) bottleneck that slowly tumbling biomacro-
Enhancement of 15N signals in a 15N-labeled polypeptide lysate with molecular weight cutoff of 10 kDa via polarization transfer from hyperpolarized water. (a) 15N NMR spectra arising from 90° 15N pulses applied over the indicated postdissolution times, reflecting the 17.9 s T1 we detect for the water protons onto the 15N signals enhanced by the exchangeable protons. The sum of six consecutive scans from this time series is displayed. (b) Sum of first 15 scans (upper trace) following the injection of hyperpolarized water compared versus a thermal equilibrium 15N spectrum (middle trace), measured by signal-averaging 10 000 scans over the course of ca. 40 h. The difference between these spectra is displayed in the bottom trace, highlighting the over-enhancement of the arginine side chains (NH2) and lysine’s (NH3+) groups and the under-enhancement of the protonless proline backbone nitrogens. The average signal enhancement of the 15N backbone amides is >500× relative to 15N thermal equilibrium signal. Other acquisition parameters are as in Figure 5.

Even with these limitations, an interesting aspect of the examined approach lies in its ability to spontaneously enhance the resonances of 15N attached to labile protons by factors in the 100–1000× range. These results are particularly promising for 15N sites that undergo rapid 1H exchange, for example, lysine side chains and amide positions in unstructured proteins at high pH; these sites cannot be efficiently enhanced by INEPT-like sequences, while thermal equilibrium 1H→15N NOE methods are inefficient in macromolecules. The spontaneous nature of the transfer is also promising for human-oriented NMR imaging setups, which are rarely equipped with full double-resonance irradiation capabilities. It is conceivable, however, that a more active INEPT-like transfer might be more effective for N–H sites undergoing intermediate proton exchange than the spontaneous transfer assayed in this study. We have carried out such tests, but preliminary results indicate that this strategy is challenged if attempting to leave the radiation-broadened reservoir of hyperpolarized H2O untouched for the sake of performing multiple 15N acquisitions. Further efforts aimed at clarifying these issues are ongoing.

The enhanced biomolecular sensitivity experiments demonstrated in this work were carried out on intrinsically unfolded systems liable to fast hydrogen exchange of their backbone protons. Additional potential targets could include structured polypeptides that are kept artificially unfolded in the NMR tube where their measurement will take place, until the arrival of hyperpolarized water triggers their sudden folding. Even in folded systems, sensitivity gains should arise from water-accessible side chains whose protons are rapidly exchanging with those of the hyperpolarized water. A different kind of experimental window that might be opened by the hyperpolarized experiments hereby described, involves measuring the rates of water exchange or water accessibility in biomolecules.38

Two kinds of water–proton exchange experiments are commonly used, depending on the range of exchange rates to be accessed. Slower exchange processes are usually determined by isotope dilution methods, whereby the volumes of proton peaks in proteins whose exchangeable sites were fully deuterated are monitored in real time as the sample is diluted by fully protonated water (or conversely, whereby peak decays are quantified as a fully protonated protein is diluted in deuterated water).39,40 Another method, better suited for studying more rapid exchange processes, relies on observing the decrease in the intensities of the labile peaks upon solvent water saturation/inversion. In this method, the signal of the individual exchangeable proton sites will depend on kex as well as on the site’s T1 value, and hence these experiments are limited to kex on the order of the site’s T1 (i.e., kex ≈ 1 s−1).41 Studying hydrogen exchange processes using the hyperpolarized water principles described in this paper has many features that complement both of these methods, both due to its real-time nature and by virtue of the various time scales that the hyperpolarization lifetime enables one to probe. In particular, the fact that a high signal contrast is not governed solely by the intrinsic T1 of the exchanging sites but rather by T1(H2O) (Figure 3C, bottom) means that it should be possible to characterize slower rates of kex than in conventional magnetization transfer methods. This research avenue is currently being investigated.
Summary of dilution factors, postdissolution polarizations, and experimental T1 values observed for different volume and dissolution conditions. Modeling of the heteronuclear signal enhancement spontaneously derived from hyperpolarized H2O injections. This material is available free of charge via the Internet at http://pubs.acs.org.

We are grateful to K. Zibrener for assistance with the experiments and to Dr. Shira Albeck (ISPC, Weizmann Institute) for the preparation of the reductase lysate. Financial support from ERC Advanced Grant #246754, EU’s BioNMR Grant #261863, DIP Project 710907 (Ministry of Education and Research, Germany), the Clore Foundation and the generosity of the Perlman Family Foundation, are also acknowledged.

The authors declare no competing financial interest.

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**ACKNOWLEDGMENTS**

The authors acknowledge the support from ERC Advanced Grant #246754, EU’s BioNMR Grant #261863, DIP Project 710907 (Ministry of Education and Research, Germany), the Clore Foundation and the generosity of the Perlman Family Foundation, are also acknowledged.

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