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Heteronuclear Cross-Relaxation under Solid-State Dynamic Nuclear Polarization

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Supporting Information

ABSTRACT: We report on the spontaneous polarization transfer from dynamically hyperpolarized ^{1}H to ^{13}C during magic-angle spinning dynamic nuclear polarization (DNP) at temperatures around 100 K. The transfer is mediated by ^{1}H−^{13}C cross-relaxation within methyl groups due to reorientation dynamics, and results in an inverted ^{13}C NMR signal of enhanced amplitude. Further spreading of transferred polarization can then occur via ^{13}C−^{13}C spin-diffusion. The resulting process is equal to the nuclear Overhauser effect (NOE) where typically continuous saturation of ^{1}H by radio frequency irradiation is employed. Here, hyperpolarization by irradiation with microwaves in the presence of typical bis-nitroxide polarizing agents is utilized for steady-state displacement of ^{1}H polarization from thermal equilibrium and perpetual spin-lattice relaxation. An effective ^{13}C enhancement factor of up to −15 has been measured. Presence of Gd(III) furthermore amplifies the effect likely by accelerated relaxation of ^{1}H. We provide experimental evidence for the proposed mechanism and show that DNP-induced cross-relaxation is a robust feature within proteins and single amino acids and discuss potential applications.

The temperature-activated dynamics of peptide side chain functional groups play an important role in dynamic nuclear polarization (DNP) enhanced magic-angle spinning (MAS) NMR spectroscopy, affecting observability and line width of respective side chain resonances. While many aromatic groups show 2-fold symmetric flips which interfere with NMR detection at room temperature but not at typical DNP temperatures of ∼100 K, 3-fold symmetric methyl group hops are fast at room temperature,[1,2] which results in intrinsically narrow ^{13}C resonances.[3] Under typical sample cooling employed in DNP, this hopping approaches an intermediate-exchange regime and often leads to broad resonances and fast relaxation.[4] This situation, however, can give rise to interesting phenomena such as heteronuclear cross-relaxation (CR) mediated polarization transfer in the solid-state.

As we will show, CR occurs between hyperpolarized ^{1}H and ^{13}C and is explained in mechanism by the nuclear Overhauser effect (NOE),[5] which has also been shown to occur in solids,[6] and especially in methyl-bearing amino acids under MAS.[7] A similar effect but occurring under substantially different experimental conditions has been demonstrated by Donovan et al. during the free return to thermal equilibrium after dissolution DNP.[8] In our case, a large deviation from equilibrium is actively maintained by dynamic hyperpolarization of ^{1}H, which perpetually drives spin−lattice relaxation. This is equivalent to the NOE, except radio frequency saturation of ^{1}H is substituted by DNP.

In a simple system constituent of ^{1}H and dipolar-coupled ^{13}C the Solomon equations can be evoked to describe this effect in the steady-state solution:[9]

$$\varepsilon_{CR} = 1 - (\varepsilon_H - 1)\frac{\gamma_H}{\gamma_C}\frac{\sigma_{HC}}{\rho_C} \quad (1)$$

Here, ε_H is the enhancement factor or polarization relative to thermal equilibrium of ^{1}H whereas ε_{CR} is the ^{13}C enhancement inflicted by CR. γ_H and γ_C are the respective gyromagnetic ratios. ρ_C and σ_{HC} are the ^{13}C auto- and ^{1}H−^{13}C cross-relaxation rates, respectively. Equation 1 is a direct result of the Solomon equations (see SI). Saturation of ^{1}H ($\varepsilon_H = 0$) leads to ^{13}C enhancement by NOE; subsequently, hyperpolarization of ^{1}H by DNP ($\varepsilon_H > 1$) would cause a net ^{13}C enhancement of opposite sign by CR.

The CR terms require significant imbalance between zero quantum and double quantum relaxation rates.[9] This can, for example, be provided by the above-mentioned methyl reorientation which features correlation times in the ns-μs range at DNP-relevant temperatures.[10−12] Furthermore, the presence of an additional paramagnetic species accelerating overall relaxation rates could amplify CR due to stochastic spin-flips of the paramagnet in the μs time scale.[13] This leads to a relaxation increase mainly of the strongly correlated ^{1}H,[14] concomitantly favoring CR over ^{13}C autorelaxation rates and potentially increasing steady-state CR enhancement.

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Here, we report on the inversion and enhancement of ^{13}C resonances by CR under typical ^{1}H-DNP conditions at a magnetic field of 9.4 T (400 MHz ^{1}H, 263 GHz e^{-}) when a bis-nitroxide polarizing agent (PA) such as TOTAPOL[15] or AMUPol[16] is used. We have observed the effect for the first time in a uniformly ^{15}N,^{13}C-labeled interleukin-1β protein where an encodable lanthanide binding tag (LBT)[17] had been introduced into the R-loop region (U−^{15}N,^{13}C-IL1β-R2). Using TOTAPOL, DNP enhancement of ^{1}H and subsequent $^{1}H-^{13}C$ cross-polarization (CP) yielded typical enhancement factors of $\varepsilon \approx 63$ for IL1β-R2 loaded with diamagnetic Lu^{3+} (f^{14}, $S = 0$). Loading with paramagnetic Gd^{3+} (f^{7}, $S = 7/2$) reduced this factor to ∼44 due to accelerated relaxation of ^{1}H (Figure 1). When the direct polarization (DP) of ^{13}C was read-out

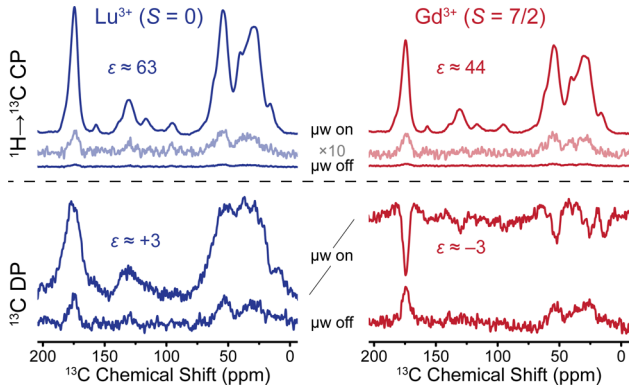

Figure 1. $^{1}H-^{13}C$ CP (top) and ^{13}C DP (bottom) spectra of U−^{15}N,^{13}C-IL1β-R2 loaded with 1 equiv of Lu^{3+} (left) or Gd^{3+} (right) with (μw on) and without (μw off) μw irradiation of 10 mM TOTAPOL. CP and DP spectra were recorded after 3 and 30 s polarization time, respectively. For the CP off-spectra, a multiplication by a factor 10 is also shown (middle trace).

using Bloch-decay, we observed strikingly different behavior between the diamagnetic protein and its paramagnetic counterpart. In both cases, the non-DNP-enhanced spectra are of similar intensity and line width. In the presence of Lu^{3+}, we observed the emergence of a broadened and positively (+)-enhanced spectrum under microwave (μw) irradiation with a small ε of ∼3. For direct ^{13}C-DNP, a close electron−nuclear dipolar contact between TOTAPOL and protein has to be present because spin-diffusion through the ^{13}C-depleted solvent is inefficient.[18] Only a small fraction of proteins are in direct proximity of a PA molecule; therefore, we witness a weighted contribution of these proteins experiencing direct ^{13}C-DNP but suffering from strong paramagnetic broadening at the same time. For Gd^{3+}, however, the broad and (+)-enhanced spectral species is less pronounced; in contrast, the emergence of an additional emissive (negatively) (−)-enhanced species with narrower lines (similar to the CP spectrum) is observed. Because of the superposition of these two species, an enhancement factor of ∼ −3 can only be given as an estimate. Upon closer analysis we also found a (−)-enhanced species in the presence of Lu^{3+} (Figure 2). Here, the contribution of the (−)-enhanced species is significantly smaller than what we observed in the Gd^{3+}-DP spectra: with increasing polarization times a dip in the region of C′ and CH_3 resonances emerged for Lu^{3+}, whereas for Gd^{3+} almost a full inversion of the complete spectrum occurred (Figure 2C). The amplitude of the inversion in the presence of Lu^{3+} is small (Figure 2B) and does

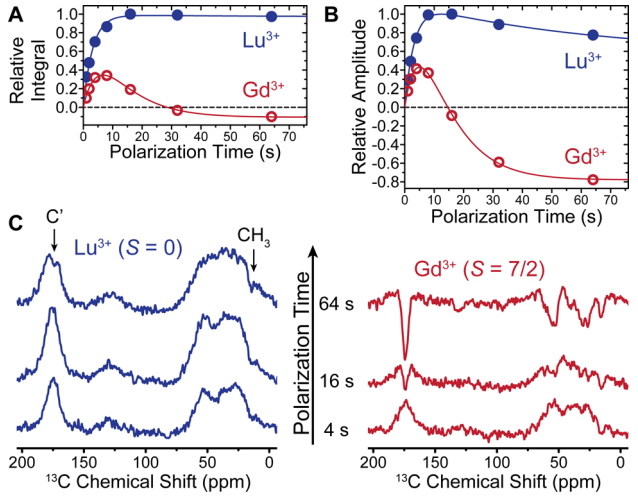

Figure 2. (A) Area under the C′ peak (integrated from 150 to 200 ppm) and (B) peak intensity of C′ resonance envelope after different polarization times measured on U−^{15}N,^{13}C-IL1β-R2 loaded with 1 equiv of Lu^{3+} (left, blue) or Gd^{3+} (right, red). (C) ^{13}C DP NMR spectra enhanced by 10 mM TOTAPOL.

not significantly influence the area under the C′-peak (Figure 2A). In the following, we will present evidence, that the reduction or inversion of ^{13}C polarization is generated by CR from dynamically hyperpolarized ^{1}H of methyl groups which are perpetually undergoing relaxation during the DP build-up period.

We have also observed the same behavior using AMUPol instead of TOTAPOL, excluding a specific PA effect. Furthermore, the presence of a lanthanide ion with large spin−orbit coupling such as Dy^{3+} (f^{9}, $J = 15/2$) does not lead to stronger inversion relative to diamagnetic Lu^{3+}, but overall signal broadening and concomitantly reduced intensity (Figure S1). Dy^{3+} has the propensity to induce paramagnetic shifts without causing significant longitudinal PRE; in contrast, Gd^{3+} shows negligible spin−orbit coupling and causes mainly longitudinal PRE.[19−21] We also recently reported on similar observations of inverse ^{13}C enhancement when ^{1}H is selectively hyperpolarized by DNP using Gd-DOTA, even in the absence of methyl groups.[22] Therefore, we assume that Gd^{3+} provides for suitable conditions that accelerate CR under these experimental conditions.

The broad line width of the (+)-enhanced signal and its fast transverse relaxation allowed us to apply a decoherence filter in form of a simple Hahn echo sequence.[23] While the broad component's phase memory is lost after a short refocusing time of 0.5 ms the long-lived coherences of the (−)-enhanced species can be detected with an excellent signal/noise ratio. The resulting emissive spectrum shows a clean baseline without distortion from the broad positive shoulders and closely resembles the CP spectrum after 180° phase shift (Figure 3). The resonances from methyl groups show a much larger enhancement than the other resonances for which $\varepsilon \approx -8$. The methyl enhancement cannot be quantified because no distinct signal can be observed within the shoulder of the alkyl side chains. Therefore, we estimate that ε of methyls is about one order of magnitude larger (Figure S2). Also, methyl inversion occurs already at much earlier times; accordingly, backbone and other side chain ^{13}C show a delayed polarization build-up due to slower spin diffusion from the few fast-inverted methyl groups over the whole protein. This led us to the conclusion

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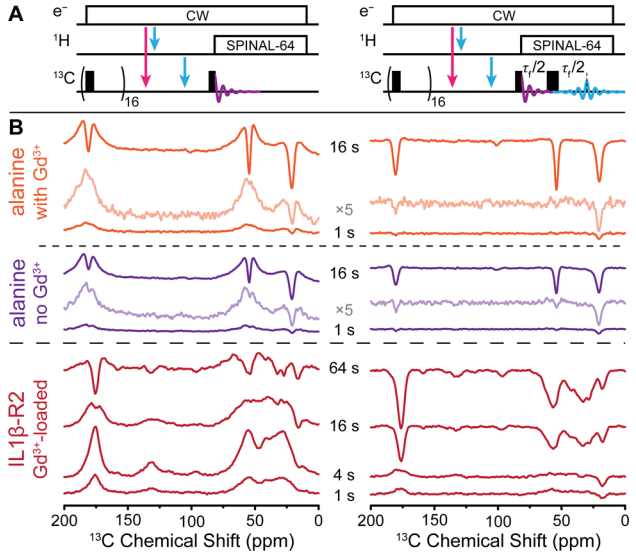

Figure 3. (A) Bloch-decay (left) and echo-filtered ^{13}C DP (right) pulse sequences. Direct ^{13}C-DNP pathway and relayed (indirect) ^{1}H-DNP/CR transfer pathways are indicated with red and blue arrows, respectively. (B) DP spectra of ∼0.4 mM U−^{15}N,^{13}C-IL1β-R2 loaded with Gd^{3+} (bottom, red); 125 mM $^{13}C_3$-L-alanine without (middle, purple) and with 0.4 mM Gd^{3+} (top, orange). Spectra recorded at varying polarization times, indicated in center, with echo-filtered spectra using τ_f = 0.5 ms. Light curves are 5-fold multiplications of shorter polarization times. 10 mM AMUPol was used.

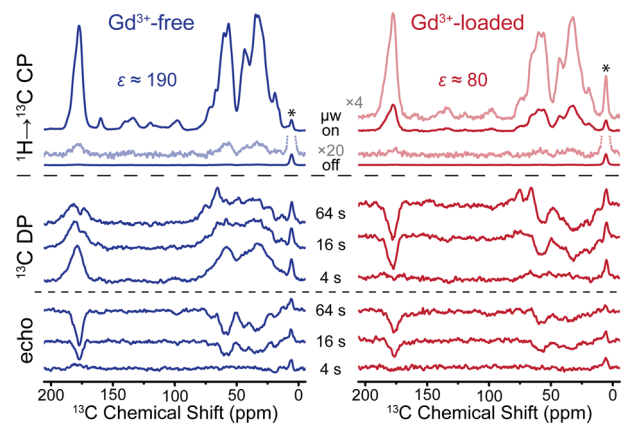

Figure 4. CP (top) and DP (bottom) spectra of U−^{15}N,^{13}C-ubiquitin-G75C-4MMDPA without (left, blue) and loaded with (right, red) Gd^{3+}. 10 mM AMUPol was used. CP off-spectra have also been multiplied by a factor 20, the on-spectrum of Gd^{3+}-loaded protein by a factor 4 (light curves). CP spectra were recorded after 2.5 s polarization time. In the case of DP, Bloch-decay and echo-filtered spectra are shown after varying polarization times given in center. Asterisks mark a signal from silicone plugs used for rotor sealing.

that CR between ^{1}H and ^{13}C of methyls is the most likely polarization transfer mechanism.

To confirm our hypothesis, we tested a small molecule dissolved in a cryoprotecting matrix. We chose $^{13}C_3$-alanine as the smallest amino acid featuring a methyl group. Indeed, we again observed a strong inversion of the DP spectrum on top of a broad (+)-enhanced species; the latter can be filtered out by implementation of a refocusing echo sequence (Figure 3). Quantification of enhancement resulted in factors between $\varepsilon_{CR} \approx -10$ for the C_β methyl resonance and $\varepsilon_{CR} \approx -4$ for C_α and C′. Addition of 0.4 mM Gd(OAc)$_3$ resulted in a ∼20% increase of these factors in magnitude, even though ^{1}H DNP enhancement decreased by a similar fraction from 170 to 140. Naturally, alanine and Gd^{3+} are homogeneously dispersed in this case so that other concentrations of Gd^{3+} might lead to significantly better boosts.

Continuous saturation of ^{1}H during the build-up period by a train of 180° pulses quenches the signal inversion, which proves that indeed ^{1}H polarization is the source of the effect (Figure S3). Furthermore, absence of methyl-^{1}H completely suppresses the inversion as we show with $^{13}C_2$-glycine as well as perdeuterated D_4-$^{13}C_3$-alanine (Figure S4). Alanine in natural isotope abundance showed exclusive inversion of $^{13}C_\beta$ without affecting C′ (Figure S5) because no dipolar pathway exists between $^{13}C_\beta$ and ^{13}C′ on different molecules. The effect occurs irrespective of deuteration or ^{13}C-depletion of the cryoprotectant: using naturally isotope-abundant glycerol we have observed $\varepsilon_{CR} \approx -15$ for alanine-C_β while enhancement close to −10 occurs for C′ and C_α even in the absence of Gd^{3+} (Figure S6). This indicates that larger ^{1}H concentrations lead to higher efficiency of inverted enhancement. This is a promising feature toward DNP in highly protonated environments such as whole cells or cellular mixtures.[24−26]

Another protein system, uniformly ^{15}N,^{13}C-labeled ubiquitin single-site mutant G75C with an attached 4MMDPA chelator tag (U−^{15}N,^{13}C-ubiquitin-G75C-4MMDPA),[22] shows CR-induced signal inversion to a large degree also in the absence of Gd^{3+}. Line widths of the (+)- and (−)-enhanced species are of similar magnitude, therefore partial cancellation of signals is observed at longer build-up periods of 64 s and clear inversion is obtained only after decay of the (+)-enhanced signal in an echo experiment (Figure 4). The addition of Gd^{3+} leads to reduction of the (+)-enhanced signal and inversion efficiency is improved even though ^{1}H-DNP enhancement factor is lowered by a factor of 2−3. Because of the presence of Gd^{3+}, the decoherence time constant is reduced; therefore, the refocusing efficiency is small and a significant amount of (−)-enhanced signal is lost after 0.5 ms. This is caused by the overall closer proximity of nuclear spins to the ion in the relatively small protein in combination with much slower electronic relaxation times of Gd^{3+} as compared to the situation in IL1β-R2. In the latter case, the LBT provides a rather asymmetric coordination sphere that induces large zero-field splitting on the order of ∼2 GHz,[27] which in turn leads to much faster electron spin relaxation.

We have shown that the peculiar reorientation of methyl groups leads to CR between dynamically hyperpolarized ^{1}H and ^{13}C. This leads to inversion and enhancement of ^{13}C polarization of the methyl resonance up to 15-fold. We expect that this factor could easily be increased in a more thorough investigation, which is currently underway in our laboratory. Once built-up on methyl-^{13}C, the (−)-enhanced polarization can easily spread through homonuclear spin-diffusion. The effect is robust and occurs in virtually every sample system containing methyl groups we have investigated so far.

The CR transfer is in competition with direct ^{13}C-DNP enhancement, which leads to broad and (+)-enhanced spectral components. Because this contribution can be potentially large, care has to be taken that direct transfer pathways between PA and analyte (e.g., by interaction between analyte and PA) is avoided. We have observed the effect equally in a matrix of natural isotope abundance as well as depleted in ^{13}C; however,

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the detailed dependence on matrix ^{13}C and ^{1}H concentration still has to be further investigated.

Presence of Gd^{3+} has shown to boost the CR enhancement factor by varying degree. This is most likely caused by general acceleration of ^{1}H relaxation including CR rates. In order to determine the exact role of Gd^{3+} and associated electron spin relaxation properties, further studies are required. Besides methyl groups and paramagnetic ions, other functional groups or molecular environments might also be highly supportive of CR in the solid-state. The analysis of DNP-driven CR would allow for elucidation of local molecular dynamics in the ns−μs range even for larger systems exclusively amenable to solid-state NMR techniques under DNP-enhancement.

We expect that this effect might become a valuable asset in the near future in order to selectively investigate species in dipolar contact with functional groups enabling such CR. For example, biomolecules situated within lipids, such as transmembrane proteins or membrane-integrating peptides, might be investigated by selective dipolar transfer from lipid tail terminating methyl groups. This would allow for detection of contacts between macromolecule and central hydrophobic region of the membrane due to specific enhancement by DNP-driven CR. Another possible scenario could be the selective enhancement of RNA regions binding methyl-carrying molecules, such as an aptamer active site being in dipolar contact with a bound ligand, or contacts between RNA and DNA or ribonucleoproteins (RNPs). Because RNA itself does not contain any methyl groups, this would be an ideal method to identify selectively and unambiguously binding sites and to study structural changes upon such binding. We are currently investigating such approaches in parallel with a detailed study of the effect on model systems and will report on our findings in due time.

Shortly before submission, we became aware that the groups of M. Hoffmann (SUNY Brockport) and G. Buntkowsky (TU Darmstadt) have jointly observed a similar effect.[28]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b08683.

Detailed experimental description and supporting figures (PDF)

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Notes

The authors declare no competing financial interest.

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