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HyperBIRD: A Sensitivity-Enhanced Approach to Collecting Homonuclear-Decoupled Proton NMR Spectra**

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Dedicated to Prof. Alex Pines on the occasion of his 70th birthday

Abstract: *Samples prepared following dissolution dynamic nuclear polarization (DNP) enable the detection of NMR spectra from low-γ nuclei with outstanding sensitivity, yet have limited use for the enhancement of abundant species like ^{1}H nuclei. Small- and intermediate-sized molecules, however, show strong heteronuclear cross-relaxation effects: spontaneous processes with an inherent isotopic selectivity, whereby only the ^{13}C-bonded protons receive a polarization enhancement. These effects are here combined with a recently developed method that delivers homonuclear-decoupled ^{1}H spectra in natural abundance samples based on heteronuclear couplings to these same, ^{13}C-bonded nuclei. This results in the HyperBIRD methodology; a single-shot combination of these two effects that can simultaneously simplify and resolve complex, congested ^{1}H NMR spectra with many overlapping spin multiplets, while achieving 50–100 times sensitivity enhancements over conventional thermal counterparts.*

While dissolution dynamic nuclear polarization (DNP) can deliver sensitivity enhancements upwards of 10000 times for the NMR spectra of slowly relaxing species like ^{13}C and ^{15}N nuclei,[1] there are still inherent limitations that prevent the extension of these methodological benefits to 1D ^{1}H NMR spectroscopy. In particular, the shorter longitudinal relaxation times T_1 of ^{1}H nuclei, challenge the times involved in the melting and shuttling of the sample from the polarizer to the NMR or MRI system. Only when using specialized hardware[2,3] or when targeting special molecules such as water,[4,5] has dissolution DNP been successfully applied to directly hyperpolarize and detect protons in solutions. Here we explore an alternative to bypass these limitations and enable the acquisition of natural abundance ^{1}H NMR spectra enhanced by dissolution DNP, while enjoying the benefits of a simplified "one-site one-peak" format.

The first of the phenomena exploited to achieve this aim, is the spontaneous ^{13}C→^{1}H polarization transfer that can be induced upon hyperpolarizing lower-γ nuclei, and rapidly transferring the sample into an NMR tube at high field as per the standard dissolution DNP protocol. Once injected into the NMR spectrometer, the hyperpolarized nuclei will begin transferring their alignment to their directly bonded protons through a cross-relaxation process.[6,7] When coupled to cross-correlation effects involving shielding and dipole–dipole anisotropies[8,9] the ensuing proton NMR spectrum will display, for each chemical site, an asymmetric "triplet" where all components have similar intensities, despite their very different abundances. The middle of these components arises from protons bonded to the 99%-abundant, NMR-silent ^{12}C isotopes, whereas the external components arise from the 1% of the ^{1}H nuclei that are bonded to ^{13}C nuclei. The splitting of these external components derives from the heteronuclear *J*-coupling, while their 100-fold enhancement derives from the transfer of the carbon hyperpolarization to its directly-bonded protons. This enhancement of the so-called ^{13}C satellites reaches a maximum value about 5–10 seconds after injection, before gradually decaying back to its 0.5% equilibrium intensity value after $5{\cdot}T_1^{C}$ times have elapsed after the injection of the sample. This sensitivity enhancement occurs spontaneously, and does not require any special sequence or pulsing.

This phenomenon is hereby exploited in combination with a second ^{13}C-associated feature, concerning a family of recently developed homonuclear ^{1}H–^{1}H decoupling sequences,[10,11] that use an isotopically selective detection of ^{13}C-bonded protons while suppressing the signals and the couplings arising from ^{12}C-bonded proton resonances.[12] Homonuclear decoupling is achieved by these bilinear-rotation decoupling (BIRD) sequences[13] using a windowed acquisition that operates synchronously with periodic inversions of the ^{12}C-bonded ^{1}H nuclei without altering the ^{13}C-bonded ones, thus effectively removing the ^{1}H–^{1}H *J*-couplings over the course of a continuous ^{1}H signal acquisition.

An optimal matching is possible between these heteronuclear polarization-transfer and BIRD-based homonuclear decoupling protocols. This complementarity rests on the fact that both the transfer and the decoupling processes, rely on aspects of the spin dynamics that arise between a naturally abundant ^{13}C atom, and its directly bonded proton. One of these aspects is the aforementioned enhancement of the

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^{1}H NMR signal that arises from the ^{13}C-bonded protons but not from their ^{12}C counterparts; the other concerns the objective of the BIRD sequence of observing only the ^{13}C-bonded protons, as these are the sole ones that will yield a homo-decoupled ^{1}H NMR signal. We refer to the resulting protocol combining ex situ hyperpolarization and homonuclear decoupling as the HyperBIRD experiment; it is here shown that when applied on prototypical organic systems, HyperBIRD can deliver an enhancement of approximately 50–100 times vis-à-vis its normal thermal counterpart, while incorporating a homonuclear decoupling that simplifies the ^{1}H NMR spectra thus enhanced, and allows one to resolve resonances from otherwise overlapping multiplets.

Figure 1 shows the experimental methodology assayed. First, the ^{13}C nuclei are hyperpolarized at cryogenic conditions (1.4 K). In our study this was done using an Oxford hypersense polarizer (Tubney Woods, UK), which irradiated samples made by co-mixing the targeted compounds with

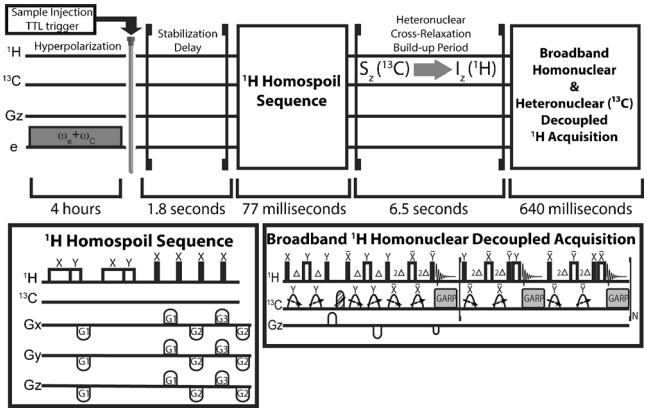

Figure 1. The HyperBIRD experiment. The ^{13}C nuclei in the sample are first hyperpolarized by ex situ DNP. Following this the sample is suddenly melted and injected into the NMR probe, and proton magnetizations are indiscriminately suppressed with a homospoil sequence like the one depicted in the box (in this study low-power pulses with durations of 5.5 and 3 ms were used, all gradients had a 1 ms duration, and gradient strengths were G1 = 30 G cm^{-1}, G2 = −13 G cm^{-1}, and G3 = 21 G cm^{-1}). Following this suppression, ^{13}C hyperpolarization is allowed to transfer spontaneously to the ^{13}C-bound protons for a few seconds, enhancing their signals by nearly two orders of magnitude. The experiment concludes with a single-shot homonuclear decoupled proton acquisition using the sequence described by Lupulescu et al.,[11] whereby ^{13}C spins are used to achieve homonuclear ^{1}H–^{1}H decoupling.

40 mM of a BDPA radical (SigmaAldrich, St. Louis) and with [D_6]DMSO as co-glassing solvent, at an electron and ^{13}C Larmor frequency sum of 94.08 GHz. Irradiation continued for 2–4 h using 100 mW of microwave power; following this the sample was suddenly melted with 4 mL of superheated methanol, and the resulting solution injected into a 5 mm NMR tube positioned in an HCN PFG-XYZ probe inside a 500 MHz spectrometer (Varian Inova, Palo Alto). Following a short sample settling period, any initial proton magnetization was saturated with a homospoil sequence incorporating low- and high-power radiofrequency (RF) pulses, and pulsed field gradients that were empirically optimized to give maximum suppression of all proton resonances and in particular of the ^{12}C-bonded proton ones. This saturation is important as the ^{12}C-bonded protons may also show a sizable enhancement, on top of their naturally more intense signals associated to their 99% abundance, following dissolution; this results from both a 1.5→300 K temperature jump and from a partial DNP enhancement that ^{1}H nuclei may undergo when hyperpolarizing ^{13}Cs. Following their post-dissolution saturation the (unsought) ^{12}C-bonded proton magnetizations will recover to their thermal Zeeman equilibrium values, while the ^{13}C-bonded ^{1}H magnetizations will grow beyond their equilibrium value, owing to the heteronuclear cross-relaxation induced by the hyperpolarized ^{13}C nuclei. This post-saturation process is illustrated in Figure 2: notice that all ^{1}H NMR resonances start being nearly null, but the heteronuclear cross-relaxation process causes the ^{13}C-bonded proton satellites to grow orders-of-magnitude in excess of their natural polarization. As these ^{13}C-coupled spectra show, ^{1}H NMR peaks bonded to ^{13}C nuclei reach a maximum approximately six seconds after sample injection, characterized by highly asymmetric doublets. A quantitative description of these ^{13}C-bonded proton

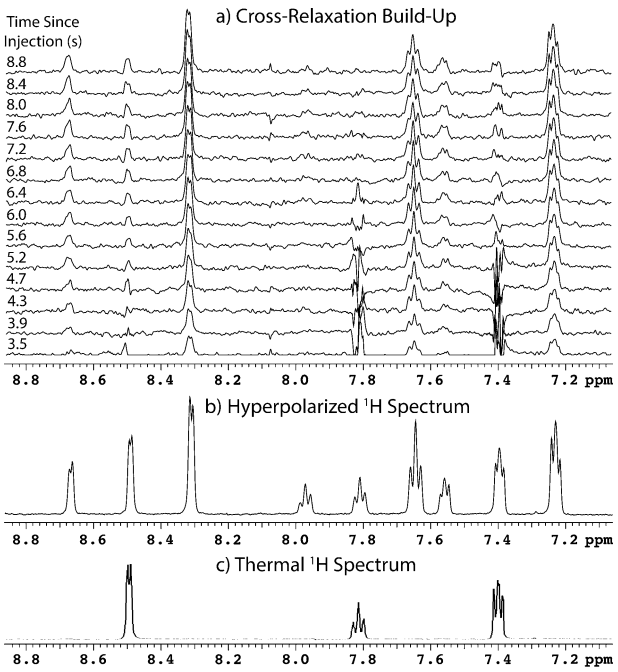

Figure 2. Heteronuclear cross-relaxation build-up affecting ^{13}C-bound protons, illustrated with a series of 1D ^{1}H NMR natural abundance pyridine spectra. a) Data acquired with small flip-angle pulses at the indicated times, following the injection of a sample that was subject to ex situ ^{13}C DNP. All proton peaks are largely suppressed at the beginning of this series by a homospoil sequence (Figure 1); the ^{13}C-bonded proton satellites subsequently grow, spontaneously and asymmetrically, as a result of heteronuclear cross-relaxation effects. b) Trace recorded for a nearly optimal ^{13}C→^{1}H transfer timing, using a 90° excitation pulse. c) Thermal spectrum acquired on the same sample following its return to thermal polarization, illustrating the positions of the ^{12}C-bonded ^{1}H nuclei. The ^{13}C-bonded satellite peaks, separated from the center peaks by $\pm J_{CH}/2$ splittings, are now invisible because of their much lower intensities.

peak trajectories[7] shows that the maximum intensity in the ^{13}C-bonded proton signals as well as the asymmetry of the doublets will be linked to the molecular correlation time, the extent of ^{13}C hyperpolarization, and the shielding anisotropy/dipolar fields contributing to the longitudinal relaxation time T_1. While it is unlikely that this set of parameters would be identical for any two inequivalent proton resonances, a suitable "average" delay can give significant enhancements on all ^{13}C-bound protons in typical organic molecules; for the present study, this spontaneous transfer delay was set to 7 seconds. By this time, the one-shot dissolution process endows the ^{1}H spectrum arising from the ^{13}C-bound protons in small- and medium-sized organic molecules, with a sensitivity enhancement of approximately 100 times vis-à-vis their thermal counterparts; this provides the ^{1}H-based experiment hereby considered with special sensitivity.

The sensitivity enhancement illustrated in Figure 2 for the ^{13}C-bound proton resonances is further exploited in the experimental protocol shown in Figure 1, for the sake of enhancing spectral resolution by ^{1}H–^{1}H homonuclear decoupling in the post-dissolution phase. This procedure exploits the fact that, beyond an enhancement in sensitivity, the presence of a ^{13}C atom can provide its directly bonded proton with a chance of distinguishing it from its surrounding neighbors. Therefore the incorporation of a bilinear rotation decoupling ("BIRD") sequence[13] based on a concatenated train of $(90)_y^{H}-0.5/J_{CH}-(180)_x^{H}$ & $(180)_x^{C}-0.5/J_{CH}-(90)_y^{H}$ pulses interleaved with periods of free evolution and data acquisition, can be used to achieve full decoupling. As further described in Ref. [11], these pulses can be used in a real-time fashion, to periodically impart 180° rotations on the ^{12}C-bound protons while leaving unchanged the ^{13}C-bound ones. One-dimensional signals collected from the latter protons while implementing this multiple-pulse procedure, leads to a robust single-shot experiment whereby ^{1}H nuclei appear as effectively homo- and hetero-decoupled.

A key to achieve such robust decoupling is, naturally, to have a sparse ^{13}C enrichment to ensure the periodic rotation of all ^{12}C-bound protons neighboring the targeted resonance. This brings about a number of limitations, including the sensitivity penalties normally associated with acquisitions on natural abundance samples. By selectively enhancing the ^{1}H resonances that are targeted by the homonuclear decoupling process, the hyperpolarization procedure illustrated in Figure 2 alleviates this, and provides an excellent complement to the BIRD-based decoupling. An example of this is illustrated by the DNP-enhanced, homonuclear-decoupled "HyperBIRD" ^{1}H spectrum shown in Figure 3. In the top trace of this figure a three-peak ^{1}H pyridine spectrum is displayed, showing similar signal intensity as a conventional thermal counterpart, but no ^{1}H–^{1}H J splittings. This single-shot spectrum enjoys the benefit of the 100 times signal enhancement derived from the ^{13}C-derived DNP process, acting in unison with a ^{13}C-based homonuclear decoupling that collapses the ^{1}H multiplet patterns into narrow singlet peaks. This can be appreciated from the BIRD-decoupled thermal counterpart in the center panel of Figure 3, which is inferior to its single-scan hyperpolarized counterpart even after 1024 averages (1 hour 40 minutes total acquisition time).

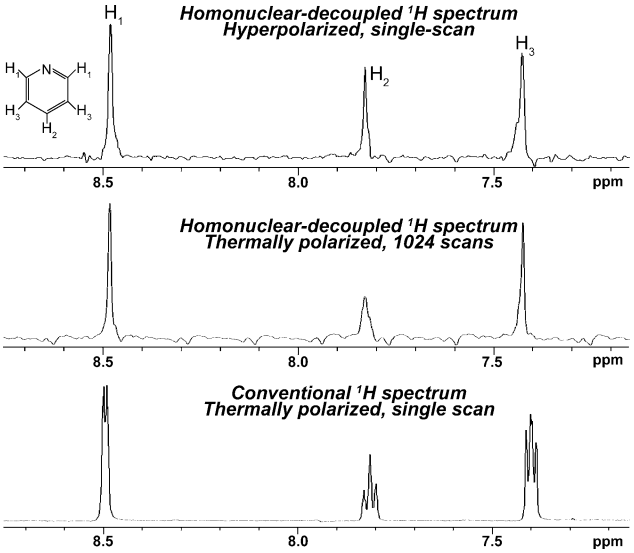

Figure 3. Demonstrating the ^{1}H-enhancing and decoupling features of HyperBIRD on a natural abundance pyridine sample. 5 μL of an equal part mixture of [D_6]DMSO and pyridine were hyperpolarized for this experiment. Dissolution was performed with 4 mL of [D_3]methanol giving a final sample concentration of about 7.8 mM. To appreciate the resolution and sensitivity enhancements of the HyperBIRD experiment this trace (top) is compared against a multi-scan BIRD spectrum collected after the sample returned to thermal equilibrium (center), as well as against a single-shot single-pulse J-coupled counterpart (bottom). The slightly larger artifacts shown by the thermally polarized BIRD spectrum reflect the challenge to completely suppress the (now much more intense) ^{12}C-bonded proton resonances.

While the HyperBIRD experiment required a longer time than its thermal counterpart if including the sample pre-polarization period, its suppression of the natural-abundance ^{12}C-bound background is also eased by the strong natural bias that the cross-relaxation effect does on behalf of the ^{13}C-bound species. Finally, both of these spectra show signals that split into the expected multiplets in a single-pulse conventional acquisition (Figure 3, bottom).

The sensitivity and resolution enhancement brought about by this procedure is further exploited in Figure 4, which demonstrates the utility of HyperBIRD for analyzing an aromatic mixture containing many overlapping proton multiplets. The thermal equilibrium single-pulse spectrum of this quinolone/toluene/pyridine/indazole sample contains many spin multiplets, with spectrally congested regions where individual resonances are hard to identify and assign. In the hyperpolarized, homodecoupled spectrum all ^{13}C-bound proton resonances are selectively enhanced and collapsed into single lines, providing an easier route to resolving the chemical shift of every site. When compared against literature values, this allows one to carry out an assignment of the origin of the chemical peaks.

Conclusions

The HyperBIRD technique as implemented here is still limited by a number of features, including an inability to decouple geminal protons and relatively lengthy sample pre-

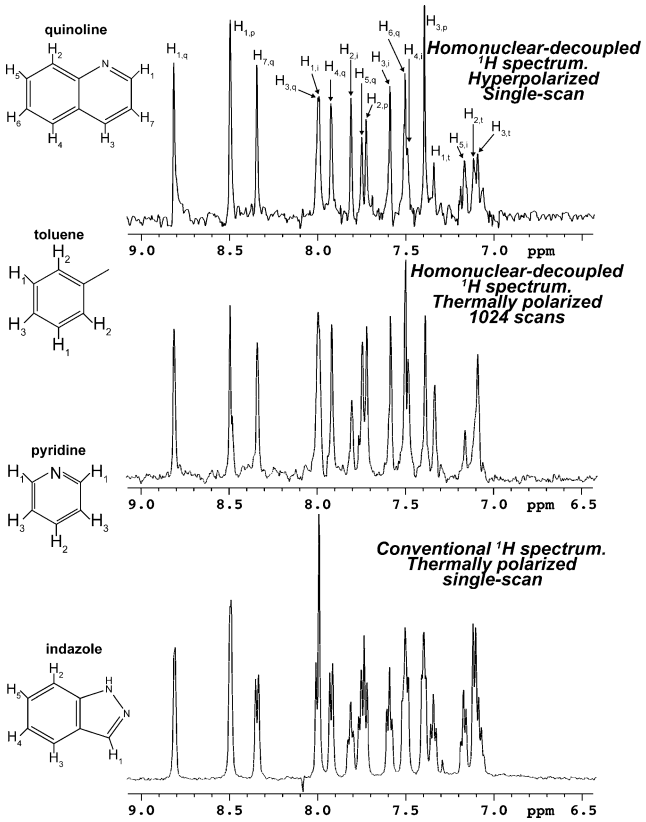

Figure 4. Application of the sequence introduced in Figure 1 to the analysis of a heterocyclic mixture with several overlapping multiplets. In the HyperBIRD spectrum (top) all individual ^{1}H resonances are resolved and tentatively assigned based on literature values; something clearly more difficult for the conventional proton spectrum (bottom). The fine residual coupling observable in the HyperBIRD spectrum around 7.1 ppm, is likely the result of insufficiently suppressed background (^{12}C-^{1}H) signals. All spectra were recorded on the same sample, prepared by combining the indicated substrates in a 50/50 mixture of sulfolane and [D_6]DMSO. 5 μL of the ensuing mixture was hyperpolarized, and dissolved in 4 mL of [D_3]methanol for the NMR observations. Final substrate concentrations were 2.53 mM for pyridine, quinoline, indazole, and 1.27 mM for toluene, all at natural abundance.

polarization times. Recent developments based on the use of homonuclear echoes[14,15] could bypass the first of these limitations, while increasingly faster DNP processes have been demonstrated with the aid of cryogenic cross-polarization.[16,17] With these and other emerging developments, the HyperBIRD technique could become increasingly promising for practical acquisitions of fully decoupled ^{1}H data. Moreover, while NMR experiments with enhanced ^{1}H sensitivity have also been demonstrated based on INEPT-based transfers of heteronuclear hyperpolarization,[18–21] the cross-relaxation-based transfer demonstrated in this report offers a number of advantages. This method is simpler sequence-wise, as it does not need to adjust millisecond-long delays based on a particular J_{CH} value and/or multiplicity as is the case with a ^{13}C→^{1}H INEPT transfer; its spontaneous nature may also make it a preferable choice when considering heteronuclei that are more exotic than carbon-13. The cross-relaxation based polarization transfer is also well suited to in vivo experiments, which tend to be performed in more hardware-limited scanners than their in vitro counterparts, and that inherently incorporate multi-second delays to allow the hyperpolarized substrate to reach the desired point of detection. Still, from an acquisition standpoint, the BIRD-based single-shot module used in this work should also be compatible with INEPT-based forms of hyperpolarization transfer. It is also interesting to consider the potential advantage of the 1D form of homodecoupled acquisition presented in this work, over single-shot 2D heteronuclear correlation experiments that starting from hyperpolarized ^{13}C or ^{15}N nuclei, have been demonstrated.[18–20] The latter have naturally the prospect of providing an enhanced resolution and information vis-à-vis the 1D ^{1}H acquisitions hereby described, yet achieve this at the expense of more complex gradient-based manipulations and with a decreased signal-to-noise ratio. Still, given the advantages inherent to fast 2D NMR sampling schemes,[22–24] it is interesting to further consider how heteronuclear cross-relaxation effects, hyperpolarization, and homonuclear BIRD decoupling concepts, could also be exploited in these kind of fast 2D experiments.

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