METHODS FOR ENHANCED NMR SENSITIVITY OF ORGANIC SMALL MOLECULES

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BACKGROUND

The first nuclear magnetic resonance (NMR) was observed in bulk matter in 1945; since then, the field has been expanded by innovative techniques. These include, among others, the nuclear Overhauser effect (1953), time averaging (1963), helium-cooled superconducting magnets (1964), and two-dimensional spectroscopy (1970s).[1] Each of these developments marked an increase in sensitivity and resolution. However, fundamental methodological improvements have slowed in recent years.

The observed NMR signal is dependent on the difference in populations of nuclear spin states based on the Boltzmann distribution. At room temperature in a 300 MHz instrument, this corresponds to only a 50 ppm difference in spin state populations. Most approaches to enhance NMR sensitivity focus on increasing the spin state population difference by decreasing the probe temperature or increasing the strength of the magnet, which further increases the spin state energy gap. Unfortunately, this approach is limited because even much larger magnets provide only modest improvements in sensitivity, and probe cooling only limits thermal noise.

Recent efforts have focused on developing hyperpolarization methods, a technique which aligns the majority of spins in a sample and has the potential to greatly increase sensitivity. This review will focus on three techniques for achieving hyperpolarized samples: optical pumping, parahydrogen-induced polarization, and dynamic nuclear polarization. Extensions of these techniques to provide >10,000-fold enhancements of the signal to noise ratio (SNR) in one- and two-dimensional spectroscopy for small organic molecules will also be highlighted.

HYPERPOLARIZATION

Hyperpolarization was first discovered in the 1950's in solid lithium samples.[2] This method efficiently orders all NMR-active nuclei within a molecule (see Figure 1); however, the induced hyperpolarization lasts only until the nucleus relaxes (governed by the longitudinal relaxation time, T_1). As a result, protons retain an enhanced signal for only a few seconds before returning to Boltzmann levels. Although it is possible in some cases to record the enhanced proton signal, most efforts have focused on low gyromagnetic ratio (γ)
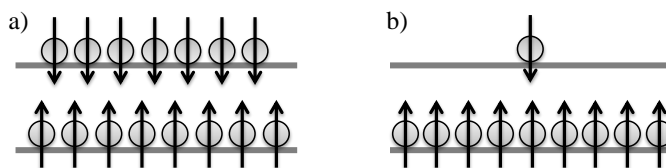
Figure 1. a) Normal polarization of spins in Boltzmann distribution. b) Hyperpolarized spin distribution.

nuclei, including ^{13}C and ^{15}N. This offers two major advantages: first, low γ nuclei tend to have longer T_1's, making it easier to observe the hyperpolarized signal; second, low γ nuclei are insensitive using current NMR methods, making hyperpolarization attractive as a complementary approach.

Initial developments focused on the transfer of polarization from circularly-polarized light to skew electronic spins of gaseous metals to non-equilibrium populations, a process known as optical pumping.[2] Later developments moved towards compounds with high levels of pre-organization and inherent polarization, including parahydrogen, to subsequently transfer polarization onto organic molecules.[2] The most dramatic work, however, has been in the introduction of dynamic nuclear polarization, a technique which uses microwaves to align the nuclear spins within an entire molecule.[2]

Optical Pumping

Optical pumping with hyperpolarized noble gases (including ^3He and ^{129}Xe) has been used in a wide range of applications, from surface and materials chemistry to quantum computing.[3] The most significant use, however, has been in magnetic resonance imaging (MRI). The hyperpolarized nucleus is extremely sensitive to changes in environment, thereby enabling tracking of the molecule throughout a biological system. In particular, hyperpolarized gases have been used extensively for the study of voids within the body, including the lung cavity, over the course of a single breath hold (20 seconds).[4] Optical pumping has found the fewest applications in solution-phase NMR because it is limited to certain noble gases; consequently, organic molecules cannot be directly visualized.

Parahydrogen-Induced Polarization

Molecular hydrogen exists in two forms: a triplet state with parallel nuclear spins (ortho) and a singlet state with antiparallel spins (para). At room temperature, these forms exist in a statistical 3:1 ratio, but when hydrogen is cooled to cryogenic temperatures (2-4 K), the more stable para form becomes prevalent. The anti-alignment of the spins in parahydrogen results in a highly ordered molecule with a net zero polarization. If this symmetry is broken by a chemical reaction, hyperpolarization is observed in those protons by a process known as parahydrogen-induced polarization (PHIP).

The earliest applications of PHIP focused on mechanistic studies of hydrogenation due to the natural reactivity of hydrogen with unsaturated systems. Unfortunately, this natural reactivity also limits PHIP to hydrogenation studies, as shown in Figure 2,[5] although some polarization can be transferred from the protons to other nuclei in the sample, allowing for enhanced detection of heteronuclei.[6]

In 2002, Duckett and coworkers illustrated that metal-hydride complexes formed from parahydrogen could be directly detected at low concentrations.[7] While this provided a method for

observing intermediates in catalytic systems, it did not provide a direct means for the analysis of small organic molecules. Extension of this system in 2007 provided a metal-binding site for pyridine, purine, and adenine.[8] Following association of the substrates with an iridium-hydride species and exposure to parahydrogen, differential signals of the hydrides could be used to infer binding of these molecules, and NOE offered a means to determine the orientation of binding. This indirect method is still limited: it cannot be extended to the characterization of unknown compounds because the resonances of the small molecule are not directly detected.

Scheme 1. Hydrogenation using parahydrogen to evaluate product mixtures
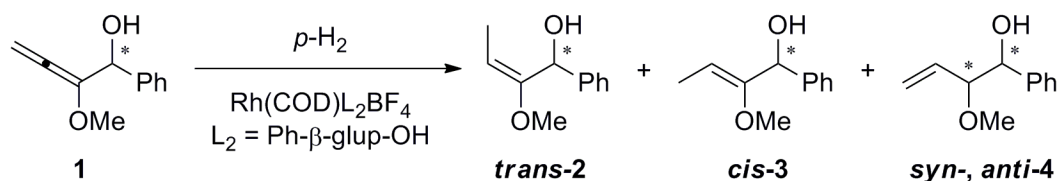
Direct detection was addressed in 2009 by the introduction of PHIP NMR-SABRE (signal amplification by reversible exchange).[9] Within a labile iridium-hydride complex (**5**), hydrides in the metal complex can be exchanged for parahydrogen (**5** to **6** to **5'**). Polarization is transferred to the substrate of interest (such as pyridine or nicotinamide, **5''**), and the substrate is released. The polarization remains on the small molecule long enough to be observed in a single scan using conventional NMR techniques. This proof-of-concept experiment provides a huge improvement over previous PHIP methods, as the parahydrogen is not included in the observed product. Additionally, PHIP NMR-SABRE allows for hydrogen, carbon, and nitrogen nuclei to be observed without inverse detection at micromolar and lower concentrations, and, in the case of carbon, at natural abundance. As in the previous system, small molecules with metal-binding domains are necessary. Despite this limitation, this method of polarization transfer through a metal complex is the most general method for hyperpolarization using parahydrogen.

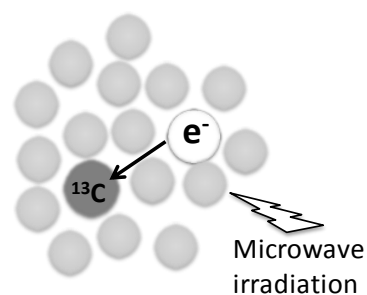**Scheme 2. Hyperpolarization transfer through iridium complex (L=metal-binding substrate).**
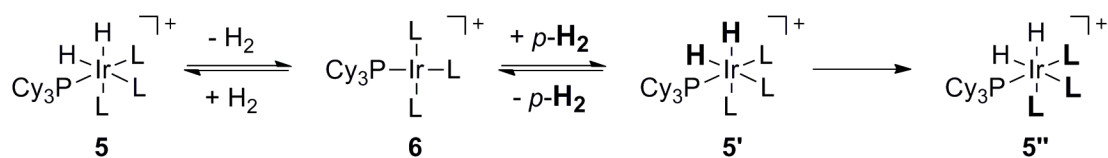
Dynamic Nuclear Polarization

One of the most broadly applicable methods for hyperpolarization of small molecules is dynamic nuclear polarization (DNP).[2] In DNP (Figure 2), spin polarization is transferred from electrons to nuclei via the Overhauser effect. Practically, this involves cooling a sample spiked with an organic radical to
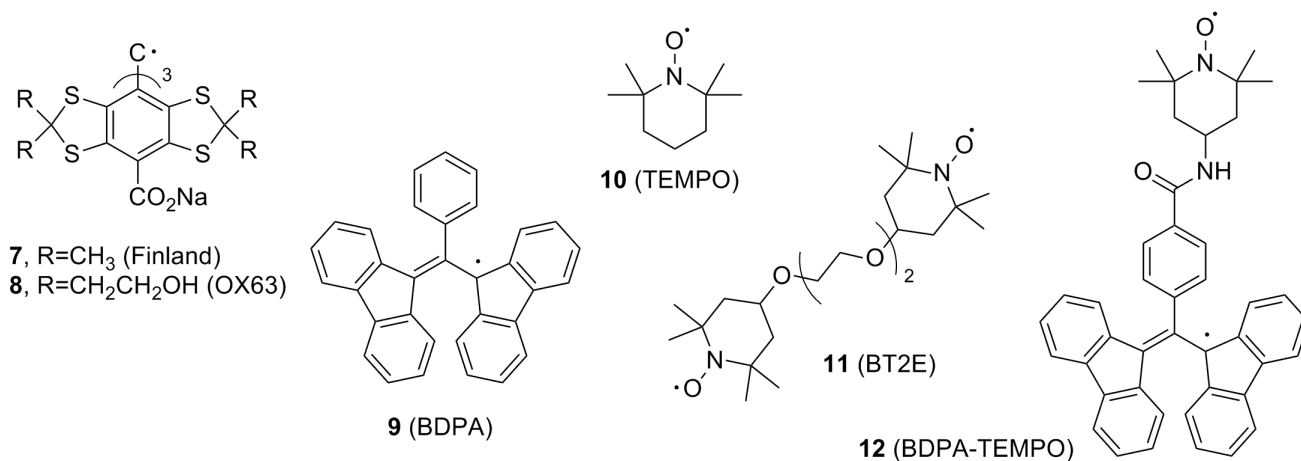
cryogenic temperatures (1-4 K) followed by microwave irradiation at a frequency close to the maximum electron paramagnetic resonance (EPR) frequency for the radical. Irradiation times vary from minutes to hours, depending on the relative energies of the radicals and nuclei, but can result in nearly complete population separation. Polarization is generally performed at lower fields (0.3 to 3 T) to increase the lifetime of the hyperpolarized material; samples are then immediately imaged.

Figure 2. Microwave irradiation of dispersed radicals leads to enhancement of nuclei in DNP.

The earliest applications focused on solid state imaging, where the sample could by hyperpolarized and immediately imaged in the same spectrometer, thereby avoiding polarization loss from temperature and field changes. Solution-phase methods, however, were in great demand in order to extend DNP to small organic molecules.

Ardenkjær-Larsen and coworkers reported the first application of DNP to liquid samples in 2003.[10] In their procedure, urea was dissolved in glycerol in the presence of a trityl radical (**7**) and cooled to 1.2 K in a low-field DNP magnet. The frozen, solid sample was irradiated for roughly 5 hours to achieve 42% ^{13}C polarization. The sample was then rapidly dissolved in a solvent within the DNP magnet, injected into a 400 MHz NMR within 6 seconds, and acquired. This procedure preserves most of the ^{13}C polarization, and allows for a single scan of 59.6 mM natural abundance urea to give an SNR of 4592. The same sample, using conventional techniques, only gives an SNR of 7 after averaging 232,128 scans, corresponding to a >10,000 enhancement of the ^{13}C signal with hyperpolarization.

7, R=CH$_3$ (Finland)
8, R=CH$_2$CH$_2$OH (OX63)

9 (BDPA)

10 (TEMPO)

11 (BT2E)

12 (BDPA-TEMPO)

Figure 3. Radicals used in DNP.

Two prongs of research have emerged from Ardenkjær-Larsen's report in 2003. The first focuses on developing molecules that can store DNP-induced polarization in long-lived states (LLS).

Polarization in an NMR-silent LLS molecule is then released and visualized when that molecule is placed in a different surrounding, such as a diester converting to a monohydrate when it is moved from a hydrophobic environment to a hydrophilic environment inside an organism.[11] LLS can also be accessed through selective pulse sequences to convert nuclear spins to stable states, thereby prolonging their T_1 and enabling manipulation of the sample prior to analysis by NMR.[12] The creation of LLS has enabled high-resolution tracking of metabolites in biological systems over a period of several minutes.

The second prong of research spurred by Ardenkjær-Larsen's 2003 report has expanded DNP to simpler radicals. The trityl radical originally described by Ardenkjær-Larsen and coworkers (**7**) is only available synthetically in a very low 0.9% yield.[13] Despite this limitation, **7** is highly soluble in both organic and aqueous solvents, and has a narrow EPR signal, making this molecule ideal for DNP. Other polarizing radicals, including TEMPO (**10**)[14] and bis-diphenylene-phenylallyl (**9**, BDPA),[15] can also be used, but are less efficient than **7**. To create systems that have the electronic advantages of trityl radicals along with the synthetic advantages of TEMPO and BDPA, the Griffin and Swager groups at MIT began to tether different radicals together. Such biradical polarizing agents can improve DNP efficiency and lower the necessary electron concentration. In the first report from these groups in 2004,[16] two TEMPO molecules were tethered with a poly-(ethylene glycol) chain (**11**). By maximizing the interaction of the two radicals, a roughly four-fold enhancement in signal was observed with the biradical **11** compared to the single radical **10**. Further efforts focused on tethering TEMPO to first a trityl radical[17] and then later to BDPA.[18] Tethered **12** showed a narrow, high-intensity EPR signal, but DNP enhancement has not yet been shown with this species. Thus, progress is being made towards new, practical sources of radicals for DNP polarization, but to date no ideal structure or synthetic method has been developed.

ULTRAFAST 2D PROCESSING

Recent developments in hyperpolarization methods, especially DNP, are enormously empowering for obtaining high resolution 1-D NMRs of unenriched samples in a single scan. Direct use of hyperpolarized samples to obtain traditional 2-D NMR spectra, however, is challenging because most 2-D methods require the nuclei to fully relax between pulses. In the case of hyperpolarized samples, relaxation returns the nuclei to their Boltzmann distribution levels, eliminating any gain from the enhanced signal beyond the first scan.

Since 2002, several groups have undertaken efforts to develop novel pulse sequences to obtain high resolution 2-D NMRs from DNP by using only a few scans. These techniques rely on encoding pulses that either utilize variable flip angles to access only part of the hyperpolarization during each pulse, or gradient pulses that polarize different cross sections of the sample to varying degrees.
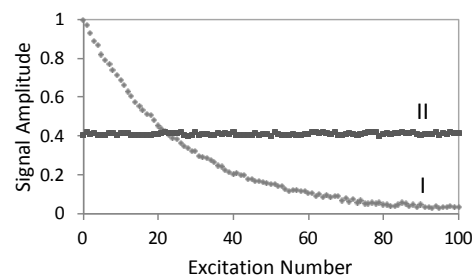
Variable Flip Angles

The first approach to expanding 2-D NMR to hyperpolarized samples focused on methods using between 3 and 30 pulses at varying angles. These methods rely on two factors. First, only a small amount of the polarization is accessed during each pulse; by comparison, in normal 2-D methods, an initial 90° pulse converts all of the spins in a sample into observable coherence. By using smaller angles, only part of the spin polarization is converted into a signal; this phenomenon is exploited in variable flip angle

Figure 4. Signal amplitude decay for hyperpolarized samples based on (I) full pulses or (II) variable flip angles.

experiments by gradually increasing the flip angle with each pulse, thereby drawing from the remaining reservoir of polarized spins (Figure 4).[19] Second, rapid pulsing prevents the sample from relaxing thermally over the course of the experiment. As mentioned above, conventional 2-D NMR experiments require the sample to fully relax between scans; removal of this delay by rapid pulsing further preserves the hyperpolarized signal.

Hilty and coworkers have recently developed two new methods for rapid 2-D NMR acquisition using variable flip angle pulse sequences. The first report, in 2008,[20] presents a process known as off-resonance coupling. Three to five scans of the proton-coupled ^{13}C spectrum are taken with off-resonance irradiation at different proton chemical shifts. The observed *J*-coupling constant varies as a function of the proton irradiation, allowing for reconstruction of what is traditionally a 2-D HMQC spectrum. In the 2009 report,[21] the Hilty group was able to acquire an HMQC spectrum directly by taking advantage of the preserved polarization following a variable angle flip. Although both of these techniques make repeated use of a single DNP polarization, diminished signal over the course of the experiment due to differences in T_1 and the corresponding ideal flip angle, makes signal processing difficult.

Spatial Encoding

The second approach for rapidly acquiring 2-D spectra from hyperpolarized samples uses gradient pulses to selectively polarize a sample spatially, thereby encoding the second dimension of the 2-D spectra along the length of the sample (Figure 5). Deconvolution of the signal upon collection allows for the direct acquisition of a high-resolution 2-D spectrum in a single scan.

The first report of spatial encoding by Frydman and coworkers was in 2002,[22] a year prior the first use of hyperpolarization with liquid samples. A single scan TOCSY of natural abundance hexapeptide (CSHAVC) showed similar connectivities in a single 0.15 second scan as in a conventional

90-minute acquisition. Similarly, *n*-butyl chloride (20% v/v) showed high resolution COSY and TOCSY spectra with acquisition times of less than one second. However, high concentrations were needed obtain this resolution since the samples were at normal Boltzmann spin distributions.

After the extension of hyperpolarization to solution-phase NMR in 2003, spatially-encoded 2-D NMR quickly became the preferred method to obtain high resolution spectra within seconds, at submicromolar concentrations.[23] For example, a DNP-enhanced single-scan HSQC with 0.47 mM natural abundance pyridine showed comparable SNR to a conventional HSQC acquired over 2.5

Figure 5. a) Excitation is offset based on the height of the tube. b) After mixing, spatially-distinct signals give a complete 2-D spectrum.

hours on 5 mM ^{13}C-enriched material, amounting to an improved sensitivity of >1000. Spatial encoding can even be extended to sequential multidimensional spectra, as long as the experiments analyze different nuclei or different types of polarization transfer (^{15}N vs. ^{13}C, or HMQC vs. HMBC), thereby preserving polarization.[24] As the resolution of the experiment relies on a variable gradient, however, only a limited region of the spectrum can be visualized during each routine. It is possible to expand the range of acquisition using spectral folding, whereby regions of the spectra are folded on top of one another during the acquisition. For example, a 60-ppm region could be compressed into a 20-ppm region following two folds; however, this is only practical if prior information is available to prevent spectral overlap. Successful application of spatial encoding has further decreased acquisition time and allowed for resolution of mixtures in a single HSQC scan.[25]

PRACTICAL APPLICATIONS AND SUMMARY

Hyperpolarization and ultrafast multidimensional NMR have numerous applications in both biological and chemical settings. Hyperpolarization of ^{13}C-labeled metabolites has allowed for *in vitro* and *in vivo* analysis of enzyme processing, enabling the high-resolution analysis of single steps in metabolic pathways; in addition, similar substrates have been used in clinical MRI.[26] DNP has been used for a number of years for protein structure analysis, both in solution and solid-phase settings.[2] Natural product identification is the newest application of this technology. Most preliminary work on hyperpolarization has used a few simple substrates, including urea and pyridine, as proof-of-principle molecules. Since 2008, extension of this methodology to more complex structures, including quinine,[27]
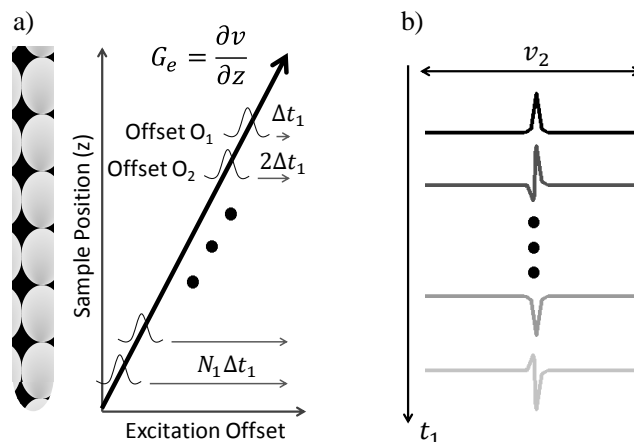
thiamine,[28] and mixtures of terpenoids,[29] suggests that these fast, high-sensitivity methods are becoming increasingly general for use with small molecules. This development is promising for application to organic chemistry, because it has the potential to allow for rapid imaging of natural abundance, low γ nuclei and to revolutionize techniques for identification and evaluation of small organic molecules.

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