High-Resolution High-Frequency Dynamic Nuclear Polarization for Biomolecular Solid State NMR

by

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B.A. Chemistry
Whitman College (2003)

Submitted to the Department of Chemistry in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

June 2011

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Submitted to the Department Chemistry May 20, 2011 in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Chemistry

Abstract

Dynamic Nuclear Polarization (DNP) has exploded in popularity over the last few years, finally realizing its potential to overcome the detrimental lack of sensitivity that has plagued performing NMR experiments. Applied to magic angle spinning (MAS) experiments, this renaissance of DNP has been primarily driven by the development of instrumentation; namely gyrotron oscillators as high-power stable microwave power sources and the NMR probes and associated equipment required to spin samples routinely below 100 Kelvin.

The first three chapters of this thesis provide an overview of the theory, instrumentation, and applications of DNP. Chapter 1 introduces the magnetic resonance Hamiltonian with a focus on interactions that are necessary to control in order to obtain high-resolution DNP spectra. Chapters 2 and 3 are published reviews of DNP. Whereas Chapter 2 targets magnetic resonance spectroscopists, Chapter 3 is intended for an electric engineering audience. Both reviews are included as the associated depth and coverage of the topics are complementary and lead to a better understanding of DNP.

The later chapters describe in detail advancements in probe, cryogenics, and gyrotron technology required to perform DNP MAS experiments, as well as the gains in sensitivity and resolution such instrumentation has permitted. Of particular importance is the development of a cryogenic sample ejection system that resulted in exquisite resolution of spectra recorded <100 K, both of crystalline peptide and the active site of membrane proteins. Such developments in instrumentation and demonstrations of resolution go towards overturning a long-held stance in the field that DNP would always suffer from broadened, unresolved spectra. Such techniques also allow us to investigate site specific dynamics of a crystalline peptide, the high resolution SSNMR structure of which is discussed in Chapter 3. DNP and the developed instrumentation is also leveraged to measure inter-atomic distances in the active site of a membrane protein with sub-angstrom precision. Chapters 7 describes the calculation of the microwave field strength across the sample in a MAS DNP probe and introduces strategies to increase it. Finally Chapter 8 introduces a major advance in the microwave source technology associated with DNP experiments. Detailed designs of a novel 250 GHz gyrotron oscillator are shown and experiments demonstrate a continuous broad 3 GHz bandwidth with >10 W across the band, which results in substantially improved DNP performance.
Thesis Supervisor: Robert G. Griffin
Title: Professor, Department of Chemistry
        Director, Francis Bitter Magnet Laboratory
DEDICATION

This thesis is dedicated to my cousin, Lt. Commander Che Barnes, who perished over the Pacific Ocean in his C-130 on a search and rescue mission in 2010.
ACKNOWLEDGMENTS

Firstly, I want to thank Robert Griffin for creating an exciting and intense laboratory full of resources and talented, ambitious individuals to learn from and work with. Above all he fosters an environment where good ideas and technologies flourish and individuals can develop into independent scientists. Through his encouragement and support I have been able to tackle projects at graduate school I would have thought totally beyond my capabilities. He has pushed me past where I thought my limits were, and I have grown to be a better scientist and stronger person because of it.

My interaction with Richard Temkin and members of his group at the Plasma and Science Center has been the singularly most exciting, challenging, and rewarding aspect of my graduate school. His caring and insightful leadership style is one I strive to emulate. His group members, Michael Shapiro, Ivan Mastovsky, Antonio Torrezan, Sudheer Jawla, Paul Woskov, Alan Cook, Elizabeth Kawalski, Brian Munroe, David Tax, and Emilio Nanni have all helped me look at DNP technology from a vacuum electronics perspective.

Judith Herzfeld has been a grounding force in my struggles with instrumentation—constantly keeping my focus on a biologically relevant target. Without my exciting conversations with her discussing the ion pumping mechanism of bR I surely could not have maintained my motivation to build probes, gyrotrons, and DNP spectrometers.

Jagadishwar Sirigiri is one of the most gifted teachers and innovative scientists I have had the pleasure to work with. With his guidance and instruction I was able to break through the intimidating barrier between chemistry and vacuum electronics. Without him, gyrotrons would have remained finicky, bizarrely complex black boxes. With him, I was able to improve on gyrotron design and now plan not only to use gyrotrons in my laboratory in the future, but indeed build my own and continue to develop this exciting technology.

Emilio Nanni is perhaps the most promising and positive scientist I have ever met. His theoretical knowledge and experimental talents as a graduate student are unparalleled and our collaboration has been rewarding and motivating. Our interaction is what I will strive to emulate in my future collaborations.

Working with Evgeny Markhasin, Loren Andreas, Eugenio Daviso, and Björn Corzilius has been a pleasure and a productive time for me. Having fun running difficult experiments with them is what I always hoped graduate school would be like. Discussing science with Andy Smith and Matthew Eddy has been invaluable to my development over the years. My interaction with them demonstrates the importance of learning from and interacting with individuals who have a different skill set and knowledge base than my own.

Ronald DeRocher and Ivan Mastovsky have been the most influential figures in my development as an engineer. They have tutored me in the equivalent of multiple semesters of classwork in drafting and design and emphasized the importance and utility of building high quality instrumentation right the first time through careful and meticulous planning.

Patrick van de Wel, Chris Turner, Tony Bielecki, Yoh Matsuki, Melody Mak-Jurkaukas, Jozef Lewandowski, Gaël De Paëpe, Ramesh Ramachandran, Vikram Bajaj,
Marc Caporini, Marvin Bayro, Thorsten Maly, Kannan Ramaswamy, Anil Mehta, Hellmut Eckert, Marko Bertmer, and Manish Mehta have all been my instructors in developing my skills as a spectroscopist over the years and I am in their debt. Without the skills and know-how of Ajay Thakar, Jeff Bryant, Michael Mullins, and Ron DeRocher I could not have built new instrumentation and would have been stuck using commercial equipment.

Blowing off steam and talking science over beers with the “Wingers” crew – Leo Gomez, Becky Nicodemus, Ziad Ganim, Marc Caporini, Sean Smith, Andy Smith, Björn Corzilius, Jason Locasale, Marvin Bayro, Galia Debelouchina, and Karsten Seidel has surely been a enjoyable and necessary part of my graduate school.

I also thank my friends at Washington University in St. Louis. Sophia Hayes was my first instructor in magnetic resonance, is the source of my interest in sensitivity enhancement, and pointed me to MIT. I aspire to combine cutting-edge research with a positive caring mindset as she does. Jacob Schaeffer has also been a valuable resource for me to talk with and learn from over the years due to his pioneering work in MAS DNP and measuring biologically meaningful distances precisely. He is one of my professional role models. Ryan Nieuwendaal and I have discussed more science in a 72-hour period than perhaps anyone can—he is an exemplary case of how much fun it can be to mix science and friendship.

The chemistry faculty at Whitman College set me on my career path and showed me how fun learning and teaching chemistry can be. Allison Calhoun was my first physical chemistry instructor and emphasized the importance of having a physical intuition of the Hamiltonian. Chuck Templeton taught me attention to detail and the value in not jumping to conclusions until you process and analyze a data set completely.

My family has provided me with emotional support and professional guidance over the years—without which I surely would have given up on my dreams. My sister has helped me with Matlab, and my brother is the source of my borderline obsessive attitude to writing up results in papers. My father instilled a spark and passion for science in me by showing me his experiments and writing out polymerase chain reactions on napkins over dinner when I was in grade school. Without him showing me how much science can be, I would not have a PhD from MIT or be the scientist I am today.

Lastly I would like to thank my committee members, Troy van Voorhis and Andrei Tokmakoff. Their sound professional guidance and support over the last year has been extremely important to my academic career.
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Chapter 1. Controlling the Magnetic Resonance Hamiltonian

Nuclear and electron spins that have dipole moments can interact with electromagnetic fields which are macroscopic in extent, such as static magnetic fields created by superconducting magnets, or microscopic in nature, such as fields originating from the individual dipole spins themselves. The sum of all of the interactions the spins have with the electromagnetic fields yields the energy of the quantum mechanical dipole spin state and is best represented by dividing such a Hamiltonian based on the nature of the interaction,

\[
H = H_Z + H_D + H_{CS} + H_J + H_Q + H_{RF}
\]  

(1.1)

In this section we introduce the form and magnitude of these interactions and comment on some of the roles they play in magnetic resonance spectroscopy. Specifically, we discuss the nature of a distribution in the magnitude of these interactions as it pertains to line broadening and degraded resolution in the type of spectra discussed in this thesis—namely dynamic nuclear polarization (DNP) enhanced magic angle spinning (MAS) experiments on peptides and proteins.

Throughout this chapter we discuss how the Hamiltonian can be controlled. Instrumentation gives the spectroscopist control over the Hamiltonian. Without RF amplifiers and associated resonance circuits, microwave sources (gyrotrons), cryogenics, and pneumatic magic angle spinning systems, etc., it would only be possible to observe the magnetic resonance Hamiltonian—not exert control over it. The renaissance that DNP is enjoying in the field of MAS NMR stems from the ability to generate microwave
nutation frequencies \((\gamma B_1)\) of \(~1\) MHz over the sample utilizing high-power gyrotrons as microwave sources\([1]\), while simultaneously spinning the rotors at the magic angle\([2, 3]\). Tremendous advancements in magnetic resonance methods have been (and will continue to be) realized by the development of instrumentation that allows spectroscopists to better control the magnetic resonance Hamiltonian.

**Zeeman Interaction and Polarization**

Nuclear and electronic magnetic moments interact with magnetic fields through the Zeeman interaction, given as:

\[
H_z = -\gamma \hbar \vec{I} \cdot \vec{B}
\]  

(1.2)

where \(\gamma\) is the gyromagnetic ratio. The gyromagnetic ratio represents both the strength of the interaction of the spin with magnetic fields and also describes the sense in which the spin precesses around the magnetic field. Gyromagnetic ratios of \(^1\text{H}\), \(^{13}\text{C}\), \(^{15}\text{N}\) spins commonly found in isotopically labeled biomolecules as well as the gyromagnetic ratio of electron spins present in paramagnetically doped DNP samples are listed in Table 1.1. In equation 1.2, \(\hbar\) is the reduced Plank’s constant, \(I\) is the spin and in the case of this thesis we limit our discussion mostly to \(I=\frac{1}{2}\) spins that can occupy \(2I+1=2\) spin states. The Zeeman interaction energy of the two states of an \(I=\frac{1}{2}\) spin is then:

\[
E_{\uparrow} = -\frac{\gamma \hbar B_0}{2}
\]  

(1.3a)

\[
E_{\downarrow} = \frac{\gamma \hbar B_0}{2}
\]  

(1.3b)

and the energy difference is

\[
\Delta E = \gamma \hbar B_0
\]  

(1.4)
where \( \omega_0 \) is the Larmor frequency, or the frequency of precession around the magnetic field. The Larmor frequency at 8.9 Tesla of nuclear and electron spins are given in Table 1.1.

Detection of the nuclear or electronic moments requires a polarization originating from a population difference in the occupation of the spin states. Although there are applications that utilize polarization stemming from the energy splitting from other terms in the Hamiltonian, such as the quadrupolar interaction (NQR detection)[4], we limit our discussion to the polarization originating from the Zeeman interaction. The population distribution is described by the Boltzmann distribution and the polarization is given by:

\[
P = \frac{\frac{-E_{\downarrow}}{k_B T} - e^{\frac{-E_{\downarrow}}{2k_B T}}}{\frac{-E_{\uparrow}}{2k_B T} + e^{\frac{-E_{\uparrow}}{2k_B T}}}
\]

or substituting in eq. 1.3a,b,

\[
P = \frac{\frac{\gamma h B_0}{2k_B T} - e^{\frac{\gamma h B_0}{2k_B T}}}{\frac{1}{2k_B T} + e^{\frac{1}{2k_B T}}} \quad (1.6)
\]

In an effort to truncate the exponentials in eq. 1.7, we note that \( k_B T \) at 80 K 1.1 x \( 10^{-21} \) J, which is much greater than the Zeeman energy (see Table 1.1). If \( k_B T \) and the Zeeman energy splitting are on the same order, the full exponential much be taken into account in
eq. 1.7 to calculate the polarization more precisely. For our purposes, we can truncate an expansion of the exponentials in eq. 1.7 as:

\[
P = \frac{\left(1 + \frac{\gamma \hbar B_0}{2k_B T}\right) - \left(1 - \frac{\gamma \hbar B_0}{2k_B T}\right)}{\left(1 + \frac{\gamma \hbar B_0}{2k_B T}\right) + \left(1 - \frac{\gamma \hbar B_0}{2k_B T}\right)}
\]

(1.8)

which simplifies as,

\[
P = \frac{\gamma \hbar B_0}{2k_B T}
\]

(1.9)

the value of which at 8.9 Tesla and 80 Kelvin of four common spins are listed in Table 1.1.

It is important to note that this polarization assumes a thermal equilibrium between the spin states has been established. The large magnetic field does not create polarization, but rather only leads to an energy splitting. The interaction of the spins with

Table 1.1. Gyromagnetic ratios of common spins discussed in this thesis and the Larmor frequency, Zeeman energy, and polarization of the spins at 8.9 Tesla and 80 Kelvin.

<table>
<thead>
<tr>
<th>I=½ spin</th>
<th>Gyromagnetic ratio (MHz/Tesla)</th>
<th>Larmor Frequency (MHz)</th>
<th>Zeeman Energy Splitting (Joules)</th>
<th>Polarization</th>
</tr>
</thead>
<tbody>
<tr>
<td>¹H</td>
<td>42.6</td>
<td>380.38</td>
<td>2.5 x 10⁻²⁵</td>
<td>1.1 x 10⁻⁴</td>
</tr>
<tr>
<td>¹³C</td>
<td>10.7</td>
<td>95.65</td>
<td>6.3 x 10⁻²⁶</td>
<td>2.9 x 10⁻⁵</td>
</tr>
<tr>
<td>¹⁵N</td>
<td>-4.32</td>
<td>-38.54</td>
<td>2.6 x 10⁻²⁶</td>
<td>1.2 x 10⁻⁵</td>
</tr>
<tr>
<td>NO• electron</td>
<td>-28049.</td>
<td>-250587.</td>
<td>1.7 x 10⁻²²</td>
<td>7.5 x 10⁻²</td>
</tr>
</tbody>
</table>
the lattice and random fluctuating magnetic fields induces transitions between the spin states that in turn generate the polarization needed for magnetic resonance detection.

**Contributions to Linewidth from the Zeeman Hamiltonian**

A very homogenous magnetic field throughout the sample leads to narrow lineshapes. Loss of spectral resolution and sensitivity can result from inhomogeneous magnetic fields across the sample which can be macroscopic in nature originating from large inhomogeneities in one of the super conducting coils, generally mis-adjusted currents in the room temperature shim coils, or the presence of material close to the sample whose susceptibility distorts the magnetic field lines throughout the sample[5]. Similarly, microscopic variations in susceptibility throughout a sample due to the presence of large domains can also perturb the magnetic field lines and lead to an inhomogeneous magnetic field and line-broadening.

**Dipolar Hamiltonian**

Dipolar couplings can be utilized both to determine biomolecular structure and to increase sensitivity of NMR via cross polarization or dynamic nuclear polarization. In addition, high-resolution NMR spectra in the solid-state are generally obtained by proper attenuation of certain dipolar couplings. Here, we focus on two spin = ½ magnetic moments, I and S, the dipolar interaction between which can be denoted as:

\[
H_D = \mathbf{I} \cdot \mathbf{D} \cdot \mathbf{S}
\]

(1.10)

Which is typically represented by:

\[
H_D = \frac{\hbar^2 \gamma_I \gamma_S}{r^3} \frac{H_0}{4\pi} (A + B + C + D + E + F)
\]

(1.11)

Where the “dipolar alphabet” is[6];
\begin{align*}
A &= (3 \cos^2 \theta - 1) I_Z S_Z \tag{1.12a} \\
B &= -\frac{1}{4} \left(3 \cos^2 \theta - 1\right) \left(I_+ S_- + I_- S_+ \right) \tag{1.12b} \\
C &= \frac{3}{2} \sin \theta \cos \theta e^{-i\phi} \left( I_+ S_Z + I_Z S_+ \right) \tag{1.12c} \\
D &= \frac{3}{2} \sin \theta \cos \theta e^{i\phi} \left( I_- S_Z + I_Z S_- \right) \tag{1.13d} \\
E &= \frac{3}{4} \sin^2 \theta e^{-2i\phi} I_+ S_+ \tag{1.14e} \\
F &= \frac{3}{4} \sin^2 \theta e^{2i\phi} I_- S_- \tag{1.15f}
\end{align*}

Here, $I_Z$ and $S_Z$ are the secular spin operators of the two spins. $I_+$ and $S_+$ and $I_-$ and $S_-$ are the raising and lower operators, and $\theta$ is the angle between the dipolar vector and the external magnetic field. Following equation 1.11, the magnitude of the relevant dipolar interactions discussed in this thesis span 6 orders of magnitude, ranging from $<10$ Hz to $\sim25$ MHz for a pair of electron spins separated by $\sim10$ Å. In the case of exogenously added radical electron spins as is generally employed in MAS DNP experiments, we commonly consider electron-nuclear dipolar interactions between a few Hz to a few MHz. Proton spins within a few angstroms of the electron spin are so strongly coupled to the electron that they can no longer participate in zero quantum (eq. 1.12b) energy conserving flip-flop transitions with bulk $^1\text{H}$ spins—thus creating a spin diffusion barrier[7, 8]. Thus the essential proton-electron couplings leading to DNP enhancement are most likely in the range of $\sim1\text{-}20$ KHz (close to the homogeneous linewidth of the $^1\text{H}$ spin reservoir).

**Contributions to Linewidth From the Dipolar Hamiltonian**
Manipulation and attenuation of dipolar couplings through mechanical rotation of the sample, applications of strong RF decoupling fields, spin dilution, and distal separation from steric hindrance can lead to high spectral resolution, that would otherwise be degraded by the presence of dipolar couplings. $^{13}\text{C-}^{13}\text{C}$, and $^{15}\text{N-}^{15}\text{N}$, and $^{15}\text{N-}^{13}\text{C}$ dipolar couplings range in magnitude from several Hz to several KHz as described by equation 1.11. In a static sample, these couplings result in a Pake pattern; the superposition of many of such patterns leads to extensively broad lineshapes. In general, a modulation of such a 2nd rank tensor interaction at frequencies several times higher than the interaction strength will partially average and sufficiently attenuate the interaction. In the case of these $^{13}\text{C}$ and $^{15}\text{N}$ dipolar couplings, mechanically rotating the sample at the magic angle ($54.7^\circ$) at $\omega/2\pi > 5$ KHz sets 1.12a and 1.12b close to zero [2, 3] and sufficiently attenuates the line-broadening associated with such $^{13}\text{C}$ and $^{15}\text{N}$ dipolar couplings.

$^1\text{H-}^{13}\text{C}$ and $^1\text{H-}^{15}\text{N}$ couplings range from several KHz to several tens of KHz depending on the distance and orientation between the spins (see equation 1.11). Although techniques such as employing recently developed magic angle spinning modules that can mechanically rotate samples at $\omega/2\pi > 50$ KHz, and extensive replacement of $^1\text{H}$ isotopes with $^2\text{H}$ can result in high-resolution spectra, a more generally employed strategy is to apply a strong $^1\text{H}$ RF decoupling fields to attenuate such $^1\text{H-}^{13}\text{C}$ and $^1\text{H-}^{15}\text{N}$ couplings. It is therefore crucial to have instrumentation that can create an RF field on the $^1\text{H}$ spins that is of several times larger than the $^1\text{H-}^{13}\text{C}$ and $^1\text{H-}^{15}\text{N}$ dipolar coupling. Initially the $^1\text{H}$-decoupling field was continuous wave irradiation. However, Bennet et al. introduced a Two Pulse Phase Modulation (TPPM) [9] irradiation scheme of
the $^1$H decoupling field that effectively decouples the extensively homogeneously broadened $^1$H spins from lower $\gamma$ nuclei. The effectiveness and importance of TPPM is demonstrated by the >1000 citations this contribution has garnered in the first 10 years after its introduction.

The nuclear spins being detected in MAS DNP experiments should not be strongly dipolar coupled to unpaired electron spins that are the source of the greater polarization. This is discussed in detail in chapter 6. However, any electron-nuclear dipolar couplings which do remain are absolutely detrimental to performing spectroscopy – namely because the dipolar interaction will lead to homogeneously broadened lines.

**Chemical Shift Hamiltonian**

Electrons in the vicinity of nuclei flow circularly in a sense that creates a magnetic field that opposes $B_0$. The magnetic field sensed by the nuclear spin is thus weaker due to this induced shielding field resulting in a precession frequency that depends on the chemical environment surrounding the nucleus. Furthermore, due to the anisotropic nature of the electron density surrounding nuclei, the induced shielding field opposing $B_0$ is dependent on the molecular orientation with respect to $B_0$. Figure 1.1 illustrates orientations that a tripeptide, APG, can take with respect to the main magnetic field. Each crystallite orientation in a static powder has a varied degree of electron shielding which manifests as a distribution of resonance frequencies and a broad lineshape. The form of this anisotropic chemical shift interaction is;

$$\gamma \left( \mathbf{I} \cdot \mathbf{\sigma} \cdot \mathbf{B} \right)$$

(1.16)
Figure 1.1. Graphical representation of an anisotropic chemical shielding tensor. The anisotropic electron density around the proline carbonyl of Alanyl-Prolyl-Glycine (APG) flows in a circular sense around the large static magnetic field, Bo. The orientation of the crystallite affects the degree of electronic shielding and thus precession frequency.

where $\sigma$ is a second rank tensor describing the anisotropy of the electron shielding around the nucleus. The anisotropic nature of the chemical shift also plays an important role in extracting structural information from biomolecules in the solid state. Where as the chemical shift tensor is averaged to its isotropic value, by fast molecular tumbling in liquid samples, the full anisotropic tensor can be extracted from spectra of biomolecules in the solid state. Thus it is important to note that the chemical shift anisotropy is not merely a phenomenon to be averaged out in order to record high-resolution spectra—it is an extremely sensitive measure of the chemical environment surrounding sites of biological interest and can be probed to gain important structural details. Chapter 6 describes an experiment that yields the precise CSA of a $^{15}$N nucleus in the active site of a membrane protein frozen in a photo cycle reaction intermediate. Additionally, the CSA is used for the determination of backbone torsion angles for the structure calculation of APG in chapter 4.

**Contributions to Linewidth From the Chemical Shift Hamiltonian**
Conformational heterogeneity in the backbone and sidechains of peptides or proteins results in a different electronic environment and extent of electronic shielding. The manifestation of such heterogeneity can be a set of discreet resonances or a broadening of the resonance, depending on the similarity of the conformations, the nature of the energy landscape of the conformational space, and the number of conformations being sampled. This becomes particularly important in cryogenic MAS spectra. Although at higher temperatures, as the conformational space is often sampled on a timescale much faster than the free induction decay, the conformational sampling can be frozen out at cryogenic temperatures resulting in multiple lines or a broadening of some resonances. Examples of such discreet or broadened resonances are shown and discussed further in chapter 6.

Non-covalent heterogeneous interactions can also lead to a distribution of electronic environments, and thus broad resonances. For example, a standard sample used for characterizing DNP enhancements is urea frozen in a glassy matrix of 60/40 glycerol/water. Such spectra are relatively broad (~350 Hz at 9 T, see chapter 5). A minor portion of this broadening can be attributed to the Zeeman Hamiltonian because of a lack of long-range (>50 Angstrom) order, varied domains, and a resulting inhomogeneous B₀ magnetic field. However, a distribution of the geometry of the first few solvent shells and intermolecular packing accounts for the majority of the broadening present in such glassy, medium-range anisotropic samples.
Scalar J Hamiltonian

Scalar J couplings are mediated through electrons present in covalent bonds and manifest as multiplet structure of high resolution spectra or a broadening of resonances in more poorly resolved spectra. The J coupling term of the Hamiltonian has the following form:

\[ H_J = \gamma_i \gamma_j \left( \mathbf{I}_i \cdot \mathbf{J} \cdot \mathbf{I}_j \right) \]  

(1.17)

For two \(^{13}\text{C}\) spins, the magnitude of which is typically \(~35-55\) Hz and \(~10-15\) Hz for \(^{15}\text{N}-^{13}\text{C}\) J-couplings.

Line Broadening from the Scalar J Hamiltonian

J-couplings are readily removed by isotopic dilution. For example the high resolution spectra shown in Chapters 4 and 6 were obtained by using natural abundance \(^{13}\text{C}\) peptide or site-specifically \(^{13}\text{C}\) labeling such that the chance of having directly bonded \(^{13}\text{C}\) spins and J-coupling is statistically very low (~1%).

Quadrupolar Hamiltonian

In the case of nuclear spins of \(I > 1/2\) a quadrupolar interaction is also part of the internal Hamiltonian. This is typically the only interaction that can be comparable in magnitude to the Zeeman interaction. Common quadrupoles encountered in biomolecular NMR include \(^{17}\text{O}\) (\(Q \sim 7\) MHz [10]), \(^2\text{H}\) (\(Q \sim 250\) KHz[11]), and \(^{14}\text{N}\) (\(Q \sim 1\) MHz).
Contributions to Linewidth from the Quadrupolar Hamiltonian

At natural abundance, the $^{14}$N isotope accounts for $\sim$99.6% of nitrogen isotopes. Thus, in the case of non-isotopically labeled peptides, the backbone $C_\alpha$ and carbonyl carbons have a directly bonded spin with a quadrupolar moment. This coupling can lead to broadening of the $^{13}$C resonance usually in the range of 15-40 Hz. Therefore in order to get the highest resolution one-dimensional $^{13}$C spectrum of a peptide backbone, often the carbon spins are present in natural abundance, but the nitrogen spins are labeled with the $^{15}$N isotope, which does not have a quadrupolar moment.

The external RF and $\mu W$ Hamiltonian

The external RF and microwave Hamiltonian has the form;

$$\gamma_h I \cdot B_1 \quad (1.18)$$

Importantly, $B_1$ must be perpendicular to $B_o$ and oscillating at the nuclear or electron Larmor frequency to induce transitions between nuclear and electron spin states. Without strong $\gamma B_1$ fields, many of the terms in the Hamiltonian (eq. 1.1) cannot be controlled. Nuclear $\gamma B_1$ fields are usually on the order of $\sim$100 kHz, thus making it possible to strongly influence all of the terms in the Hamiltonian besides the Zeeman and quadrupolar interactions—which are generally $\gg$100 kHz. As described in detail in chapter 7, the electron $\gamma B_1$ is $\sim$1 MHz in MAS DNP experiments.
References

Chapter 2. High Field Dynamic Nuclear Polarization for Solid and Solution Biological NMR


Abstract

Dynamic Nuclear Polarization (DNP) results in a substantial nuclear polarization enhancement through transfer of magnetization from electrons to nuclei. Recent years have seen considerable progress in the development of DNP experiments directed towards enhancing sensitivity in biological NMR. This review covers the applications, hardware, polarizing agents, and theoretical descriptions that were developed at the Francis Bitter Magnet Laboratory at MIT for high field DNP experiments. In frozen dielectrics, the enhanced nuclear polarization developed in the vicinity of the polarizing agent can be efficiently dispersed to the bulk of the sample via $^1$H spin diffusion. This strategy has been proved effective in polarizing biologically interesting systems, such as nanocrystalline peptides and membrane proteins, without leading to paramagnetic broadening of the nuclear resonances. Gyrotrons have been used as a source of high power (5-10 Watts) microwaves up to 460 GHz as required for the DNP experiments. Other hardware has also been developed allowing *in situ* microwave irradiation integrated with cryogenic magic angle spinning solid state NMR. Advances in the quantum mechanical treatment are successful in describing the mechanism by which new
introduction

as abragam and goldman[1] pointed out in their seminal review of dynamic nuclear polarization (dnp), three of the major breakthroughs in materials science in the 20th century involved access to low temperature[2], high pressure regimes[3] and the production of highly polarized nuclei. the latter was first targeted for a wide variety of physical and chemical applications. among the known methods for enhancing nuclear polarization, microwave (mw) driven dnp, which involves transfer of magnetization from electron to nuclear spins, has proven to be one of the most effective and versatile in its ability to polarize a wide range of systems. this is a consequence of the strong coupling of the electron spin to the surrounding nuclei. the corresponding increase in polarization is a natural complement to the rapidly developing field of solid state and solution nmr as a structural tool in biology. the inherent lack of sensitivity in nmr is a major limiting factor in its extension to larger and more interesting biological systems. thus, dnp experiments that achieve even a fraction of the theoretical maximum sensitivity enhancement of 660 could revolutionize many aspects of nmr spectroscopy.

in 1953 overhauser originally proposed that it was possible to transfer polarization to nuclei from electrons in metals by saturating the electron transition[4]. the idea that saturating one transition could increase the polarization in another was counter-intuitive and not widely accepted until experimental verification by carver and

biradical polarizing agents yield larger enhancements at higher magnetic fields. finally, pulsed methods and solution experiments should play a prominent role in the future of dnp.
Slichter[5]. These studies served as the foundation for modern DNP, and were soon expanded to solid dielectrics by Abragam[6] who first characterized the solid effect (SE). In the 1980's Wind, Yannoni, Schaefer and their colleagues performed DNP in conjunction with magic angle spinning (MAS) solid state NMR (SSNMR)[7-9]. However, extending DNP to higher fields proved challenging due to a lack of high power μw sources above 94 GHz. One approach to circumvent this problem, pioneered by Dorn[10], is to polarize samples at lower magnetic fields and then transfer the sample to a higher field where the NMR spectrum is recorded. However, such methods employing field cycling have their technical limitations. Applying in situ high power μw irradiation at the detection field strength without the need for field cycling has been the focus of our experiments at MIT. This approach has proven an effective means for gaining enhanced polarization in SSNMR aimed at biological solids. For other implementations of DNP, we direct the reader to other articles in this volume and to the recent review by Maly et al.[11].

Developments in instrumentation for high power μw sources and cryogenic MAS and theoretical and chemical advancements involving the cross effect (CE) and biradicals have allowed the expansion of DNP to higher magnetic fields as needed for modern biological SSNMR. This review describes applications demonstrating the ability to acquire contemporary multidimensional SSNMR spectra on various biological systems as well as the instrumental, theoretical, and chemical developments that lead to the first DNP/SSNMR experiments performed at high magnetic fields (5 and 9 Tesla). However, the continuous wave (CW) methods discussed below exhibit an inverse field dependence ($\omega_0^{-1}$ to $\omega_0^{-2}$). Thus, extension of DNP to even higher magnetic fields will likely entail
development of pulsed experiments to increase the efficiency of the polarization transfer process. Accordingly, we review a number of time domain methods that have been explored at fields up to 5 T.

This review is organized as follows. In Section 2 we discuss applications of CW DNP techniques to biological systems that provide a rationale for the development of DNP experiments. Section 3 describes the instrumentation required for the experiments including gyrotron oscillators which are the source of the subterahertz radiation used to irradiate the electron paramagnetic resonance (EPR) spectrum and the low temperature probes used in the experiments. In Section 4 we discuss the cross effect and the biradical polarizing agents that have thus far proven most effective in producing large enhancements ($\epsilon \sim 290$) in solids at 90 K. Finally, in Section 4 we discuss two areas for future development – temperature jump DNP experiments for solution spectroscopy and time domain experiments. Both of these areas should undergo considerable development in the future.

**Solid state applications to biological samples**

**Frozen glass-forming solvents**

DNP on frozen glycerol/water solutions doped with biradicals has been proven widely applicable as a means of providing signal enhancements on the order of $10^2$-$10^3$ in a range of biological systems such as virus capsids and lipids[12], amyloidogenic peptides[13], and large membrane proteins[14, 15]. An aqueous solvent mixture consisting of deuterated glycerol, D$_2$O, water, and a soluble biradical polarizing agent[16,
provides a glass forming matrix that distributes the polarizing agents uniformly and at the same time acts as a cryoprotectant. Experiments on non-glass-forming solvent matrices, which form crystals upon freezing, were found to yield significantly smaller DNP enhancements. This appears to be caused by an aggregation of the polarizing agents along the edge of the crystalline domains, concomitant with a degradation of the spectral resolution due to poor long-range homogeneity.

The DNP experiments described in this review rely on an efficient polarization transfer from electrons to nearby protons, relayed by nuclear spin diffusion among the proton network leading to an efficient and uniform signal enhancement of the bulk nuclei of interest[18]. Partially deuterated solvents yield larger enhancements because the biradicals can polarize the smaller proton reservoir more completely, and there is minimal broadening of the nuclear resonances because the radicals are not in direct contact with the bulk nuclei. The experiment is typically performed at cryogenic temperatures (≤90 K) to lengthen the nuclear spin-lattice proton relaxation time (T_{1n}) allowing efficient pumping from the electron spin reservoir and appropriate \(^1\)H spin diffusion. This approach yields enhancements that are a function of temperature and magnetic field, and \(\varepsilon=400\) is achieved at 20 K and 5 Tesla[19]. In addition, enhancements of 10-25 are observed in some samples at room temperature[7, 20].

The low temperatures also affect the Boltzmann equilibrium, resulting in a Boltzmann population that is about three times higher at 90 K than at 270 K, thus giving a further increase in sensitivity. Even lower temperatures (∼20 K) accessible using helium as a spinning gas, result in more polarization but also a longer T_{1n}. We note that the important parameter for improving signal to noise in SSNMR is not only the
magnitude of polarization, but rather the signal intensity recorded per unit time\(^{1/2}\), which is dependent on the \(T_{1n}\) and thus the recycle delay. Bloembergen et al. \cite{21} first showed that the presence of paramagnetic ions in a solution shorten the \(T_{1n}\), an observation which has been more recently exploited in SSNMR experiments to reduce the low temperature proton \(T_{1n}\) from 15 seconds to 0.7 s, thus increasing the data collection rate by a factor of nearly 20\cite{22}. Thus, polarizing radicals serve not only as centers for DNP, but also as relaxation agents because the magnetization between experiments is replenished from electron-nuclear polarization transfer and proton spin diffusion. Typical recycle delays in our experiments are 5-10 seconds.

Figure 2.1 Spin diffusion spectrum of U-\(^{13}\)C,\(^{15}\)N proline in 40 mM TEMPO and glycerol/water. The DNP enhancement is 9, at 5 Tesla and a temperature of 90 K.
We note that we refer to the enhancement, $\varepsilon$, as the ratio of the NMR signal intensities with and without microwaves. Other experimental parameters, such as the temperature, are identical between the two measurements (unless indicated otherwise). However, in the discussion of solution DNP experiments below we use the enhancement $\varepsilon^+$ that includes a contribution from the Boltzmann temperature factor.

Multi-dimensional experiments increase the resolution of NMR spectra[24] but require repeated acquisitions, and thus a high level of experimental stability and reproducibility. Although the initial experiments discussed in the introduction showed a marked signal enhancement, they were not suitable for recording multi-dimensional spectra mainly due to the difficulties of spinning with helium for extended periods. Rosay et al. [23] first used nitrogen as both a spinning and cooling gas to perform long-term 2D DNP experiments at 90 Tesla. Figure 2. shows a 2D spectrum of a frozen solution of U-$^{13}$C,$^{15}$N-proline recorded with DNP at 5 Tesla (a higher magnetic field than earlier experiments). This was the first successful demonstration of the integration of DNP-based polarization enhancement with a standard 2D SSNMR pulse sequence. These data involved enhancement of a solute in intimate contact with the bulk solvent (containing the radicals). More recent versions of this experiment have achieved $\varepsilon \sim 200$[25].

**Macroscopic particles and aggregates**

Rosay et al.[12] showed that nuclei inside a virus capsid, which are not in direct contact with the bulk solvent, can be efficiently polarized through DNP. These experiments demonstrated equal signal enhancements for $^{15}$N in the coat proteins (on the
outside) and $^{31}$P nuclei in the DNA inside the capsid. These data indicated that $^1$H spin diffusion can act as an efficient mechanism for the uniform transfer of polarization into macromolecular assemblies on the order of 6 nm in size. The virus capsid experiments were performed at 15 K in the absence of sample spinning, and with low µw power from a Gunn diode, resulting in a DNP enhancement of 26 for both $^{15}$N and $^{31}$P. This also demonstrated that polarizing the proton bath and then relying on proton spin diffusion allows the enhancement of many different nuclei, such as $^{31}$P, $^{15}$N, and $^{13}$C via Cross Polarization (CP) [26].

Recent MAS SSNMR/DNP experiments by van der Wel et al.[13] also established that $^1$H spin diffusion allowed the highly polarized $^1$H magnetization from DNP to penetrate into even larger domains devoid of radicals. This is important since one attractive application of DNP in the solid state is the study of nanocrystalline or fibrillar macroscopic aggregates of proteins or peptides. Since these polypeptide aggregates are of a finite size, such samples can involve a spatial separation of the compounds of interest from the frozen solvent matrix that contains the radicals acting as sources of electron polarization. As discussed above, earlier experiments demonstrated the ability to efficiently polarize the matrix and the molecules contained therein. Enhancement of the polypeptide signals inside macroscopic crystals is however dependent on the ability of the enhanced polarization to (a) cross the boundary between the matrix and the sample aggregates and (b) the ability for the polarization to diffuse into the bulk of these aggregates.

These issues were directly addressed in DNP experiments performed on nanocrystalline aggregates made from a peptide fragment, GNNQQNY$_{7-13}$, originating
Figure 2.2 DNP experiments on [20% U-13C,15N-GNNQ]QNY nanocrystals in d$_8$-glycerol/D$_2$O/H$_2$O (60/30/10) with 10 mM TOTAPOL (a) Illustration of heterogeneously mixed DNP sample of crystalline peptide and the DNP solvent matrix, based on Transmission Electron Microscope (TEM) data on GNNQQNY nanocrystals. Arrows illustrate diffusion of enhanced polarization from the matrix into the crystals. (b) Differential polarization enhancement buildup of peptide and matrix carbon signals. The signal intensities are normalized to the equilibrium off signal. (c) DNP-enhanced 2D $^{13}$C-$^{13}$C DARR/RAD correlation spectrum of the same sample. Adapted from and reproduced with permission ref.[13] (Copyright 2006 American Chemical Society)

from the yeast prion protein Sup35p. This peptide forms either nanocrystals or amyloid-like fibrils, and has been studied by X-ray crystallography [27, 28] and solid state NMR [29] with the goal of elucidating the nature of amyloid fibril structure. The crystals used in the DNP experiments were 100-200 nm wide and were shown to be impenetrable by the TOTAPOL biradicals used in the experiments. The crystals were prepared in the absence of the radicals and subsequently mixed with a standard DNP solvent system, designed to be highly deuterated as well as glass-forming due to the presence of glycerol. Hence, efficient DNP enhancement would require polarization to diffuse into the crystals (see Figure 2.2). Indeed, it was found that the glycerol carbons in the frozen matrix were
highly and rapidly enhanced ($\varepsilon \sim 160$ with a time constant of 7-8 s), whereas the peptide signals were enhanced less and more slowly ($\varepsilon \sim 120$ with a time constant of 15.5-17 s), as shown in Figure 2.2b. These results consistent with a transfer of polarization from the bulk solvent into the crystals via $^1$H-$^1$H spin diffusion. The DNP enhancement allowed the rapid acquisition of a 2D $^{13}$C-$^{13}$C experiment as illustrated in Figure 2.2c. The results suggest that sensitivity enhancement via DNP is in principle (and in practice) possible for heterogeneous, hydrated protein crystals as well as fibrils, and should be an essential contribution to these areas of biological solid state NMR.

**Membrane proteins; Bacteriorhodopsin**

The most explicit demonstration of DNP in conjunction with SSNMR to address biologically relevant questions was recently performed on the membrane protein, bacteriorhodopsin (bR)[14, 30]. bR is a 26.6 kDa, 248-residue membrane protein acting as a light-driven ion pump. Initial DNP experiments on bR performed by Rosay et al.[15] demonstrated an enhancement of 50 at 90 K and 5 Tesla. Extending these experiments to 9 Tesla, corresponding to 380 MHz proton frequency, provided sufficient resolution to gain high quality 2D spectra and DNP expedited data acquisition permitting experiments which would not be possible without DNP.

Figure 2.a shows the clear improvement in signal-to-noise that DNP provides in the active site of a membrane protein. The single $^{15}$N resonance of the Schiff Base is barely visible above the noise in the non-enhanced spectrum even with 3.5 days of signal averaging, but can clearly be seen after only 30 minutes of acquisition time using DNP. Figure 2.3b shows cross-peaks between the Schiff base $^{15}$N and $^{13}$C-15 of the retinal chromophore. Four cross-peaks are observed where only two are expected, providing
clear evidence for structural heterogeneity in the dark-adapted state of bR that has not been detected previously. Note also that Figure 2.3b shows negligible, if any, broadening of the resonances in the active site of bR due to the conditions used for DNP. Extensive assignments of resonances in mixed photointermediates of bR further show the usefulness of the excellent sensitivity available using DNP (V.S. Bajaj et al., unpubl.).

Figure 2.3. a) $^{15}$N MAS spectra of light adapted $\zeta^{15}$N-Lys-bR. Top: Spectrum acquired on a 317 MHz spectrometer using a 5 mm ZrO$_2$ rotor with a 160 µL sample volume, 10,000 scans, 3.5 days (~5000 min) of data acquisition, $T = 200$ K. Bottom: Spectrum acquired with DNP–250 GHz µw irradiation using a 4 mm sapphire rotor, 40 µL sample volume $T = 90$ K, 384 scans, 30 min of data acquisition. The resonances from left to right are: protonated Schiff base $^{15}$N at 165 ppm; natural abundance amide backbone at 130 ppm; natural abundance guanidine–HCl at 80 ppm (only in the 380 MHz spectrum); six free $\zeta^{15}$N-Lys signals at 50 ppm. $\omega_0/2\pi = 7$ kHz. b) 2D nitrogen carbon correlation spectrum of dark adapted bR recorded with 250 GHz DNP showing heterogeneity which cannot be resolved in the 1D nitrogen spectrum. Reprinted from ref. [14]. Copyright 2007, with permission of Elsevier.

**Instrumentation**

**Probe Design**

Performing modern SSNMR experiments with concurrent high frequency microwave irradiation for DNP required the development of several new pieces of instrumentation. MAS SSNMR necessitates both the mechanical rotation of the sample
rotor in the kilohertz regime and the ability to apply strong $B_1$ fields at the nuclear and electron Larmor frequencies.

Generation of strong $B_1$ fields at the nuclear Larmor frequencies is usually accomplished with solenoid RF coils. The coil and MAS apparatus complicate the design of a $\mu$w resonator cavity and hence limit the quality factor, $Q$. This contrasts to $Q$ values in EPR resonators which are $\sim 10^3$, whereas the $Q$ for the $\mu$w circuit in our SSNMR DNP experiments is closer to unity. The inside of the stator cavity is coated with a thin layer of silver, which reflects the microwaves and increases the $Q$ slightly. The large $Q$ available in EPR probes permits the use of lower power microwaves (milliwatts), to create strong $B_1$ fields near the electron Larmor frequency. In our SSNMR DNP cavities, the lower $Q$ value means there is a need for a much larger $\mu$w power. Both the use of gyrotron oscillators as a high power $\mu$w source, as well as designs of corrugated waveguides needed for such DNP/SSNMR experiments will be discussed below.

In DNP experiments the $\mu$w's can be introduced either along the axis of the MAS rotor[7], or perpendicular to the rotor axis[31] (see Figure 2.). Note that perpendicular irradiation together with sample rotation also exposes more of the sample to the microwaves, which have a limited penetration depth, leading to more complete polarization. Using a high power $\mu$w source, rather than a resonance cavity structure, separates the requirements for spinning samples at kilohertz frequencies and cryogenic temperatures from creating a $B_1$ for the electrons strong enough to saturate the DNP transition. Advances in the last decade in probe technology and other hardware needed for cryogenic MAS have allowed stable spinning of the MAS rotor down to 85 K and $\omega_r/2\pi \sim 10$ kHz. Using cryogenic nitrogen as the bearing and turbine gas[12] provides
important advantages compared to systems employing a separate variable temperature
gas stream. Namely, the gradient of the sample temperature is smaller and it is easier to
fit all of the necessary lines into the limited space available in the bore of the magnet.
The high amount of flow needed to spin the sample also provides a high cooling capacity
that far exceeds losses due to conductive, convective, and radiative heat transfer. This
heat loss still must be minimized to achieve temperatures down to 80 K at the sample,
while preventing ice formation in the RF tuning box. Fiberglass vacuum jacketed
cryogen transfer lines, plated stainless steel RF transmission line, fiberglass insulation,
and a fiberglass and aluminum dewar thermally isolate the probe. RF transmission lines
separate the cryogenic environment in the vicinity of the sample from the tuning elements
located outside the magnet bore.[32]

The bearing and drive pressure of the MAS nitrogen gas is regulated at room
temperature with a Bruker MAS unit, after which it passes through a heat exchanger with
a pressurized heat transfer can, allowing for continual filling and operation[33]. The cold
cryogens next move through a series of vacuum jacketed transfer lines, bayonet
connections, and heaters for precise temperature control. Sapphire rotors that are nearly
transparent to the $\geq 140$ GHz $\mu$w radiation are routinely spun at $\sim$10 KHz and 85 Kelvin
with this apparatus.

**Gyrotron Oscillators**

The high power, millimeter wave radiation required by the low Q of the sample cavity is
generated with a gyrotron oscillator. Prior to the use of a gyrotron, there was a dearth of
high power sources operating in the 100-600 GHz regime this fact more than any other
has impeded the development of DNP experiments for magnetic fields used in contemporary high resolution NMR experiments. In a gyrotron the electron beam is launched from an annular cathode and accelerated through the field of a strong superconducting magnet. The field profile is designed to compress the beam as it moves through the vacuum tube to a resonant cavity that converts the transverse kinetic energy from the helical motion of the electrons into microwaves. A quasi-optical mode converter

Figure 2.4. Drawings of a cryogenic SSNMR DNP probe a) probe showing 1) probe-head 2) cut-out of vacuum dewar 3) tuning elements of the RF circuit located in the box 4) corrugated waveguide from gyrotron 5) concave and flat mirrors to direct microwaves into the vertical waveguide 6) vacuum jacketed transfer lines for the bearing and drive cryogens. b) probe-head for 4 mm rotors. 1) stator housing 2) sample rotor within RF coil (at the 'magic angle') 3) metal mirror miter 4) the inner conductor of the coaxial RF transmission line is corrugated on the inside and serves as an overmoded waveguide 5) outer conductor of the RF coaxial line is stainless steel for thermal isolation but is coated with silver and gold for good electrical performance.
couples the radiation to the output window of the device. Details of the physics and engineering of gyrotrons have been described recently[34, 35].

Gyrotrons are appropriate for use with high field DNP because they are fast-wave millimeter devices, differing from slow-wave sources that rely on structures that scale with the microwave wavelength and become prohibitively fragile for generation of high power levels at higher frequencies required for DNP. Gyrotrons employ a resonator that can be overmoded with dimensions larger than the operation wavelength, allowing for high power output for extended periods due to reduced thermal and ohmic losses in the resonator walls. Gyrotrons capable of generating watts of CW power at 140 GHz[20],

Figure 2.5 a) Cross-sectional schematic of the cylindrically symmetric 460 GHz gyrotron tube, not shown to scale, indicating key components. Adapted from ref.[36] (Reproduced by permission of IEEE). b) 250 GHz line layout for DNP experiments. Adapted from ref.[37] (Reproduced by permission of IEEE)
250 GHz[38] and 460 GHz[36] have been built for integration into DNP spectrometers. The availability of these sources enables DNP experiments to be performed in situ at high fields and avoids the necessity of shuttling as is described in other experiments in this volume.

**Corrugated transmission lines**

Transmitting the microwaves from the gyrotron to the sample in the probe with minimal loss, and monitoring the microwave power output is another challenge that has been addressed by Woskov et al.[37] Fundamental mode waveguides have unacceptable insertion losses, and do not couple to a free-space Gaussian beam that is used for quasioptical manipulation of the beam demanded by the physical geometric restrictions of the experiment. A corrugated overmoded waveguide with a cross-sectional diameter greater than the wavelength supports the efficient HE\textsubscript{11} mode. The HE\textsubscript{11} mode has a very low insertion loss and couples efficiently to a free-space Gaussian beam. A schematic diagram of the apparatus implementing such waveguides, mirrors, and metal miter bends is shown in Figure 2.b resulting in a minimal 0.8 dB power loss from the gyrotron to the rotor. A quartz directional coupler was also developed for use in our 250 GHz apparatus to monitor the forward and reflected power output of the gyrotron.

**Cross Effect and Biradicals**

One of the important experimental findings of our group that allows efficient high frequency DNP is that the cross effect (CE)[39-43] mechanism together with the use of biradicals always lead to improved polarization enhancements when compared to and
thermal mixing (TM)[9, 44] and the solid effect (SE)[6]. All of these CW polarization schemes show an inverse magnetic field dependence of the observed enhancements. Since the SE depends on excitation of forbidden electron-nuclear transitions, the enhancements are inherently small and the mechanism shows a $\epsilon \sim B_0^{-2}$ dependence, where $B_0$ is the external magnetic field. Therefore, this mechanism is not routinely used in current DNP applications. DNP experiments based on the CE mechanism have a less pronounced field dependency ($\epsilon \sim B_0^{-1}$) but still show an attenuated enhancement at increasing magnetic fields. However, the enhancements remain sufficiently large that they permit many experiments that are otherwise impossible. Thus, as discussed in more detail below, the CE mechanism is the primary polarization mechanism exploited for biological DNP experiments at high magnetic fields (5 and 9 T).

**DNP mechanisms at high magnetic fields**

Recently Hu[45] introduced a quantum mechanical description of the high field DNP processes that have been used at MIT over the last decade[12, 14-18, 23, 46-48]. This theoretical treatment is different from the existing literature on DNP processes that relied on equations of motion that correspond to macroscopic quantities averaged over an ensemble of spins[9, 44, 49, 50]. The historical semi-classical approach, although suitable at low magnetic fields, is less appropriate for the current experiments at higher magnetic fields where the electron spin reservoir is inhomogeneously broadened due to dilute radical concentrations and the fact that the g-anistropy is large compared to the electron-electron dipole coupling. This new quantum mechanical description allowed the same authors to explain in detail the SE and CE mechanisms and in particular the improved
CE-DNP transfer reported at high magnetic fields when using biradicals as polarizing agents [16, 17].

Figure 2.6. Quantum mechanical diagrams of the electron-nuclear transitions (dashed arrows) in (a) the SE, (b) the CE and (c) TM mechanisms, which involve single, paired and multiple electron spins, respectively. Note that the probabilities of electron-nuclear transitions are always small in the SE but could be large in the CE and TM, especially when there is degeneracy between states with alternating nuclear spin quantum numbers. Adapted from ref. [45]

Considering both experimental results and this new theoretical description, we will now briefly discuss the three CW DNP mechanisms found in solid dielectrics. The SE[6], CE[39-43] and TM[9, 44] mechanisms are distinguished by the number of electrons involved: single, paired and extended networks of electron spins, respectively. Figure 2.6 shows the energy level diagrams corresponding to these three DNP processes. The dominant DNP mechanism can be determined by a direct comparison of the EPR linewidth $\delta$ of the paramagnetic polarizing species with the nuclear Larmor frequency, $\omega_i$. When $\delta < \omega_i$ the polarization transfer is principally driven by the SE and involves a
polarization transfer between a single electron and nuclear spin described by the following time-independent Hamiltonian[45]:

\[
H_0^{SI} = \omega_{0S}S_z - \omega_{0l}I_z + A S_z I_z + B S_z I_z
\]  

(2.1)

where \(\omega_{0S}S_z\) and \(\omega_{0l}I_z\) are the electron and nuclear Zeeman terms in the lab frame, and A and B the secular and non-secular part of the hyperfine coupling interaction. This can be represented in the product spin bases (PSB) shown in Figure 2.6a. The DNP effect relies on the non-secular hyperfine coupling (last term of (1)) which by mixing the states \(|1\rangle\) and \(|3\rangle\) and the states \(|2\rangle\) and \(|4\rangle\) allows the \(\mu\)w irradiation to drive the DNP polarization transfer. Two resonant effects can be achieved when the microwave frequency matches \(\omega_{0S} + \omega_{0l}\) or \(\omega_{0S} - \omega_{0l}\), leading to a negative ZQ or a positive DQ enhancement, respectively. The mixing factor that describes the probabilities of the above transitions is proportional to \(\omega_{0l}^{-2}\) as it arises from a second order perturbation with respect to the spin interactions. Thus, the efficiency of the SE scales with \(\omega_{0l}^{-2}\).

Figure 2.7. (a) Illustration of the EPR spectrum of monomeric TEMPO nitroxide at 5 Tesla. Note that the breadth of the spectrum is \(\sim\)600 MHz and is large compared to that of the \(^1\)H Larmor frequency (211 MHz). The arrows indicate the approximate frequencies of
two electron spins, separated by $\omega_i/2\pi$, expected to participate in the CE/TM DNP enhancement process (b) Illustration of the $\mu$W-driven three-spin process associated with TM or CE DNP where two coupled electrons undergo an energy conserving flip-flop process that leads to enhanced nuclear spin polarization. (c) The molecular structure of the BTnE biradicals where $n$ is the number of ethylene glycol units that tether two nitroxide radicals (TEMPO). The dots represent the two unpaired electrons whose displacement is approximated as the oxygen-oxygen distance, $R_{o,o}$. Reproduced with permission from ref. [48] (Copyright 2004 American Chemical Society).

In contrast to the SE, which involves magnetization transfer from a single electron, the other two mechanisms involve two (or more) electrons in close proximity, with EPR frequencies that are separated by $\omega_{0i}$. For a single (mono) radical, this can only occur if its EPR spectrum line width is large compared to the nuclear Larmor frequency (i.e. $\delta > \omega_{0i}$). This is illustrated in Figure a for monomeric TEMPO as the polarizing agent: two (close) TEMPO molecules at appropriate orientations relative to the magnetic field have the required difference in resonance frequency. When these electrons are dipolar coupled, irradiation at the resonance frequency of the first electron produces a simultaneous spin flip of both the second electron and the nucleus, yielding the generation of nuclear spin polarization (Figure 2.7b). Under these conditions, the SE is actually reduced due to the overlap of the positive and negative DNP enhancements that cancel each other [51]. In the CE there are two participating electrons and a single nuclear spin, and there are now eight energy levels to consider (Figure 2.6b). Microwave irradiation can drive polarization transfer by saturating the transitions (dashed lines in figure). This transfer is made possible because of the coupling between states $|2> \text{ and } |7>$ (or states $|3> \text{ and } |6>$ if $\gamma < 0$), which is a consequence of the combined effect of the electron–electron and electron–nucleus interactions. The DNP effect is further maximized when the levels $|2> \text{ and } |7>$ become degenerate, i.e. when the matching
condition \(|\omega_{0S1}-\omega_{0S2}|=\omega_{01}\) is fulfilled.[45] For a monomeric radical having a broad EPR spectrum, this condition is fulfilled when the different g-tensor orientations of the two electrons result in a frequency mismatch equal to the nuclear Larmor frequency. However, another important requirement is sufficient proximity of the electrons such that the e-e dipolar coupling can induce the mixing of states |2> and |7>. Increasing the monomeric radical concentration is one way to achieve this, but as shown by Hu et al.[16], the use of biradicals (see Figure 2.7c) as polarizing agents is a much more efficient way to achieve efficient polarization transfer. The two unpaired electrons associated with the two radical moieties of a biradical correspond to the two electrons required for the CE and as shown by Hu[52], a quantum mechanical treatment of the spin dynamics of an electron-electron-nucleus system allows one to understand in detail the improved DNP mechanism[45]. Note that the two electrons involved in the process need not be from a single type of radical. Recent work by Hu et al.[53] demonstrated the use of a mixture of two radicals, TEMPO and trityl, as DNP polarizing agents. This approach also provided a significant improvement in the DNP enhancement over using TEMPO by itself. This is due to the fact that the EPR spectrum of trityl is offset from the \(g_{22}\) maximum of the TEMPO spectrum, aside from being much narrower (less anisotropic), by a difference that approximates the nuclear Larmor frequency. These features increase the number of radicals with the proper frequency separation (rather than shorten) and average inter-electron distance and thus improves the DNP transfer via the CE mechanism.

The TM mechanism differs from the CE by the number of electron spins involved in the polarization transfer, namely two electrons for the CE and multiple for TM, which
also translates into an EPR spectrum that is mainly inhomogeneously or homogeneously broadened, respectively. The TM can be seen as an extension of the CE where couplings among electrons induce manifolds of states (see Figure 2.6). In TM energy overlap between manifolds is required for maximizing the electron-nuclear transfer. However, TM requires a high concentration of paramagnets to achieve a homogeneously broadened spectrum and this in turn is inconsistent with the goal of high resolution in the MAS spectrum.

**Biradicals**

As pointed out in the previous section, the CE mechanism can be greatly enhanced with the use of biradicals as polarizing agents[48]. These new biradicals consist of two 2,2,6,6-tetramethylpiperidinyl-1-oxyl (TEMPO) radicals tethered by a poly-(ethylene glycol) chain (Figure 2.7c), and referred to as BTnE, for bis-TEMPO-n-ethylene glycol, where \( n \) is the number of ethylene glycol units in the tether. The size of the \( e^-e^- \) dipole coupling is directly controlled by the length of the chain, and can therefore be chosen to optimize the DNP enhancement. The \( e^-e^- \) couplings in the biradicals discussed here vary between 10 and 30 MHz as opposed to the \( \sim 0.3 \) MHz coupling present when monomeric TEMPO is present at the same electron concentration (\( \sim 10 \) mM).

The DNP enhancement results obtained with different polarizing agents are summarized in Figure 2.8. Note that the BTnE biradical polarizing agents yield a factor of \( \sim 4 \) larger signal intensities over those obtained with monomeric TEMPO. In addition, larger enhancements are obtained at significantly lower electron concentrations (40 mM
for TEMPO and 10 mM for BTnE respectively), thereby reducing the paramagnetic broadening present in the NMR spectrum. Finally, as can be seen in Figure 2.8, within the BTnE series of biradicals a reduction of the tether length improves the observed DNP enhancement. Unfortunately, the BTnE series is sparingly soluble in H2O/glycerol solutions used in cryoprotecting samples for DNP experiments. These observations motivated the design, synthesis and characterization of the improved polarizing agent, 1-(TEMPO-4-oxy)-3-(TEMPO-4-amino) propan-2-ol (TOTAPOL) (Figure 2.b) consisting of two TEMPO molecules tethered with a three-carbon chain [17] copiously soluble in aqueous media.

![Figure 2.8](image-url)

**Figure 2.8.** Histogram of DNP enhancements (with error bars) in 4 mm (light gray) and 2.5 mm (dark gray) rotors with TOTAPOL biradical, a series of BTnE biradicals, and monomeric TEMPO. The data illustrate that TOTAPOL yields the largest enhancement, especially when the microwave’s penetration depth is optimal for 2.5 mm rotors. Reproduced with permission from ref. [17]. Copyright 2006 American Chemical Society.

Figure 2.8 compares the DNP enhancement obtained with TOTAPOL (6 mM electron concentration) to results with the BTnE ($n = 2, 3, 4$) series and monomeric...
TEMPO (10 and 40 mM electron concentration respectively). Reducing the number of atoms separating the two TEMPO moieties increases the electron-electron dipolar interaction and the observed enhancement. The enhancement is larger using TOTAPOL than BT2E. The underlying reason for this result is partly due to the shorter e\(^{-}\)-e\(^{-}\) distance, but could also be related to differences in the electronic relaxation times of the two biradicals, or the relative orientations in which the two TEMPO moieties are frozen.

Figure 2.8 also shows different enhancements for 4 and 2.5 mm rotors. We attribute this to the penetration of the microwaves into the bulk of the sample and possibly the differential attenuation of the microwaves by the sapphire rotors with different wall thicknesses. For systems that are sample-volume limited the smaller rotor offers significant advantages in sensitivity. However, when this is not the case the 4 mm system yields improved signal-to-noise, as even if the enhancement value is smaller, the increased volume still provides a stronger signal overall.
Figure 2.9. (a) Illustration of the growth of the nuclear polarization as a result of μw irradiation using the biradical TOTAPOL (b) as polarizing agent. Integration of the spectral intensities with and without irradiation yields a $^1$H enhancement of $\varepsilon \sim 290$ measured indirectly through the $^{13}$C CP signal using the pulse sequence shown in the inset (c). The measurements were performed on a sample of 3 mM TOTAPOL and 2 M $^{13}$C-urea in $d_6$-DMSO/D$_2$O/H$_2$O (60:34:6 w/w/w) at 90 K, 5 T, and $\omega_c/2\pi = 7$ kHz MAS (spinning sidebands marked by asterisks). The time constant associated with the growth is $\sim 9$ s, approximately the nuclear $T_1$ of the sample. Reproduced with permission from ref. [17]. Copyright 2006 American Chemical Society.

Figure 2.9 shows DNP enhancement buildup obtained on a 2 M $^{13}$C-urea using TOTAPOL as the polarizing agent. The pulse sequence for DNP-enhanced $^{13}$C-CPMAS NMR experiments is shown in inset (c) of the figure. The $^1$H polarization is initially saturated by a series of $90^\circ$ pulses followed by a delay of $3 T_2$. Subsequently, microwave irradiation is applied to dynamically polarize the $^1$H’s or, in the absence of microwaves, the thermal equilibrium polarization is allowed to develop. Finally, the $^1$H polarization is transferred to $^{13}$C via cross polarization and observed in the presence of TPPM decoupling.[54] Figure 2.9 shows the resulting spectra for a range of microwave irradiation periods, leading to an enhancement factor for $^1$H polarization $\varepsilon \sim 290 \pm 30$.
determined by comparing the saturated NMR signals after a 40 s delay with and without microwaves. Note that the error for the enhancement factor was determined primarily by the uncertainty in measuring the intensity of the non-enhanced NMR signal.

Hence at 140 GHz microwave frequency this yields a maximum enhancement of ~290, while the electron concentration is reduced by a factor of 6 from the typical level of 40 to 6 mM. Very importantly, TOTAPOL has hydroxyl and secondary amine moieties on the tether and these functional groups increase the solubility of the biradical in aqueous media so that it is compatible with a variety of biological systems where DNP experiments are currently performed.

New perspectives

Previous sections have focused on MAS SSNMR experiments that have established DNP as a routine technique for improving sensitivity, but extending these experiments to even higher fields, solution NMR, and room temperature requires advancements in instrumentation and theory. This section is devoted to recent developments in solution DNP using an in situ temperature cycling approach.

Temperature Jump Solution DNP

Solution-state DNP relies on completely different processes than the solid-state DNP mechanisms. In this case, none of the solid-state DNP methods, i.e. SE[6], CE [39-41] and Thermal Mixing (TM)[9, 44], can be used directly. The only available mechanism is the Overhauser effect [4, 5, 55], but its efficiency drops considerably at high magnetic fields. In particular, the Overhauser effect is provides efficient polarization
transfer for small molecules with rotational or translational correlation times of $\sim 10^{-12}$ s in low magnetic fields where the condition $\omega_S \tau_c < 1$ is satisfied ($\omega_S$ being the electron Larmor frequency and $\tau_c$ the correlation time). However, in the high field regime commonly employed in modern NMR experiments ($\omega_S > 2\pi \times 100$ GHz), and the rotational and translational spectral densities become vanishingly small. Thus, the Overhauser effect is no longer able to provide usable nuclear polarization enhancements.

With this in mind, Joo and coworkers recently introduced an original solution to this problem in which they perform $^1$H dynamic nuclear polarization at low temperature ($\sim 90$ K), and subsequently detect the solution state NMR spectrum after rapidly melting the sample through in situ irradiation with an IR laser.[56] The experiment, referred to as temperature jump DNP (TJ-DNP), can be recycled using the workflow diagram shown in Figure 2.10a. The sample is cooled to 90 K and polarized in the solid-state using a CE DNP mechanism with the recently introduced biradical TOTAPOL as the source of electron magnetization[17, 48]. The $^1$H polarization is then transferred to low-$\gamma$ spins via cross polarization and the sample melted in situ with an infrared laser pulse. The enhanced signal is finally observed in the presence of $^1$H decoupling. The entire cycle can be repeated in situ and signal averaging performed as in regular solution-state NMR experiments. Note that if the polarization step were performed at a lower temperature a larger enhancement factor would be observed.
Figure 2.10. (a) Pulse sequence used in the TJ-DNP experiment. The samples are irradiated with 140 GHz microwaves at 90 K, polarizing the $^1H$ spins in the sample. Enhanced $^1H$ polarization is then transferred to $^{13}C$ via cross polarization. During the heating period using a 10.6 μm CO$_2$ laser, the $^{13}C$ magnetization is stored along the z-axis of the rotating frame. The $^{13}C$ spectrum is detected following a 90° pulse in the presence of WALTZ $^1H$ decoupling. (b) Experimental spectra obtained for U-$^{13}C$ urea: solid state MAS spectrum produced after 40 s DNP time at 90 K ($\epsilon \sim 290$) and the liquid state spectrum (red) with an enhancement $\epsilon^+ \sim 400$, after a 1.2 s melting period. Note that the DNP spectrum was acquired in a single scan, whereas the black RT spectrum required 256 scans. (c) $^{13}C$ TJ-DNP NMR spectra of (a) Na[1,2-$^{13}C_2$,2-$^2H_3$]-acetate in 60% $^2H_8$-glycerol and 40% water (80% $^2H_2O$/20% H$_2$O) after 40 s polarization and 1 s melting, and (b) [U-$^{13}C_6$,2-$^2H_7$]-glucose in H$_2$O after 30 s polarization time and 1.5 s melting period. Samples contained 3-5 mM TOTAPOL biradical polarizing agent, corresponding to 6-10
mM electrons. The TJ-DNP spectra (the top traces in each figure) were recorded with a single scan, while the room-temperature spectra were recorded with (a) 128, and (b) 512 scans, respectively. Reproduced with permission from ref.[56]. Copyright 2006 American Chemical Society.

Figure 2.10 b) and c) show the TJ-DNP-enhanced $^{13}$C NMR spectra of $^{13}$C-urea, Na-[1,2-$^{13}$C$_2$,2$^2$H$_3$]-acetate, and [U-$^{13}$C,$^2$H$_7$]-glucose. The top traces represent the TJ-DNP-enhanced spectra whereas the bottom traces reflect the signal intensity obtained with $^1$H-decoupled Bloch decays. Deuteration of the samples was employed in order to circumvent $^1$H mediated $^{13}$C relaxation in the viscous solution phase. The enhancements observed in these spectra are $\varepsilon^+ \sim 400$ for urea, $\varepsilon^+ \sim 290$ for sodium acetate, and $\varepsilon^+ \sim 120$ for glucose. Note that the enhancement $\varepsilon^+$ is determined by the intensity of the DNP enhanced signal relative to the signal due to the Boltzmann polarization at 300 K as this represents the relevant enhancement in the case of solution-state DNP experiments. This enhancement can be separated into two distinct contributions due to (a) the polarization gain due to the electron-nuclear polarization transfer and (b) a relative polarization increase due to changes in the Boltzmann polarization at different temperatures. This implies that enhancements reported in the literature for solid-state and liquid-state experiments often differ by the factor ($T_{\text{obs}}/T_{\text{wave}}$), since for solution DNP experiments that include a melting step there can be a large difference between the microwave irradiation ($T_{\text{wave}}$) and the NMR measurement temperature ($T_{\text{obs}}$). For instance, Ardekjær-Larsen and co-workers[57] achieve an enhancement $\varepsilon^+ = 44,400$ by polarizing at 1.2 K, which corresponds to $\varepsilon \sim 178$ and ($T_{\text{obs}}/T_{\text{wave}}$) $\sim 250$. Their experiments reflect an alternate approach to achieve DNP in solution, where the polarization step takes place at low field and very low temperature, followed by dissolution and dilution of the sample.
with superheated water, and transfer to a higher field for detection. Note that this is in
contrast with TJ-DNP where the melting occurs in situ, which allows efficient recycling
of the experiment. Another noteworthy distinction is the polarization time in both
approaches: $\geq 80$ min in the experiment of Ardenkjær-Larsen et al. [57] compared to
typically 40 s for the TJ-DNP approach. This difference is due primarily to the fact that
in the dissolution experiment $^{13}$C is polarized directly whereas in TJ-DNP the $^1$H’s are
first polarized and this polarization is transferred to $^{13}$C.

Figure 2.10 hence shows that significant signal enhancements in the range of 120-
400 can be obtained for low-$\gamma$ spins such as $^{13}$C and $^{15}$N using the TJ-DNP approach as
long as the $^{13}$C $T_1$ is long compared to the melting period and sufficiently long in the
solution phase. The resolution is not degraded by the presence of a paramagnetic
polarizing agent: the $^{13}$C-$^{13}$C J-coupling is resolved in the acetate spectrum (Figure
2.10c).

The TJ-DNP experiment should clearly find a wide application in metabolic
screening, a subject that is of intense interest in the pharmaceutical industry. Moreover
the TJ-DNP experiment could be performed together with multidimensional fast
acquisition techniques allowing acquisition of multidimensional experiments in a few
seconds or even a fraction of a second [58]. Finally, TJ-DNP should be applicable on
proteins and nucleic acids that are robust with respect to the freezing and thawing
process. This is an area of current investigation.

**Pulsed DNP**

Although the CE mechanism provides large enhancements ($\varepsilon \sim 10^2$), it requires
relatively long polarization transfer times on the order of $T_{1n}$ (the nuclear spin-lattice
relaxation time) and becomes less efficient as the $B_0$ field is increased (see Figure 2.1b). The expected $1/B_0$ dependence has recently been confirmed experimentally by comparing enhancements at 5 and 9 Tesla. It is therefore important to investigate and develop different polarization DNP schemes in this high field regime.

In the late 1980’s three alternative DNP techniques were introduced at low magnetic fields: Nuclear Orientation Via Electron Spin Locking (NOVEL), the Integrated Solid Effect (ISE) and the Nuclear Rotating Frame-DNP (NRF-DNP). These three DNP schemes rely on the use of coherent pulses (either RF or microwave) and fall into the class of pulsed-DNP methods as opposed to the CW-DNP methods mentioned above (SE, CE, TM). In principle they should be of interest for applications at high magnetic fields (11-20 T) currently used in biological NMR. More recently the dressed state solid effect (DSSE) was proposed as a fourth time domain DNP mechanism.[59, 60]

The magnetic resonance community encountered a similar barrier for transferring polarization from protons to low $\gamma$ nuclei, the solution to which was a doubly rotating frame pulsed experiment now known as Cross Polarization (CP).[61] This experiment, introduced in 1973, consists of a double on-resonance irradiation and alleviates the field dependency problem. In conventional NMR-CP experiments involving $I=1/2$ nuclei, the rf fields are sufficiently strong that they dominate all other interactions in the Hamiltonian of the spin system. The entire NMR spectrum for both species can be efficiently excited and spin-locked. The Hartmann–Hahn matching condition is then met by matching the rf field strengths $\omega_{15} = \omega_{11}$, allowing an efficient, field independent ZQ polarization transfer to occur.[62] This is illustrated in Figure 2.11a.
The situation is however completely different for pulsed DNP experiments where the electron g-anisotropies and hyperfine couplings are of the order of several or tens of MHz whereas nuclear interactions and rf/μw fields are 1 to 3 orders of magnitude smaller. This leads to different polarization transfer mechanisms. The other major difference with SSNMR CP is that the number of nuclei spins to polarize is much larger than the number electron spins (3-4 orders of magnitude) leading to small single passage polarization transfer. Fortunately, the much shorter electron relaxation time $T_{1e}/T_{1n} \sim 10^3$–$10^4$ allows for a multiple contact experiment within the nuclear relaxation time $T_{1n}$, thereby accumulating nuclear polarization approaching the large ratio of the electron and $^1$H $\gamma$-factors.

The first pulsed DNP experiment we discuss is Nuclear Rotating Frame DNP (NRF-DNP), the corresponding pulse sequence is shown in Figure 2.12a. The principle of NRF-DNP was originally demonstrated in 1958 by Bloembergen and Sorokin for a single crystal of CsBr in which transverse Cs magnetization was built up by cooling the rare $^{133}$Cs spins in the rotating frame with a $^{133}$Cs spin-lock pulse, while irradiating the abundant $^{79}$Br spins with an RF field of frequency $\omega = \omega_{Br} \pm \gamma_{Cs} B_1^Cs$.\[63] Wind and coworkers performed the first NRF-DNP experiment at low field (B$_0$=1.4 T) to enhance the $^1$H magnetization by irradiating electrons during a $^1$H spin-lock pulse. \[64]
Figure 2.11. Energy level diagram comparing various polarization transfer schemes such as (a) CP, (b) Laboratory Frame Solid Effect (LFSE), (c) Nuclear Rotating frame Solid Effect, (d) Dressed State Solid Effect. The polarization transfer is achieved when either allowed or forbidden transition are irradiated (dashed lines). The underlined terms represent interactions that mix eigenstates and allow rf/μw irradiations to drive polarization transfer.
In the nuclear rotating frame the nuclear energy splitting decreases which consequently improves the degree of eigenstate mixing between a single electron-nuclear (e-n) spin pair. This is implicit in equation 3.2a that gives the degree of dipolar mixing $q_L$ and $q_R$ in the laboratory and rotating frames respectively:

$$q_L = -\frac{3}{4} \frac{\gamma_e \gamma_n h}{\omega_{\text{lab}}} \frac{1}{r^3} \sin \theta \cos \theta \exp(-i\phi),$$  \hspace{1cm} (3.2a)

$$q_R = \frac{1}{4} \frac{\gamma_e \gamma_n h}{\omega_{\text{rot}}} \frac{1}{r^3} \left(1 - 3\cos^2 \theta\right),$$  \hspace{1cm} (3.2b)

where $\gamma_e$ and $\gamma_n$ are the electron and nuclear gyromagnetic ratios, $h$ is the Planck’s constant, $\omega_n$ is the nuclear Larmor frequency in either the lab or rotating frame, $r$ is the electron-nuclear interspin distance, and $\theta$ and $\phi$ are the polar coordinates that specify the orientation of the dipole vector in the lab/rotating frame. The increase of nuclear eigenstate mixing in the nuclear rotating frame allows the $\mu$W irradiation to efficiently drive the polarization transfer to the nuclei during a relatively short combined RF and $\mu$W irradiation period ($< 100$ ms).

Using this approach, Farrar et al.\[65\] reported a high field (5 T) NRF-DNP $^1$H single-shot enhancement $\varepsilon$ of 0.89, for a frozen solution of 15 mM trityl radical in 40:60 water/glycerol at 11 K. While this value of the enhancement reflects an effective signal attenuation (compared to thermal equilibrium), the transfer is governed by a very small nuclear rotating frame spin-lattice relaxation time ($T_{1p}=27$ ms) which thus allows signal averaging with a repetition rate orders of magnitude faster than in the CW-DNP experiments, especially at (very) low temperatures. The repetition rate is therefore not limited by the lab frame spin-lattice relaxation time $T_{1n}$, which is typically many minutes.
at 10 K for non-paramagnetically doped samples. The increased repetition rate allowed by the NRF-DNP experiment translates to a real-time signal enhancement of $\varepsilon_r \approx 197$, where $\varepsilon_r$ is the ratio of the signal-to-noise per unit time $^{1/2}$ with and without $\mu$w irradiation. The NRF-DNP experiment allows one to leverage the increased sensitivity provided by low temperatures (since $\varepsilon$ is close to 1) while avoiding the limitations of slower repetition times due to the very long nuclear spin-lattice relaxation times induced by the cryogenic temperatures for systems. Note that this is only valid if nuclear spin diffusion is not the rate limiting process to polarize the bulk nuclei.

An important point to highlight is that this DNP experiment was performed with a low power Gunn Diode source (power at the sample $\approx$1-5 mW) and no resonant structure. Under the same experimental conditions, the NRF-DNP does indeed compare favorably with optimized lab frame CE-DNP due to its faster recycling time and hence improved signal-to-noise per unit time $^{1/2}$ ($\varepsilon_{\text{ref}} / T_{\rho}^{1/2} = 4.4$ for 15 mM trityl radical and $\varepsilon_{\text{lab}} / T_{1n}^{1/2} = 1.7$ for 25 mM 4-amino TEMPO radical respectively)

By rewriting the e-n Hamiltonian (see 3.1) and the on-resonance RF irradiation, in the tilted nuclear rotating frame, one can understand the nature of the polarization transfer:

$$H_0^{SI} + H_i^{RF} = \omega_{0z}S_Z - \omega_{ii}I_Z + AS_zI_x,$$  \hspace{1cm} (3.3)

From this equation, it is clear that the “secular” term of the hyperfine coupling in the lab frame, $A$, is no longer secular with respect to the electron lab frame and nuclear rotating frame Larmor frequencies. In this experiment, the non-secular hyperfine coupling has been neglected as $\omega_{0i} \gg B$ in the high field regime considered in this
review. Equation 3.3 shows that the secular hyperfine coupling $A$ mixes the eigenstates in the nuclear tilted frame and allows the µw irradiation to drive the polarization transfer. By writing the µw irradiation in the tilted nuclear rotating frame, we obtain the matching condition for the nuclear rotating frame SE (see Figure 2.12c):

$$\omega_{\text{mw}} = \omega_{05} \pm \sqrt{(A/2)^2 + \omega_{rf}^2},$$

(3.4)

Where $\omega_{\text{mw}}$ represents the frequency of the µw irradiation.

In the limit of a weak RF field strength ($\omega_{rf}/2\pi \sim 100$ kHz) compared to the hyperfine coupling to protons in trityl (~1 MHz), the above expression simplifies:

$$\omega_{\text{mw}} = \omega_{05} \pm A/2.$$  

(3.5)

The NRF-DNP experiment (as is also indicated by 3.2) is not dependent on the static magnetic field strength, suggesting that large DNP enhancements should be obtainable even at magnetic fields larger than 5 T. This definitely appears as a significant advantage over the lab frame DNP experiments which all exhibit an inverse dependence with the $B_0$ magnetic field.

The second high field pulsed DNP mechanism we discuss was introduced in 2000 by Weis et al.[59, 60] as the Dressed State Solid Effect (also referred to as electron nuclear CP (e-NCP)) and explained as a solid effect mechanism in the electron “dressed state” (defined by the µw irradiation).[67, 68]
Figure 2.12. (a) NRF-DNP pulse sequence. The NRF-DNP enhancements are determined from a comparison with the lab frame signal obtained with a solid-echo pulse sequence and no $\mu w$ irradiation or nuclear spin-lock. (b) Pulse sequence for the indirect detection of electron-nuclear cross polarization. The electron spin echo intensity is monitored after an electron spinlock and a refocusing $\pi$-pulse. The echo intensities of two sequences with and without rf pulses are subtracted and recorded as a function of the rf frequency, $\omega_{rf}$. Reprinted from refs. [65, 66]. Copyright 2000 and 2006, with permission of Elsevier.

This experiment relies on a simultaneous $\mu w$ spin-lock of the electron magnetization and an off-resonance rf irradiation of the protons that can be set to match the strong $\gamma B_{1e}$ electron Larmor frequency in the rotating frame. The interaction of the electron spin with the $\mu w$ field is treated as an electron spin dressed state. In contrast to the customary laboratory frame solid effect, it is possible to obtain nuclear polarization with the dressed state solid effect DSSE even in the absence of non-secular hyperfine coupling.

Efficient, selective excitation of forbidden dressed state transitions (see Figure 2.11d) generates nuclear polarization in the nuclear lab frame on a time scale of $\mu s$. 
depending on the strength of the electron-nuclear coupling, the \( \mu \) and rf irradiation, resulting in a repetition rate comparable to \( T_{1e}^{-1} \), where \( T_{1e} \) is the electronic spin lattice relaxation time. These frequencies fall in to the radio-frequency range and are given by the following matching condition:

\[
\omega_{\sigma} = \omega_{0l} \pm \sqrt{\left(\frac{A}{2}\right)^2 + \omega_{ls}^2}
\]  

(1)

where \( \omega_{\sigma}, \omega_{0l}, \omega_{ls} \) and \( A \) stands for the rf field frequency, the nuclear Larmor frequency, the electron rotating frame Larmor frequency and the hyperfine coupling constant.

The pulse sequence of the DSSE experiment is displayed in Figure 2.12b. The electron spin echo intensity is monitored after an electron spin-lock and a refocusing \( \pi \)-pulse. The echo intensities of two sequences with and without rf pulse are subtracted and recorded as a function of the rf frequency \( \omega_{rf} \) and thus represent an indirect detection of electron-nuclear CP. Experimental results obtained on a perdeuterated BDPA-d_{21} radical in a protonated polystyrene matrix are shown in Figure 2.11 using a 3 \( \mu \)s on-resonance spin-lock irradiation.

The EPR spectra display three lines representing the three dressed state transitions. The positions of the outer peaks change with the \( \mu \) power applied and converge towards the nuclear Larmor frequency \( \omega_{0l} \) as the \( \mu \) is decreased, which is in agreement with (1). The upper traces of Figure 2.11 show the variation of the transferred polarization for two rf field strengths (50 and 350 W, \( \omega_{11}/2\pi\sim100 \) kHz at 350 W) and a
fixed μw field strength. A change in the rf power does not result in a detectable shift or broadening of the CP matching condition which is expected from (15). However, a decrease in signal intensity is observed upon reduction of the rf power indicating that the polarization transfer is faster for larger rf fields, and a detailed analysis of the time evolution supports this observation [43].

**Conclusions**

We have reviewed experiments on a wide range of samples that demonstrate large NMR signal enhancements available using a common technique of polarizing the bulk nuclei with DNP and subsequent spin diffusion. In 2000 we reported enhancements of ~10 in MAS spectra recorded at 90 K. Subsequently we have improved that figure to 290 with advances in theory, polarizing agents and instrumentation. Future DNP instrumentation developments together with theoretical treatments of the SE and CE, represent an avenue for extending DNP of biologically relevant systems to even higher magnetic fields, and improving enhancements that approach the theoretical maximum of 657. These experiments should lay a foundation for conducting magnetic resonance experiments at higher fields and on systems that have heretofore been inaccessible due to low sensitivity.
Figure 2.11. DSSE/eNCP experiment on perdeuterated BDPA for various settings of the μw (ω₁/2π ~ 1.75, 0.9 and 0.5 MHz) and rf field strengths (ω₁/2π ~ 100 kHz at 350 W). The CP contact time was set to 3 μs. Reprinted from ref.[65, 66]. Copyright 2006, with permission of Elsevier.

Acknowledgments

A.B.B. acknowledges receipt of a NSF graduate research fellowship. This research was supported by the National Institutes of Health through grants EB001960, EB002804, EB002061, EB004866, and EB002026.
References


Chapter 3. THz Dynamic Nuclear Polarization NMR

Adapted from “THz Dynamic Nuclear Polarization NMR” by Emilio A. Nanni, Alexander B. Barnes, Robert G. Griffin, and Richard J. Temkin. Accepted for publication in the inaugural issue of IEEE THz, 2011.

Abstract

Dynamic Nuclear Polarization (DNP) increases the sensitivity of NMR spectroscopy by using high frequency microwaves to transfer the polarization of the electrons to the nuclear spins. The enhancement in Nuclear Magnetic Resonance (NMR) sensitivity can amount to a factor of well above one hundred, enabling faster data acquisition and greatly improved NMR measurements. With the increasing magnetic fields (up to 23T) used in NMR research, the required frequency for DNP falls into the THz band (140 to 600 GHz). Gyrotrons have been developed to meet the demanding specifications for DNP NMR, including power levels of 10 to 100 Watts; frequency stability of a few MHz; and power stability of 1% over runs that last for several days to weeks. Continuous gyrotron frequency tuning of over 1 GHz has also been demonstrated. The complete DNP NMR system must include a low loss transmission line; an optimized antenna; and a holder for efficient coupling of the THz radiation to the sample. This paper describes the DNP NMR process and illustrates the THz systems needed for this demanding spectroscopic application. THz DNP NMR is a rapidly developing, exciting area of THz science and technology.
Introduction

Nuclear Magnetic Resonance (NMR) is the preferred spectroscopic approach for the solution of problems in many areas of science, including physics, chemistry, biology, materials science, and medicine. The excellent resolution of NMR is a consequence of long nuclear relaxation times that are in turn due to the small magnetic moments of the nuclear spins that couple weakly to the surrounding lattice. The small size of these magnetic moments however leads to reduced sensitivity in NMR experiments.

Dynamic nuclear polarization (DNP) increases the sensitivity of NMR by transferring the large spin polarization from stable paramagnetic centers to the nuclear spin reservoir [1]. In 1953 Overhauser [2] proposed that it was possible to transfer polarization to nuclei from electrons in metals by saturating the electron transition. This technique was experimentally verified by Carver and Slichter [3]. The DNP approach to NMR was extended to solid dielectrics by Abragam and Proctor [4] who first characterized the solid effect (SE). In the 1980s, Wind, Yannoni, Schaefer and colleagues [5-7] performed DNP in conjunction with magic-angle-spinning (MAS) NMR.

Microwave driven DNP experiments are now recognized as a powerful method of enhancing signals in solid state and solution NMR and imaging. DNP improves the sensitivity of NMR spectra by about a factor of 100, thus reducing the acquisition time in multidimensional NMR experiments by roughly $10^4$ [8]. This enhancement permits studies of larger molecules, reaction dynamics or high-throughput screening [9]. The theoretical maximum enhancement in the case of $^1$H spins is given by the relationship $\gamma_s/\gamma_i \approx 660$ [2] where $\gamma_s$ and $\gamma_i$ are the electron and nuclear gyromagnetic ratios, indicating further developments to DNP methods and instrumentation should result in still higher...
enhancements. The power level desired from the THz source in DNP is highly dependent on several experimental factors including the mechanism of polarization, sample temperature, sample volume, polarizing agent and coupling efficiency of the source. Fundamentally, the power level needed is related to the electron relaxation rate which must be overcome in order to transfer polarization to the nucleus [10, 11]. Experimentally, the theoretical maximum enhancement from DNP is not achieved and is often limited by the available power.

These early, successful efforts in microwave driven DNP NMR could be extended to magnetic fields of up to about 3.4T using commercially available sources, such as solid state multipliers, klystrons, etc., at a frequency of up to 94 GHz [12]. However, modern NMR spectroscopy has pushed to much higher magnetic fields, where much better spectral resolution is achieved. The extension of DNP NMR to high magnetic fields had to wait for the development of THz sources of radiation at the relevant frequencies. Fortunately, the development of the THz gyrotron enabled this extension of DNP NMR into the high magnetic field regime.

The first DNP NMR experiments using a gyrotron were conducted by Becerra et al. at the MIT Francis Bitter Magnet Laboratory at a magnetic field of 5 T, corresponding to 211 MHz NMR frequency and 140 GHz microwave frequency [13]. This successful advance was followed at MIT by extension of the technique to 9T with the development of a 250 GHz gyrotron for use in a 380 MHz spectrometer [14] and later extension to 16 T with the development of a 460 GHz gyrotron for a 700 MHz spectrometer [15]. Research on the gyrotron also led to the discovery of broadband tuning of the gyrotron.
source, which could be useful in partially covering the electron spin resonance spectrum in DNP NMR experiments [15, 16].

As described by Griffin and Prisner in a recent article, DNP NMR is truly enjoying a “renaissance” at the present time [9]. Some examples of recently developed THz DNP NMR systems include a 260 GHz / 400 MHz spectrometer in operation in Frankfurt, Germany [17, 18] and a 395 GHz / 600 MHz spectrometer operating at the Osaka Institute in Japan [19]. Instrumentation for a 263 GHz / 400 MHz DNP NMR system has been developed by industry and used in DNP NMR research [18, 20, 21].

This paper is organized as follows. In Section II, the THz DNP NMR technique is described in detail. Section III describes the source technology used in THz DNP NMR. Section IV describes the transmission lines for transporting the radiation from the source to the sample. Section V describes the sample probe, sample holder and the coupling of the THz power into the sample. Section VI is the Summary and Conclusions.

**THz Dynamic Nuclear Polarization**

The structure and function of biomolecules are strongly correlated. Determining three-dimensional structural detail of proteins at an atomic resolution is crucial to understanding how they work as machines, how they catalyze chemical reactions, how they bind to each other, as well as their interaction with drugs and signaling molecules. By far, the most powerful technique available for structure determination is X-ray diffraction. However, there are certain drawbacks of X-ray crystallography; namely, the highly ordered crystalline environment required to record diffraction patterns is not a truly biological environment. In vivo, proteins are in solution, bound to or embedded inside the cellular membrane, intrinsically disordered, or arrange themselves into amyloid
fibrillar strands. For such molecules in a biologically realistic environment, X-ray diffraction is not applicable and the magnetic resonance of nuclear and electron spins can be leveraged to determine structural detail. In particular, the site-specific signals of nuclear spins can reveal sub-angstrom level structural detail of proteins and molecules. Due to the excellent resolution of NMR spectra, NMR has evolved as the preferred spectroscopic approach for the solution of problems in many areas of science, including physics, chemistry, biology, materials science, and more recently medicine.

Nevertheless, the sensitivity of NMR experiments is low when compared to other spectroscopic approaches. Furthermore, since both high resolution solid and solution state NMR are utilized with increasing frequency in structural studies of macromolecular biological systems-proteins, nucleic acids, etc.-sensitivity continues to be an issue of paramount importance in the successful application of the technique.

DNP has successfully improved the sensitivity in NMR experiments by factors of 20-400 (corresponding to 400-160,000 in acquisition time) \[23, 24\], depending on the experimental conditions such as temperature, solvent composition, deuteration levels, radical type and concentration, etc. The increased signal intensity shortens the acquisition time, reduces the amount of sample required and allows the acquisition of multidimensional spectra with high signal to noise. The following subsections illustrate the mechanism of polarization transfer for efficient DNP at high fields and show that large DNP polarization enhancements are consistently obtained at high field. These results demonstrate applications to structural studies of biologically significant systems which would not be feasible without DNP.
Figure 3.1. Common DNP polarization transfer mechanisms in solid dielectrics. a) In the SE, one electron and one nuclear spin flip simultaneously with excitation from THz radiation, whereas the CE involves two electron spins and one nuclear spin. b) EPR spectra of the radicals that are the polarization source in THz DNP experiments. (left) The SE utilizes narrow line radicals such as trityl. (right) EPR spectra of nitroxide radicals such as TEMPO or TOTAPOL are much broader and used for the CE. c) Field/frequency dependent profiles of the two mechanisms. In the SE (left) the THz source is set to $\omega_{01}$ away from the center of the EPR line. (right) For the CE, the THz radiation is on resonance with one of the two electrons involved. d) chemical structures of typical radicals used for the SE; trityl (left) has a mostly isotropic environment around the unpaired electron density which results in a similar resonance frequency (narrow line) across the powder average of the crystallites. TOTAPOL (right) for the CE, more anisotropic chemical environments around the electron density yield broader lines such that two electrons are separated by $\omega_{01}$ and fulfill the matching condition. (Adapted from [22])
Mechanisms of DNP

DNP was first proposed and performed in the 50's [2, 3], and in the 80's DNP experiments were incorporated into MAS experiments with a goal of increasing signal intensities in the spectra [6, 7]. MAS of samples in solid-state NMR (SSNMR) spectroscopy reduces line broadening in spectra by spinning at the “magic angle” of 54.7 degrees with respect to the magnetic field, B_0. These initial MAS DNP experiments used 40 GHz microwaves (1.5 T) and the solid effect (SE); signal enhancements of 25 were observed at ~300 K [5, 25-28]. Subsequently, DNP experiments were initiated at MIT at 140 and 250 GHz (5 and 9 T fields) [13, 14, 29-33] using gyrotrons.

The two mechanisms that are most important for continuous wave (CW) DNP processes in MAS experiments are the solid effect (SE) and the cross effect (CE). Which of the two mechanisms dominate (SE or CE) depends on the size of the inhomogeneous breadth, Δ and the homogeneous breadth, δ of the electron paramagnetic resonance (EPR) spectrum, compared to the nuclear Larmor frequency, ω_0

The SE is a two spin process (Figure 3.1a left) that governs the polarization process when ω_0 > Δ,δ and thus requires a radical with a narrow line such as trityl (Figure 3.1b, d left). The frequency dependence of the enhancement profile for trityl is illustrated in Figure 3.1c (left) and shows minima and maxima when the irradiation frequency is ω_0±ω_0, where forbidden electron-nuclear flip-flops are excited leading to a negative or positive enhancement. Since the SE utilizes forbidden transitions the enhancements scale as ω_0^{-2} and are therefore attenuated at higher fields [34].

The CE, a three spin effect (Figure 3.1a, right), is a DNP mechanism that has been shown to yield more efficient transfers at high-field. It relies on the fact that the
resonance frequencies of two electrons, $\omega_{0s1}$ and $\omega_{0s2}$ in the EPR spectrum, satisfy the condition $\omega_{0s1}-\omega_{0s2}=\omega_{01}$ (Figure 3.1b,c right) and as such the enhancements scale as $\omega_{0}^{-1}$. Furthermore, the CE works well with a large set of radicals such as nitroxides (TEMPO and its relatives), in which $\Delta>\omega_{01}>\delta$ at high field. Assuming dipole-dipole couplings exist amongst the two electrons and the nucleus, THz irradiation near either $\omega_{1s}$ or $\omega_{2s}$ flips one of the electrons up, and a subsequent three spin cross relaxation process with the energy difference between the two electron spins going into polarizing the nuclear spin.

The efficiency of CE depends on two spatial factors: (1) the distance between the electron spins, which determines the electron-electron dipolar coupling and (2) the relative orientation of the two radicals, which determines, via the g-anisotropy tensors, the frequency separation $\omega_{0s1}-\omega_{0s2}$. For the case of biradical polarizing agents, both of these factors can be optimized by design of the molecular linkage tethering two nitroxide groups such as that found in 1-(TEMPO-4-oxy)-3-(TEMPO-4-amino)propan-2-ol (TOTAPOL) [35] (Figure 3.1d, right) and bis-TEMPO-bis-ketal (bTbK) [23]. The magnitude of the maximum enhancement obtained with the water-soluble TOTAPOL radical is ~175 at 212 MHz with 6 W of THz power [35], an improvement by a factor of ~4 from monomeric nitroxides, like TEMPO. Since the tether connecting the two TEMPO groups is relatively flexible, their relative orientation is not tightly constrained and many biradicals do not have the correct geometry corresponding to the desired separation frequency. Therefore, with a more rigid tether that can lock the two TEMPOs at a desired relative orientation, it is possible to further improve the performance of a polarizing agent. Enhancements of 250 with 4 W of THz power are achieved using bTbK, a new biradical connecting two TEMPOs with a rigid bis-ketal tether [23]. Thus,
Figure 3.2. Electron micrographs and 0.263 THz DNP spectra of GNNQQNY showing a signal enhancement of a) 20 on the nanocrystals and b) 35 on the amyloid fibrils. (Adapted from [37], [38], and [39])

significant gains in enhancements have been realized by optimization of polarizing agents for high-field DNP.

An important point illustrated in Figure 3.1c is that magnetic field (B₀) has to be swept to the appropriate point in the EPR spectrum in order to optimize the DNP enhancement. In present day DNP spectrometers, this is accomplished with a superconducting sweep coil that was installed on the magnet at the time it was manufactured. In NMR magnets that lack a sweep coil, a tunable source of THz radiation would be useful, see discussion in Section III.F.
Other spectroscopic techniques are enabled by double resonance (NMR and microwave) methods. The Overhauser effect (OE) [2] is important for conducting solids (metals, etc.) and solution samples. Thermal mixing involves a homogeneously broadened EPR spectrum [36] that is present at low magnetic field and as such is not applicable for THz DNP.

Applications of THz DNP in Solids

DNP has been demonstrated to be an extremely versatile technique, resulting in drastic gains in sensitivity in a wide range of biological systems ranging from nanocrystalline peptides [37] and amyloid fibrils [39], to proteins in virus capsids [40], soluble proteins [31], and membrane proteins embedded in lipid bilayers [31]. Similar DNP strategies have recently been extended to materials research and inorganic chemistry applications to enhance signals from nuclear spins on surfaces [41]. This section describes in more detail some of these initial applications of THz DNP.

Amyloid Fibrils and Nanocrystals

Proteins are chains of amino acids strung together through covalent peptide linkages. After synthesis, they must fold into the correct three-dimensional structure to perform their biological function. Proteins that miss-fold can aggregate and cause numerous diseases such as Alzheimer's and cystic fibrosis [42, 43]. DNP NMR is well suited to determine the atomic resolution structure of these pathogenic aggregates [44], sometimes referred to as amyloid fibrils or prions. Understanding the structure can yield insight into how and why they form and in turn help combat the associated diseases.

GNNQQNY$_{7-13}$ (where each letter stands for a single peptide) is a polypeptide originating from the yeast prion protein Sup35p that can form either nanocrystals or amyloid fibrils. At 212 MHz $^1$H frequency and 90 K, 0.140 THz DNP yields a
substantial enhancement factor of 120 on nanocrystals of GNNQQNY, permitting the acquisition of 2D spectra in only 20 minutes due to the excellent sensitivity available with DNP [37]. The biradical polarizing agent, TOTAPOL, is too large to penetrate into the nanocrystals, yet leads to $^{13}$C and $^{15}$N spectra with enhancements of 120 for the peptide resonances and 160 for the solvent, illustrating that $^1$H spin diffusion effectively distributes the enhanced polarization throughout the lattice. These initial experiments have been extended to higher fields in a study using a 263 GHz commercial Bruker spectrometer [20] which demonstrated an enhancement of 20 on nanocrystals (Figure 3.2a) and 35 on amyloid fibrils of GNNQQNY (Figure 3.2b). This drastic gain in sensitivity may be used for structural determinations of GNNQQNY and other fibrils.

**Membrane Proteins**

Bacteriorhodopsin (bR) is a membrane protein which functions as a light-activated H$^+$ pump. A retinal chromophore in the center of the protein that is at equilibrium between two conformations (Figure 3.3b) absorbs a photon of light that initiates a photocycle consisting of distinct intermediates (Figure 3.3a). Despite the fact that there are now ~70 crystal structures available for this protein, the mechanism for its function as a pump is not yet established. The X-ray structures have thus far been unable to resolve essential structural details such as torsion angles in the retinal chromophore in the photocycle intermediates [48] and to discriminate if the protein is an H$^+$ pump (as assumed for 30 years) or a backward directed OH$^-$ pump.
Figure 3.3. a) Photocycle illustrating the intermediates examined with DNP enhanced MAS spectra. b) The conformational equilibrium of the retinal chromophore in the resting state. The yellow box highlights the nuclei in the spectra in the lower panels. c) 2D spectra of the active site of bR showing correlation peaks between C14 and Ce recorded in 7 hours with THz DNP and 5 days without THz DNP even with 10 times more sample. d) build-up profiles of the correlation between C14 and Ce. The profiles are fit and yield a distance of 3.11±0.22 Å between the C14 and Ce in bR555 and 3.90±0.08 Å in the bR568 trans conformation. e) $^{15}\text{N}_{5}^{13}\text{C}15$ correlation experiments provide assignments of the retinal-C15 resonance in each state: (left) the dark-adapted state; (right) the mixture of L intermediates. (Adapted from [45], [46] and [47])
SSNMR structural methods are capable of measuring torsion angles and internuclear distances with high precision, but the relative insensitivity of traditional SSNMR is a severe limitation with large proteins. Recording a single 2D data set on a protein with a large effective molecular weight of 32 kiloDaltons typically requires 10 days of signal averaging. However, with DNP enhancements even as low as ε=20, it can be done in less than an hour. This in turn permits the measurement of a variety of torsion angles and distances in the resting state and in photointermediates that will elucidate the pumping mechanism of this protein. Accordingly, experiments confirm that DNP is capable of enhancing the NMR signals in the active site deeply buried in bR by a factor of 40 [49]. Figure 3.3c,d shows 2D correlation spectra of a sample which has specific labels at the C14 and Cε position in the active site (yellow box of Figure 3.3b). With THz DNP in only 7 hours it is possible to record a spectrum showing correlation peaks between the C14 and Cε carbons [45]. With the sensitivity boost from THz DNP, it was possible to record multiple correlation spectra in which the polarization transfer time between the two carbon sites was varied. The dipolar coupling strength, which is directly related to the distance between the two spins, can then be extracted by the build-up profiles of the correlation peak intensity (Figure 3.3d). In addition to these experiments that measured inter-nuclear distances to sub-angstrom precision, THz DNP also revealed heterogeneity in the different conformational states of bacteriorhodopsin [47].

In the resting state of the protein, what was previously thought to be only one conformer, bR555 clearly is resolved as two resonances in the 2D spectrum (Figure 3.3e, left). Furthermore, the L intermediate (blue box of Figure 3.3a) was shown to actually
consist of four states (Figure 3.3e, right), three of which are shunt states that do not result in a proton being pumped across the membrane [47].

**DNP for Solution State NMR**

There is considerable interest in applying DNP techniques to high field solution NMR experiments. Recently, significant progress has been made in performing DNP directly in the solution state using a 263 GHz gyrotron [17]. However, it is also possible to perform a polarization step and melting *in situ*, the latter being performed with CO₂ laser radiation. This experiment is referred to as *in situ* temperature jump DNP (TJ-DNP) [24].

A schematic representation of the TJ-DNP cycle is shown in Figure 3.4. In the experiment, the DNP NMR probe was maintained at 90 K, and the frozen sample was polarized with microwave irradiation at 140 GHz. Polarization was then transferred by cross polarization from \(^1\text{H}\) to \(^{13}\text{C}\). The sample was then rapidly melted by a CO₂ laser pulse (10 W for 1.2s, \(\lambda=10.6 \mu\text{m}\)), and a \(^{13}\text{C}\) solution NMR signal was immediately recorded with \(^1\text{H}\) decoupling. Figure 3.4b shows the solution spectrum (right) as well as the SSNMR \(^{13}\text{C}\) spectrum obtained with DNP but without melting. For this sample (\(^{13}\text{C}\)-urea in DMSO/water), the sensitivity enhancement that was observed was 400±15 relative to the normal solution NMR experiment carried out entirely at room temperature [24]. Figure 3.4 shows a spectrum in which TJDNP is integrated into a 2D \(^{13}\text{C}\)-\(^{13}\text{C}\) experiment.
**Gyrotrons for DNP NMR**

**THz Sources**

As DNP NMR experiments move to higher magnetic fields, several requirements make gyrotrons the most practical source for THz radiation. As seen in Table I, the THz source should ideally produce a minimum of 20 W CW. When coupled with typical transmission line efficiencies of 50 to 80% from the source to the sample, the available power at the sample will be sufficient for saturation of the enhancement in most cases. It is highly desirable to have the output beam in a nearly Gaussian free space mode capable of direct injection and matching into the waveguide transmission line. For a Gaussian
beam, the ideal waist size is equal to about 0.64 of the waveguide radius of the transmission line. Table I also lists the frequency and power stability needed for the duration of the NMR signal acquisition period ($\geq 36$ hours). Additionally, the life time of the device must also be considered, due to the major capital and personnel investments involved in NMR research.

The generation of millimeter, sub-millimeter and THz radiation at high power has proved to be a significant challenge. In Figure 3.5, updated from the review article [50], the state of the art capabilities for solid state and vacuum electron sources is shown. As seen in Figure 3.5, solid state devices in the THz range suffer from scalability and efficiency issues, that lead to limited output powers. However, they have proved useful in some DNP NMR experiments that use small sample sizes and metallic resonators [6, 51-53] suitable for aqueous and static (non-MAS) DNP experiments. These experiments have relied on sample volumes that occupy a small fraction of the resonator. However, to optimize S/N in MAS DNP experiments, large sample volumes are required. Furthermore, metallic resonators or rotating metallic components are not permissible in the stator as they would produce eddy currents and levitation effects.
Figure 3.5. State-of-the-art of solid state and vacuum electron devices. (Adapted from[50])

<table>
<thead>
<tr>
<th>Requirement</th>
<th>Requirement Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency (~2B₀ GHz)</td>
<td>~28*B₀ GHz</td>
</tr>
<tr>
<td>(B₀ = NMR field in T)</td>
<td>(B₀ = NMR field in T)</td>
</tr>
<tr>
<td>Output Power</td>
<td>≥ 20 W</td>
</tr>
<tr>
<td>Power Stability</td>
<td>± 0.5 %</td>
</tr>
<tr>
<td>Frequency Stability</td>
<td>&lt; 2 MHz</td>
</tr>
<tr>
<td>Run Time</td>
<td>&gt; 36 Hours</td>
</tr>
<tr>
<td>Lifetime</td>
<td>&gt; 50,000 Hours</td>
</tr>
<tr>
<td>Voltage Regulation</td>
<td>0.1%</td>
</tr>
<tr>
<td>Current Regulation</td>
<td>1%</td>
</tr>
<tr>
<td>Output Mode Purity</td>
<td>&gt;90%</td>
</tr>
</tbody>
</table>
Classical microwave tubes, e.g. klystrons and traveling wave tubes, can produce high power (hundreds of Watts) electromagnetic radiation up to 100 GHz, but these slow wave devices require physical structures in the interaction cavity that are much smaller than the wavelength of operation. This small element size produces difficulties with thermal damage and manufacturing of the interaction cavity when the frequency is extended into the THz range. Furthermore, at THz frequencies slow wave devices suffer from reduced lifetime due to electron beam interception and high heat loads. The Extended Interaction Klystron (EIK) is a possible source for THz radiation needed in DNP experiments. As an oscillator it has achieved an average output power of 7 W at 220 GHz, and an average output power of 0.3 W at 280 GHz [54, 55]. The EIK is promising for DNP applications, but has limited power and may have limited lifetime at THz frequencies.

Electron cyclotron resonance masers (gyrotrons) are capable of producing high average power in the microwave, millimeter wave and THz bands [56]. Due to the overmoded cavities used in gyrotrons, they remain the only demonstrated, highly stable device capable of producing adequate power levels (a minimum of 10 W CW) with an adequate lifetime (about 100,000 hours), in the frequency range of interest for DNP NMR.

Fundamentals of Gyrotrons
Figure 3.6. Cross-sectional schematic of a typical cylindrically symmetric gyrotron tube used in DNP NMR experiments, not shown to scale, indicating key components. (Adapted from [15])
In gyrotrons the emission of coherent THz radiation results from the resonant interaction between the eigenmodes of a cavity, typically cylindrical, and a mildly relativistic electron beam that is gyrating in a constant axial magnetic field. The basic configuration of a gyrotron, Figure 3.6, consists of a magnetron injection gun (MIG) that launches an annular electron beam into the hollow bore of a solenoidal magnet. The magnetron injection gun is located at the bottom of Figure 3.6. The axial magnetic field is produced by a DC superconducting magnet. A second magnet can be added at the location of the MIG to provide additional control of the electron beam compression and velocity ratio. In the MIG, the orientation of the DC electric field that extracts the electron beam from the cathode produces a beam that has both a perpendicular and parallel velocity component with respect to the axial field produced by the solenoidal magnet. As the electron beam travels into the central bore, it undergoes adiabatic compression that increases its orbital momentum. The electrons enter a metallic cavity that has an eigenmode resonance that is close in frequency to a harmonic of the gyration frequency of the electron. The electron beam surrenders some of its kinetic energy to the electromagnetic mode through stimulated emission. The electrons exit the cavity and are deposited on a metallic collector. The gyrotron resonator supports a high order cavity mode that is difficult to transport over long distances. It is often convenient to convert the wave into a Gaussian beam that can be efficiently extracted in a transverse direction using mirrors.

In order for the electron beam to interact with the electromagnetic mode in the cavity, a harmonic of the relativistic electron frequency must be close to the frequency of oscillation for the cavity mode [57]. The relativistic cyclotron frequency is
\[ \Omega_c = \frac{eB_o}{\gamma m_e} \] (3.1)

where \( e \) is the charge of the electron, \( B_o \) is the DC axial magnetic field, \( m_e \) is the electron mass and \( \gamma \) is the Lorentz factor \((1-v^2/c^2)^{-1/2}\), where \( v \) is the velocity of the electron, \( c \) the speed of light. In a cylindrical cavity, of radius \( r_w \) and length \( L \), the dispersion relation for TE modes is

\[ k^2 - k_r^2 = 0 \] (3.2)

where \( k = \omega/c \) is the wave vector in free space, \( \omega \) is the frequency of the THz wave, \( k_r = \nu_{mn}/r_w \) is the transverse propagation constant, \( \nu_{mn} \) is a root of the Bessel function \( J'_m(x) \) and \( k_z \) is the axial propagation constant (approximately \( q\pi/L \), where \( q \) is an integer).

The cyclotron beam mode dispersion relation or Doppler shifted resonance condition is

\[ \omega - k_z v_z - s\Omega_c = 0 \] (3.3)

where \( v_z \) is the axial velocity of the electrons and \( s \) is the integer cyclotron harmonic number. When these dispersion relations are equally satisfied, as seen in Figure 3.7, oscillation and stimulated emission can occur. The dotted/dashed line and the dashed line in Figure 3.7 are the cyclotron beam mode dispersion relation for \( s = 1 \) and \( s = 2 \), respectively. The black circle is a fundamental forward wave oscillation, the square
is a second harmonic forward wave oscillation and the diamond is a fundamental backward wave (BW) oscillation. Traditionally, gyrotrons interact with the fundamental forward wave [13, 14, 20], however the second harmonic forward wave is useful for higher frequency experiments [15, 16, 58, 59] and the BW interaction is used at the first or second harmonic to provide the frequency tunability in gyrotrons for DNP NMR experiments.

The cavity in a gyrotron typically consists of a central cylindrical region, with a down taper on the gun side and an uptaper on the collector side. A cross section of the cavity is shown in Figure 3.8. Superimposed over the cavity is the axial electric field profile for the mode of the cavity shown, which is a TE$_{4,3,1}$ mode. The interaction
between the electron beam and the cavity mode occurs only at the radius of the annular electron beam, \( r_a \), as shown in the insert of Figure 3.8. Because the cavity modes are non-uniform in the radial direction, the strength of the interaction between the beam and a cavity mode is determined by the radial placement of the electron beam. This can be advantageous (especially in second harmonic experiments) by allowing for the selection of a desired mode.

**Nonlinear Theory of Gyrotrons**

For an exact understanding of the operation of a gyrotron oscillator, the interaction between an energetic electron and the electromagnetic mode confined in the interaction cavity (resonator) must be described. In a gyrotron, kinetic energy is extracted from the electron beam as it passes through the interaction cavity. However, a gyrotron does not extract any energy from the axial component of the velocity, only from the transverse component. This energy extraction is made possible because an electron traveling in a circular path orthogonal to a DC magnetic field will feel an accelerating or decelerating force from the resonant oscillating electric field that can deposit or extract energy from the electron. The initial applied force from the cavity mode results in some electrons gaining energy and some electrons losing energy. The more energetic electrons rotate slower and the less energetic electrons rotate faster, because of the dependence of \( \gamma \) and \( \Omega_c \) on velocity and energy. The different rotational frequencies produce phase bunched electrons that act coherently to deposit energy into the electromagnetic mode of the cavity. This process can be described by the pendulum equations that relate the change in energy and momentum for the electron to the electric and magnetic fields that are present. The equations of motion for the electron are
\[
\frac{\partial e}{\partial t} = -ev \cdot E
\]
\[\text{(3.4)}\]

\[
\frac{\partial p}{\partial t} = -e(E + v \times B)
\]
\[\text{(3.5)}\]

where the electron energy is \(e\), the momentum \(p\), the THz electric field is \(E\), the DC magnetic field is \(B\) and the velocity of the electron is \(v\). The instantaneous kinetic energy and momentum of a relativistic electron are \(\gamma m_e c^2\) and \(|p| = \gamma \beta m_e c\) respectively, where \(\gamma = (1 - \beta^2)^{-1/2}\) and \(\beta = v/c\).

Figure 3.8. Geometrical profile of a cylindrically symmetric 330 GHz gyrotron cavity and the electric field profile of the excited TE_{4,1} mode. The insert shows the transverse electric field profile of the cavity mode. The electron beam radius is labeled as \(r_e\). (Adapted from [60])
It is useful to convert these equations into normalized variables for the practical case of a cylindrical resonator by taking into account the strength of the coupling between the electron and electromagnetic mode. The conversion to the normalized pendulum equations is covered in detail by [57, 61] and results in

\[ \frac{\partial u}{\partial \zeta} = 2Ff(\zeta)(1-u)^{n/2} \sin \theta, \]  

(6)

\[ \frac{\partial \theta}{\partial \zeta} = \Delta - u - nFf(\zeta)(1-u)^{n/2} \cos \theta. \]  

(7)

where the normalized variables are the electron energy \( u \), the electron phase \( \theta \), the axial position \( \zeta \), the field strength \( F \) and the magnetic field detuning \( \Delta \). With a cavity length \( L \), the normalized cavity length \( \mu \) and assuming a Gaussian axial field profile

\[ f(\zeta) = e^{-(2\zeta/\mu)^2}, \]  

(8)

\[ \mu = \pi \frac{\beta_\perp^2 L}{\beta_z^2 \lambda}. \]  

(9)

The beam-wave coupling has been incorporated into \( F \) [61]. These coupled differential nonlinear equations of motion describe a gyrating electron interacting with an electromagnetic field, by tracking the normalized transfer of energy and the phase of the electron. Equations (6) and (7) are only functions of three parameters: \( F, \mu \) and \( \Delta \). In a gyrotron an electronic efficiency of up to 70% is achievable with the optimization of these parameters [61].

**Development of THz Gyrotrons**
Table 3.2

<table>
<thead>
<tr>
<th>140 GHz Gyrotron Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Operating Mode: TE_{031}</td>
</tr>
<tr>
<td>Voltage: 12 kV</td>
</tr>
<tr>
<td>Current: 35 mA</td>
</tr>
<tr>
<td>Beam Radius: 1.82 mm</td>
</tr>
<tr>
<td>Output Power: 15 W</td>
</tr>
<tr>
<td>Magnetic Field: 5.12 T</td>
</tr>
<tr>
<td>Beam Velocity Ratio: 1.6</td>
</tr>
</tbody>
</table>

The discovery of the cyclotron resonance instability by R. Twiss [49], J. Schneider [62] and A. Gapanov [63] was followed by the first detailed experimental demonstration by Hirshfield and Wachtel [64]. The first high power gyrotron in the THz band produced a kilowatt of power at a frequency near 300 GHz [21]. Pulsed THz gyrotron oscillators with several hundred kW of power were also developed [65-67], reaching almost 1 MW of power at 300 GHz [68]. In the past two decades, progress has been made on providing useful millimeter and sub-millimeter wave sources for Electron Cyclotron Resonance Heating (ECRH) of plasmas [56, 60]. At very low power levels, step tunable gyrotrons have been demonstrated at frequencies in the THz band for spectroscopy [69-71]. Gyrotrons were developed at power levels of 10 to 100 watts for application to DNP NMR and EPR experiments [13-16, 20, 58, 59]. The gyrotrons for DNP NMR must meet exacting specifications for reliability, efficient power conversion, frequency bandwidth and signal stability, as listed in Table I. With interest in developing this technology for higher frequency NMR experiments, these THz sources present a challenge to THz technology for sources, transmission lines and related components. A comprehensive review of the history and present state of THz vacuum electronics is provided in this journal issue [72].
DNP Experiments with Gyrotrons

In 1992 the first use of a gyrotron for a DNP NMR experiment was reported by Becerra et al. [13] using a 140 GHz CW gyrotron capable of producing 20 W. Signal enhancements for polystyrene doped with BDPA of 10 for $^1$H and 40 for $^{13}$C were reported when comparing NMR spectra recorded with and without microwave power. The 140 GHz gyrotron operated in the TE$_{031}$ mode of a cylindrical resonator with a 42 kV, 10-100 mA electron beam producing 20 W CW and 200 W pulsed. This was the first gyrotron with enough stability for phase-coherent magnetic-resonance spectroscopy. The power is converted internally to the TE$_{01}$ mode and extracted from the gyrotron after a miter bend through a window. A transmission line and mode converters provide the power to a WR-8 waveguide that goes to the sample and radiates the power along the axis of rotation of the MAS sample. Recently, the gyrotron was upgraded to be operated with a lower voltage MIG [30, 73] with the key parameters of the 140 GHz gyrotron listed in Table II. The bandwidth of the gyrotron has been measured using a heterodyne receiver system. The emission bandwidth of the gyrotron was found to be 1 MHz at 60 dB below the carrier [74, 75]. This is the most sensitive measurement to date of noise in a gyrotron and the results assure that parasitic modes do not participate in the excitation of the electron spins in DNP NMR spectroscopy. This gyrotron has remained in service for nearly two decades of operation.
Figure 3.9. Photo of the 250 GHz gyrotron oscillator (left), the 380 MHz NMR spectrometer (right).

Figure 3.10: Commercial DNP/NMR spectrometer with the gyrotron and magnet shown at the left and the control and cooling systems for the gyrotron shown on the right. (Adapted from [20])
A 250 GHz DNP NMR system [14, 76] was commissioned at MIT in 2000 with a design that improved upon the 140 GHz spectrometer. This gyrotron produces 15 W with a 12 kV, 35 mA electron beam in the TE$_{032}$ mode. $^1$H Enhancements of up to 170 have been observed for 1-$^{13}$C-glycine at temperatures of 20 K using a MAS probe [14]. The gyrotron, transmission line and NMR setup are shown in Figure 3.9. The 250 GHz gyrotron is designed to operate in a vertical orientation in order to simplify support and alignment. A radial bore in the magnet allows the transmission of the microwave power through a side vacuum window of the tube. As a result, the electron beam and microwave power can be separated just after the interaction in the gyrotron cavity. An internal Gaussian beam mode converter was included in the design. This reduces ohmic losses in
the output waveguide and simplifies the design of the beam collector and microwave transmission line. A specially built corrugated transmission line with very low loss brings the power from the gyrotron to the sample [77]. The transmitted beam had a measured Gaussian beam content of 93% [78]. The gyrotron output power is monitored and controlled by feedback stabilization.

In recent years, gyrotrons have been built for DNP NMR research by several groups throughout the world. The first commercial DNP system using a gyrotron is a 263 GHz/400 MHz NMR spectrometer that was developed in 2009 by Bruker BioSpin [20], shown in Figs. 10 and 11.
More recently, gyrotrons that operate at the second harmonic of the electron cyclotron frequency ($\omega = 2\Omega_c$) have been developed for DNP NMR experiments due to the reduced requirements placed on the magnetic field of the gyrotron (half the magnetic field required for fundamental operation). Unique physics and engineering challenges arise at the second harmonic due to reduced efficiency and fundamental mode competition. These difficulties were overcome by careful design of the resonator and choice of the operating mode which is relatively isolated in the mode spectrum. The first completed second harmonic gyrotron for DNP NMR applications was a 10 W, 460 GHz gyrotron oscillator for NMR at 700 MHz [15, 16, 74, 79]. The electron beam parameters
Figure 3.14. First observation of continuous tuning in a NMR DNP gyrotron. (Adapted from [15])

during operation are a voltage of 12 kV, current of 140 mA, with a beam radius of 1.03 mm. The gyrotron is shown in Figure 3.12.

With the completion of two additional second harmonic sources in 2009 [58, 59] the first DNP enhancements using second harmonic gyrotrons were reported in 2010 at 260 GHz [17] and 395 GHz [19]. The 260 GHz experiment utilized a 20 W gyrotron and was able to produce signal enhancements of -79 on an aqueous solution of Fremy's salt [80], far exceeding previous results using a solid state source [17, 53]. The 395 GHz gyrotron is capable of producing 40 W using the TE06 mode of a cylindrical cavity with an enhancement of 10 for 1 M 13C6-glucose and 10 mM TOTAPOL [19]. A second harmonic gyrotron for DNP has also been built at 330 GHz [16].

**Tunability**

Due to the extremely sensitive frequency requirements of DNP, either a NMR magnet equipped with sweep coils or a THz source that is continuously tunable can be very valuable. Fabricating a gyrotron cavity to operate at a predetermined frequency with \( \Delta f_{set} \approx 50 \) MHz accuracy would be a major engineering requirement due to the extremely
tight tolerance placed on the cavity radius, as can be seen from (2). It is also difficult and expensive to alter the magnetic field of the NMR experiment to match the gyrotron with such accuracy. With a tunable source that is able to vary the frequency over a range much greater than $\Delta f_{set}$ this engineering challenge is alleviated. The frequency of the THz radiation must match the absorption condition for the appropriate DNP mechanism in order to provide a signal enhancement. If the frequency is not correct a drastic change in enhancement will be observed, as seen in Figure 3.1c. Furthermore, sweeping the frequency over the entire range of the EPR spectrum is of interest to the user. In general the width of the EPR spectrum grows linearly with the magnetic field of the NMR experiment adding an additional challenge to higher frequency (typically second harmonic) sources.

Continuous tunability in gyrotrons with TE$_{mna}$ cavity modes can be achieved by keeping the transverse mode numbers (m,n) constant and varying the axial mode number (q). By keeping the transverse index the same, nearly continuous tuning can be achieved by either voltage or magnetic field tuning without affecting the mode converter and transmission line performance. Tuning is improved by increasing the cavity length resulting in a decreased frequency shift from adjacent axial modes via their hybridization. Continuous tunability in a gyrotron for DNP was first reported by Hornstein et al. [15]. In this gyrotron the increased cavity length to lower the oscillation start current for the desired second harmonic mode, led to the hybridization of axial modes, with 2 GHz of tuning reported for fundamental modes near 230 GHz and 50 MHz for the second harmonic mode at 460 GHz, as seen in Figure 3.14. When the 460 GHz gyrotron was
Figure 3.15. Measured start oscillation current (solid line) and measured frequency (diamonds) of the operating mode TE\textsubscript{-4,3} as a function of magnetic field. The theoretical start currents for the first six axial modes TE\textsubscript{-4,3,q}, where \( q = 1, 2, 3, 4, 5, 6 \), are shown as dash-dotted lines and they were computed using linear theory. (Adapted from [60])

later rebuilt to operate in the TE\textsubscript{11,2} mode, over 700 MHz of tuning was observed at the second harmonic [16].

This technique for achieving continuous tuning has also been implemented in a 330 GHz second harmonic oscillator [60]. A continuous tuning range of 1.2 GHz was observed experimentally via a combination of magnetic, voltage and thermal tuning. Thermal tuning is achieved by the precise control of the cavity cooling, allowing the cavity to expand or contract as needed. An additional advantage to long cavities is that the start current for oscillations is lowered for all axial modes allowing for operation with smaller beam powers. For a beam voltage of 10.1 kV the oscillation start current for the 330 GHz gyrotron is shown in Figure 3.15.
Figure 3.16. Tuning with magnetic field from a 395 GHz fundamental mode gyrotron. (Adapted from [86])

Tuning of over 1 GHz has recently been achieved in a 395 GHz gyrotron [59, 81]. The 1.6 GHz of tuning shown in Figure 3.16 demonstrates how switching axial mode numbers allows for nearly continuous tuning with magnetic field even with fundamental mode experiments at very high frequencies. On the left side of Figure 3.16 the gyrotron is interacting with a forward wave and on the right side of the Figure 3.16 a backward wave.

**Internal mode converters**

Extracting THz radiation efficiently from gyrotrons in a manner useful for DNP NMR spectroscopy is very challenging. Internal mode converters that provide high quality Gaussian beams provide the most utility due to the attractive qualities that Gaussian beams provide with respect to transmission, as discussed in Section 4. Internal
mode converters are a topic of great interest in the gyrotron community at large due to their power extraction efficiency. Internal mode converters in the form of step cut launchers for azimuthally symmetric modes and helical cut launchers for rotating modes were pioneered by Vlasov [82, 83]. Modern mode converters include the use of quasi-parabolic mirrors to correct for the ellipticity of the beam produced by the launcher. Designs are also optimized using numerical codes, such as the electric field integral equation code Surf3d [84]. One example of an internal mode converter is shown in Figure 3.6, labeled as item 6, and in Figure 3.17a. This mode converter was designed for use with a TE\(_{11,2}\) mode at 460 GHz [85] with a helical cut launcher, parabolic mirror and two flat mirrors shown in Figure 3.17a. The profile of the gyrotron output beam measured with a Spiricon Pyrocam III pyroelectric camera is shown in Figure 3.17b. The resulting beam is 92% Gaussian with a waist of \(w_x = 4.1\) mm and \(w_y = 4.6\) mm. Similar mode converters have now been implemented on most DNP gyrotrons [14-16, 20, 58].

**Transmission Lines**
The power generated by the THz source (gyrotron) must be transmitted to the sample with low loss. In the THz band, it is not possible to use conventional single mode rectangular waveguide for transmission, since the ohmic loss in such guides is much too large. The location of the source, typically a few meters from the NMR spectrometer, requires that the loss per meter be kept as low as possible. Transmission lines for terahertz radiation are an area of intensive research at the present time [12]. Since the frequency bandwidth needed for DNP NMR systems is usually very small, the transmission line can be optimized for a single frequency, thus simplifying the problem significantly. The number of approaches to the design of the transmission line is quite extensive. Free space beaming techniques rely on propagation of a Gaussian-like beam via a series of mirrors and/or lenses. This approach has low loss but may have issues of safety and the stability of alignment. The most common approach is the use of overmoded waveguides, in which the guide radius is much larger than the wavelength. Such waveguides can be corrugated metallic waveguides; dielectric waveguides or
dielectric lined metallic waveguides. Reviews of the properties of overmoded waveguides are given by Bhartia and Bahl [49] and Thumm and Kasparek[87].

Figure 3.18a shows a schematic of a corrugated metallic waveguide. The guide has grooves of depth $d$ that are one quarter wavelength. The radius $a$ is much larger than a wavelength. The corrugations are optimized when the groove depth $d \approx \lambda/4$, period $w_1 \approx \lambda/3$ and tooth width $w_2 < w_1/2$. Figure 3.18b shows the transmission line used in the MIT 250 GHz DNP NMR system [77]. Figure 3.18b illustrates some of the key features of a low loss transmission line. The main transmission line is a 22 mm diameter ($a = 11$ mm) metallic corrugated waveguide made of aluminum. The corrugations $d$ are one quarter wavelength in depth ($0.3$ mm), one quarter wavelength wide ($w_2 = 0.3$ mm) with a period of $w_1 = 0.4$ mm. Two 0.254 m waveguide sections, fifteen 0.124 m waveguide sections and one 0.064 m directional coupler block were assembled with outer diameter clamps to achieve the desired waveguide length.
Figure 3.18. a) Illustration of corrugated metallic waveguide of radius $a \gg \lambda$, groove depth $d \approx \lambda/4$, period $w_1 \approx \lambda/3$ and tooth width $w_2 < w_1/2$. (Adapted from [89]) b) Schematic of the transmission line used in the MIT 250 GHz DNP NMR system. (Adapted from [77])

The modes and ohmic loss in a corrugated waveguide have been developed using the theory of Doane [88]. The linearly polarized output of the gyrotron excites a linearly polarized mode (or modes) in the waveguide [89]. The lowest order mode is the hybrid mode $\text{HE}_{11}$, whose transverse electric field is given by:

$$ E^+ (r, \varphi) = \left( X_{01}/a \right) J_0 \left( X_{01} r/a \right) $$  \hspace{1cm} (3.10)
where $X_{01}$ is 2.405 and $J_0$ is the zero order Bessel function. This expression is valid when the corrugation depth is approximately one quarter wavelength and the radius $a$ is much larger than the wavelength.

The estimated ohmic loss in the overmoded waveguide of Figure 3.18b is less than $10^{-3}$ dB per meter and is thus negligible. The measured loss for the 22 mm diameter waveguide portion of the transmission line of Figure 3.18b is shown as open circles in Figure 3.19 and amounts to less than 1% per meter.

For perfect excitation of the $HE_{11}$ mode on the transmission line, the overall transmission line loss is expected to be dominated by loss at miter bends and by tilts and offsets in the fabrication and alignment of the waveguide components. However, in practice, the loss may be dominated by the imperfections in the THz beam entering the transmission line and poor coupling of the beam to the line. When the output beam of the gyrotron is not a perfect Gaussian beam or the output beam is not perfectly matched (due to tilts and offsets) upon insertion into the waveguide, high losses may be found on the transmission line [90].

![Figure 3.19. Measured loss of the transmission line in Figure 3.18. (Adapted from [77])](image)

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The transmission line shown in Figure 3.18b has directional couplers for measuring forward and reverse power. They have been fabricated by stretching metal wires across the waveguide surface area and have only a 2.6% insertion loss, as shown in Figure 3.19. This approach was found to be superior to using a quartz coupler. A smaller diameter waveguide in the probe is necessitated by the limited space in the probe, this transition can be performed with waveguide tapers or mirrors. Bends in the transmission line may be accomplished using a right angle bend, called a miter bend, in waveguide or using a mirror box. The transmission line of Figure 3.18b has only one right angle bend and it is accomplished using two mirrors. The mirrors also match the beam from the 22 mm waveguide into the 8 mm waveguide used in the NMR probe. Due to losses induced by imperfections in the Gaussian beam quality of the gyrotron and by errors in the alignment of the components of the transmission line, the transmission efficiency from the gyrotron output window to the sample is typically in the 50 to 80% range [78, 91].

The transmission line shown in Figure 3.18b is only one example of successful transmission lines used in DNP NMR research. Recently, a very long transmission line using corrugated waveguides and switches has been successfully implemented on a 260 GHz gyrotron system for DNP NMR [92, 93]. A fully optical (mirror) transmission line using a mirror relay line has also recently been implemented for a DNP NMR spectrometer system [94]. Future research will concentrate on making these transmission lines simpler and more efficient.

**Versatile Probes for DNP**

MAS DNP probes can couple frequencies spanning eight orders of magnitude of the electromagnetic spectrum to samples under study. The DNP probe for experiments at
a 9T magnetic field shown in Figure 3.20 integrates an RF circuit that resonates an inductor surrounding the sample at 38, 96 and 380 MHz to control and detect $^{15}\text{N}$, $^{13}\text{C}$, and $^1\text{H}$ spins, respectively. 250 GHz and 532 nm radiation channels illuminate the sample to excite the EPR and visible spectra [45]. Additionally, probes also house a pneumatic spinning module to mechanically rotate the sample at frequencies up to 8 KHz at an angle of 54.7° (the Magic Angle) with respect to the $B_0$ NMR field. This motion partially averages anisotropic chemical shift and dipolar tensors that would otherwise result in broad NMR line shapes and poorly resolved spectra.

**Cryogenic Magic Angle Spinning**

Electron spin relaxation times, $T_{1e}$, in MAS DNP experiments increase at lower temperatures. Electron spins relaxing faster than the rate of transfer of polarization to the nuclear spins result in inefficient DNP transfers. Utilizing polarization agents with long ($T_{1e}\sim1\text{ms}$) longitudinal relaxation at ambient temperatures [26] is a strategy to circumvent this issue, and these $T_{1e}$'s are generally observed in radicals such as trityl with narrow powder patterns. However, these systems lead to a SE mechanism that does not scale well at higher magnetic fields. A more general strategy is to dope the sample with radicals that have a larger g-anisotropy that supports the CE mechanism, and to perform the experiments at low temperatures (~90 K), where the relaxation times of both the electron and nuclear spins are longer. After the initial electron to nuclear polarization step, inherent proton spin-lattice relaxation mechanisms compete with the microwave driven polarization of the nuclear spins. The DNP enhancement thus has a strong dependence on temperature, ranging from <5 at 160 K [20], 100 at 85 K [45], and 170 at 20 K [14], for model samples frozen in a glassy matrix and at magnetic fields of 9-9.5T.
Achieving a uniform cryogenic sample temperature without exposing sensitive elements in the probe to a harsh environment is accomplished by thermally isolating the sample chamber and using the bearing and turbine gases needed for MAS spinning also as variable temperature cryogens [45]. A vacuum jacketed dewar (Figure 3.20b) and the use of insulating material in the probe body reduces heat transfer from the sample chamber to the probe box and magnet bore which are maintained at ambient temperature.

In MAS DNP experiments, cooling to near 80 K can be achieved through a heat exchange process with liquid nitrogen. Since the DNP enhancement is extremely sensitive to temperature in the range of 80 K, the temperature typically must be regulated to within 0.5 K. Furthermore, the generation and trapping of photointermediates in photoactive proteins such as bR requires temperature control over a wide temperature range from 90 K – 273 K.[95]

**Cryogenic Sample Exchange**

Due to the complicated nature of overmoded transmission lines, as discussed in Section IV, misalignment during sample exchange can result in excessive down time and reduced performance. In addition, the magic angle and shim coils become misadjusted when the probe is physically moved out and back into the magnet, which can lead to degradation of spectral resolution (Figure 3.20c). Sample eject systems operate at low temperatures and avoid physical adjustments to the transmission line, warming and cooling the probe, while at the same time retaining much better experimental stability [45].
Figure 3.20. Cryogenic MAS and sample exchange. a) Overview of the system architecture of the cryogenic sample spinning and exchange system. Thick black lines indicate vacuum jacketed transfer lines. b) Elements of the probe design required from cryogenic spinning. c) Spectra of a tripeptide with the cryogenic sample exchange and broad unresolved spectrum without the sample exchange. (Adapted from [45, 96])
RF circuit

RF circuits such as that in Figure 3.21 couple power from RF amplifiers and the NMR spectrometer to an inductor surrounding the sample. The RF circuit is used to detect the NMR signals which are ultimately the data that result from an NMR DNP.

Figure 3.21. Solid model rendition of a typical 4 channel RF-THz circuit showing transmission lines, sample inductor, isolation elements and variable tuning and matching capacitors, corrugated waveguides and quasioptics. (Adapted from [45])
experiment. The resonant frequencies of the RF circuit must match that of the nuclear spins to efficiently utilize the RF power and generate strong fields orthogonal to the main magnetic field of the superconducting NMR magnet. The tuning, matching, and isolation elements in the RF circuits can either be located near the coil or utilize a transmission line to physically separate the elements from the harsh cryogenic environment near the sample [97, 98]. The severely constrained spacing inside the bore of NMR magnet has led to the use of the THz waveguide as the inner conductor for the RF transmission line which carries the 3 RF frequencies to the RF coil.

**THz coupling to sample**

The sensitivity enhancement in DNP experiments depends strongly on the intensity of the millimeter waves in the sample of interest. Specifically, it is important to know the magnitude of the THz field, $B_{1s}$, that is orthogonal to $B_0$ and oscillates at the frequencies leading to polarization transfer. The THz beam is brought through the probe in a small diameter waveguide, as shown in Figure 3.21. The THz radiation is launched from the end of the corrugated waveguide in a Gaussian power distribution generated from the $HE_{11}$ mode of the corrugated transmission line, as shown in Figure 3.18. However, the RF coil, large sample volume, and other physical requirements of the MAS NMR experiment hinder the illumination of the sample with the THz waves, and indeed the implementation of a high quality factor resonator surrounding the sample.
Figure 3.22. THz coupling to sample. a) HFSS calculation of the THz wave intensity throughout the sample and spinning chamber. b) solid model depicting the THz antenna, spinning module, and sample. (Adapted from [78])

Other strategies of coupling the THz power axially down the rotor (rather than the perpendicular coupling shown in Figure 3.22b) have been explored by other groups at 39 GHz [5] and 394 GHz [19]. Although successful at lower frequencies, axial coupling at higher frequencies (>200 GHz) is rather inefficient due to the dielectric constant and loss tangent of the sample [78]. Optimization of THz coupling and RF performance is crucial to overall quality of the probe [99].

Recently, High Frequency Structure Simulator (HFSS) was used to calculate how the THz radiation travels from the antenna, diffracts through the coil, and is distributed across the sample, as shown in Figure 3.22 [78]. Simulations were performed using a linearly polarized Gaussian beam as the input with a sample dielectric constant extrapolated from measurements at 140 GHz. The sample is dissolved in a water/glycerol mixture inside of the 2.4 mm internal diameter rotor and extended the full length (12.45 mm) of the RF coil. In the sample the calculated average $B_{1S}$ THz field is $13 \mu T/W^{1/2}$. The $B_{1S}$ in the sample varies quite drastically from 3 to $50 \mu T/W^{1/2}$ due to diffraction effects.
In comparison, EPR and ENDOR resonators are able to achieve much higher average $B_{1S}$ values per $W^{1/2}$ and improved homogeneity. Specifically, by using low order metallic resonant structures and sample volumes that occupy a small fraction of the resonator, $B_{1S}$ values on the order of 1 mT/$W^{1/2}$ have been reported [51, 100, 101]. As discussed previously these metallic resonators are not suitable in MAS DNP experiments, but alternate methods for increasing the Q-factor of large volume samples could prove beneficial.

The enhancement observed in a DNP CE experiment can be described using the calculated $B_{1S}$ and a simplified expression for the steady state enhancement derived by Wollan [10]. Since the microwave field, $\omega_{1S}=\gamma B_{1S}$, is inhomogeneous, as demonstrated in Figure 3.22, the expression must be integrated over the field dependence for the sample volume

$$\varepsilon = \frac{1}{V} \varepsilon_{\text{max}} \int \frac{\alpha \omega_{1S}^2}{1 + \alpha \omega_{1S}^2} dV$$

(3.11)

where $\alpha=(T_{1S}T_{2S})/2$ and $T_{1S}$ and $T_{2S}$ are the electronic longitudinal and transverse relaxation times. The factor of 1/2 converts from the time averaged linearly polarized $\omega_{1S}^2$ to the time averaged circularly polarized component that interacts with the electrons.
$T_{1S}$ for a Nitroxide radical is expected to range from 10-400 $\mu$s in the 90 Kelvin temperature regime, and $T_{2S}$ is a strong function of radical concentration being in the range of 10 to 200 ns for the 20 mM electron concentration in our sample. The magnitude of the product $T_{1S}T_{2S}\omega_{1S}^3<<1$ to obtain the quadratic dependence observed at low $\omega_{1S}^2$.

It then saturates at high $\omega_{1S}^2$. Figure 3.23 is a plot of the experimental enhancement of 1 M $^{13}$C-urea and 10 mM TOTAPOL dissolved in d$_8$-glycerol/D$_2$0/H$_2$0 (60/30/10% by volume) [102, 103] vs. $\omega_{1S}/2\pi$ at 250 GHz for the sample and probe described in Figs. 20 and 21 and the field distribution shown in Figure 3.22. An excellent fit to the experimental data is obtained for the parameters $e_{\text{max}} = 230$, $T_{1S}T_{2S}=8\times10^{-12}$ $\text{s}^2$.

Calculating the value of $\gamma_{1S}B_{1S}$ at the level of a mesh element volume defined by the HFSS

Figure 3.23. DNP enhancement vs. $\omega_{1S}/2\pi$ (bottom) and power (top) for a fully packed rotor of 1 M $^{13}$C-urea and 10 mM TOTAPOL dissolved in d$_8$-glycerol/D$_2$0/H$_2$0 (60/30/10% by volume). (Adapted from [94])

model is crucial to understanding the DNP enhancement data and, indeed, to modeling the enhancement more generally obtained from the cross effect [10].
Conclusions

Dynamic Nuclear Polarization has become firmly established as a powerful technique for enhancing signal intensities in NMR spectroscopy. In DNP NMR, the required frequencies for microwave irradiation of the sample have increased as NMR has moved to progressively higher magnetic fields due to the improved resolution and sensitivity. Modern NMR spectrometers are presently operating at proton frequencies close to 1 GHz, corresponding to fields of 23 T. The microwave frequencies needed for these spectrometers enter the terahertz regime as the magnetic field exceeds 10.7 T. Stated another way, for NMR spectrometers operating above 450 MHz, the required microwave frequency for DNP NMR is in the terahertz range.

The gyrotron is capable of achieving the power and stability requirements for THz DNP NMR. The number of gyrotron-based DNP NMR systems was only a single system in 1990 and increased to two systems only in 2000 (both at MIT). However, as pointed out by Griffin and Prisner [9], DNP NMR is now enjoying a renaissance. There are now approximately 20 THz DNP NMR systems either operating or under construction [104] and more systems will surely follow. THz DNP NMR will thus be recognized as a major present-day application of THz technology.

The lack of THz sources has led to complex schemes such as dissolution or shuttle DNP [12, 105, 106], where the sample is polarized in a lower magnetic field region and then transported to the high field region for signal acquisition. Low power solid state sources have also been employed in static DNP experiments [6, 51-53, 107, 108] that would likely benefit from higher power THz sources [80]. The appeal of
resonant structures can be illustrated by looking at an expression for the Quality factor (Q) of the sample region defined as:

\[ Q = \frac{\omega W}{P} \]  

(3.12)

where \( \omega \) is the resonant frequency, \( W \) is the energy stored in the cavity, and \( P \) are the electric and magnetic energy, \( V \) is the input power. We can assume that \( P \) and \( V \), where \( V \) is the volume. With these simple substitutions we can rewrite Eqn. (12) for the quantity of interest in DNP

\[ \langle B_{1s}^2 \rangle \propto \frac{QP}{V}. \]  

(3.13)

Eq. 13 shows that in order to minimize the required power \( P \) it is advantageous to work with samples of small size (small \( V \)) contained in high \( Q \) cavities. In NMR experiments, however, it is useful to use larger samples since the NMR signal strength increases with the sample size (or volume, \( V \)). In addition, it is not easy in MAS NMR to construct optimized resonators with the highest possible \( Q \). One limitation on the \( Q \) is the finite power dissipation (loss) of the sample itself. In addition, the resonators cannot be with metallic walls since metallic objects will interfere with the NMR signal. For these reasons, in present day experiments, power levels in the ten to twenty watt range are used to saturate the enhancement in DNP/NMR experiments. Novel coupling schemes and dielectric cavities could prove useful in the future to raising the \( Q \) of the sample chambers and alleviating the stringent requirements on the THz power level.

Further utilization of THz DNP NMR will likely be strongly coupled to advances in THz technology. The THz source power level of ten to one hundred Watts needed for THz DNP NMR is currently only achievable by gyrotrons. One can however envision the
possibility of achieving the required power levels at THz frequencies with advanced versions of klystrons or traveling wave tubes. These might bring down the cost and complexity of the THz DNP NMR system. When coupled with commercial development, THz DNP NMR could become the preferred approach to many solid and liquid state NMR experiments.

**Acknowledgements**

This research was supported by the National Institutes of Health through grants EB001965, EB004866, EB001960, EB002804, EB003151, and EB002026. A.B.B. was partially supported by graduate research fellowship from the National Science Foundation.

**References**


Chapter 4. High-Resolution Solid-State NMR Structure of Alanyl-Prolyl-Glycine


Abstract

We present a de novo high-resolution structure of the peptide Alanyl-Prolyl-Glycine using a combination of sensitive solid-state NMR techniques that each yield precise structural constraints. High quality $^{13}$C-$^{13}$C distance constraints are extracted by fitting rotational resonance width ($R^2W$) experiments using Multimode Multipole Floquet Theory and experimental chemical shift anisotropy (CSA) orientations. In this strategy, a structure is first calculated using DANTE-REDOR and torsion angle measurements and the resulting relative CSA orientations are used as an input parameter in the $^{13}$C-$^{13}$C distance calculations. Finally, a refined structure is calculated using all the constraints. We investigate the effect of different structural constraints on structure quality, as determined by comparison to the crystal structure and also self-consistency of the calculated structures. Inclusion of all or subsets of these constraints into CNS calculations resulted in high quality structures (0.02 Å backbone RMSD using all 11 constraints).
Introduction

Determining the high-resolution structures (backbone RMSD < 0.1 Å) of uniformly labelled polypeptides with solid-state NMR is often prohibitively difficult due to a lack of precision available with homonuclear broadbanded recoupling techniques such as RAD[1], DARR[2] and TSAR[3]. Although these experiments can define the secondary and tertiary structure and yield a clear global structure of the protein, the lack of high-precision measurements can leave detail of the active site and mechanisms that are of broader interest to the biological community still undefined. More precise techniques such as REDOR[4], RFDR[5], DRAWS[6], and local field correlations yield sub-angstrom precision, but are often limited to spin-pair labelled systems.

A previous study on the polypeptide, N-f-MLF-OH, demonstrated the use of FS-REDOR, and torsion angle constraints to determine a high-resolution de novo structure[7]. Since the publication of that structure, additional high-resolution techniques applicable to uniformly $^{13}$C, $^{15}$N labelled systems have been developed, such as Rotational Resonance Width (R$^2$W) [8, 9] and DANTE-REDOR [10]. DANTE-REDOR can yield high quality restrictions on torsion angles, in addition to heteronuclear distance constraints. R$^2$W utilizes band selective R$^2$ to recouple homonuclear spin pairs and can separate weaker dipolar oscillations (from long distance, structurally relevant spin pairs) from relaxation processes using a constant mixing time strategy and has been recently applied to measure distances in uniformly labelled proteins [11]. However, Ramachandran et. al [12] discussed the effect of the relative CSA orientation of the two spins in extracting accurate distances from R$^2$W experiments. To make R$^2$W a truly de
novo, precise, and accurate technique for making homonuclear distance measurements, one must obtain the relative CSA orientation of the spin pair experimentally.

We demonstrate the utility of an iterative approach to structure determination of uniformly labelled polypeptides using various solid-state NMR methods. Namely, \( \psi \) torsion angle and \( {^{13}}C-{^{15}}N \) distances from NCCN [13] and DANTE-REDOR measurements are first used to establish a structure from which approximate relative CSA orientations are available. Those orientations are then utilized into the fitting routines of R\(^2\)W profiles to also obtain precise and accurate \( {^{13}}C-{^{12}}C \) distances. Finally all experimentally determined homonuclear, heteronuclear, and torsion angles constraints are used in a final energy minimization that yields a high-quality de novo structure. We further discuss the effect of each sub-group of structural constraints on the structural quality as judged by self-consistent backbone RMSD and comparison to the known crystal structure.

**Results and Discussion**

Figure 4.1 shows the 11 constraints determined for APG listed in Tab. 1. Comparison with the X-ray crystal structure indicates the level of accuracy achieved in each measurement. Two of the restrictions on \( \varphi \) dihedral angles were determined with a local field NCCN experiment[13]. DANTE-REDOR was used to determine the glycine \( \varphi \) angle, and 4 \( {^{13}}C-{^{15}}N \) internuclear distances. The four carbon internuclear distances were extracted using R\(^2\)W.
Figure 4.1. Crystal structure of APG[14] showing the 11 SSNMR experimental constraints used in the structural refinement. Torsion angles are displayed in violet, REDOR distances in orange and R²W constraints in black. Note that the proline ring has a split occupancy between two conformers in the crystal structure.

Table 4.1. Experimental solid-state NMR constraints used in the structure calculation and corresponding values from the crystal structure from all NMR experiments; DANTE-REDOR (orange), NCCN torsion angles and R²W distances (black).

<table>
<thead>
<tr>
<th>Torsion Angles</th>
<th>X-ray</th>
<th>SSNMR</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\psi_{Gly}$</td>
<td>$178^\circ$</td>
<td>$180^\circ \pm 15^\circ$</td>
</tr>
<tr>
<td>$\psi_{Ala}$</td>
<td>$153^\circ$</td>
<td>$152^\circ \pm 5^\circ$</td>
</tr>
<tr>
<td>$\psi_{Pro}$</td>
<td>$157^\circ$</td>
<td>$162^\circ \pm 5^\circ$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Distances</th>
<th>X-ray (Å)</th>
<th>SSNMR (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro N - Ala Cβ</td>
<td>3.2</td>
<td>3.2 ± 0.1</td>
</tr>
<tr>
<td>Gly N - Pro Cβ</td>
<td>3.2</td>
<td>3.2 ± 0.15</td>
</tr>
<tr>
<td>Pro C' - Pro Cγ</td>
<td>3.3/3.6</td>
<td>3.9 ± 0.39</td>
</tr>
<tr>
<td>Ala C' - Pro Cβ</td>
<td>3.6</td>
<td>3.8 ± 0.38</td>
</tr>
<tr>
<td>Ala C' - Pro Cγ</td>
<td>3.6</td>
<td>3.9 ± 0.39</td>
</tr>
<tr>
<td>Gly N - Ala C'</td>
<td>4.1</td>
<td>4.1 ± 0.6-0.3</td>
</tr>
<tr>
<td>Gly N - Pro Cγ</td>
<td>4.5/4.3</td>
<td>4.2 ± 0.6-0.3</td>
</tr>
<tr>
<td>Pro C' - Ala Cβ</td>
<td>4.7</td>
<td>4.4 ± 0.44</td>
</tr>
</tbody>
</table>
CSA Parameters for Precise $R^2 W$ Fitting

The CSA magnitude and relative orientation of the two spins being recoupled by $R^2$ has a profound impact on the spin dynamics[15] and accuracy of the distances extracted using $R^2 W$ [12]. For example Figure 4.2b shows the distribution of distances resulting using different CSA orientations taken from an ensemble of structures generated from simulated annealing without experimental constraints. The range of motion displayed in unconstrained simulated annealing is large enough even in this small molecule to cause substantial deviation in the final $R^2$ calculations, which demonstrates the importance of first calculating a lower resolution structure.

As a starting point in the $R^2 W$ fitting routine, an initial structure based on REDOR and torsion angle measurements was first calculated. The relative CSA orientation from this structure was then used as an input for the $R^2 W$ fitting routine, which is derived from a multipole multimode Floquet theory (MMFT) treatment of the two spins[16]. The $^{13}C-^{13}C$ distances from these $R^2 W$ fits were then used together with the REDOR and torsion angle constraints to calculate a final structure. $R^2 W$ fits generated with relative CSA orientations found in the final structure yielded the same distance as those generated from the initial REDOR-torsion angle structure. Thus we found it unnecessary to perform further iterations in which the structure generated with initial $R^2 W$ distance constraints is used to refine these constraints.

The default values from SIMMOL were used for relative CSA - molecular orientations, the span and skew of carbonyl carbons were extracted from a 4 kHz CP-MAS spectrum according to Herzfeld and Berger[17], and aliphatic carbon span and skew values were taken from Ye et al. [18]
Carboxyl Terminus

The carboxyl terminus is not well defined due to the absence of $^{13}$C-$^{13}$C constraints to the Gly carboxyl carbon (see Figure 4.1). This is predominantly due to intermolecular and dipolar truncation effects complicating any $^{13}$C-$^{13}$C distance.
measurements between the Gly C' and the Pro Cα and Pro Cβ. The Pro Cβ carbon to Gly C’ carbon intermolecular distance is 5.5 Å in the crystal structure, but there are 3 other intermolecular Pro Cβ to Gly C’ separations that are less than 5.4 Å (See Figure 4.3). The crystals consisted of 10% 13C-labeled peptide diluted with natural abundance APG to reduce the number of intermolecular contacts, however there still appears to be enough coupling between intermolecular Pro Cβ - Gly C’ pairs to substantially effect the R²W profile and result in a fitting of the data to 4.7 Å rather than 5.5 Å seen in the crystal structure.

Furthermore, the one-bond Pro C’ – Pro Cα dipolar coupling truncates the Pro C’-Gly Cα coupling which would also constrain the carboxyl terminus. Even though R²W is a band-selective recoupling technique and thus usually does not suffer from dipolar truncation [20], the single bond coupling is an order of magnitude stronger than the coupling of interest and is also recoupled when the spinning frequency is set to the N=2 recoupling condition for the Pro C’ to Gly Cα interaction; the stronger coupling dominates and insufficient polarization is transferred to the Gly Cα. We note that the dipolar truncation should not be as pronounced at higher magnetic fields where the resonances are more separated and the recoupling condition is thus more selective.
Figure 4.3. Crystal lattice of APG showing competing intermolecular distances to the long-range internuclear distance that could be used for structural refinement.
Figure 4.4. 20 lowest energy structures of APG using simulated annealing (CNS) a) no constraints b) 2 φ angles from NCCN measurements c) 4 $^{13}$C-$^{13}$C distance constraints d) 4 $^{13}$C-$^{15}$N heteronuclear constraints e) all 11 constraints f) crystal structure
Constraints Effect on Structural Quality

The standard annealing protocol in the program CNS [21, 22] was used to generate 40 APG structures in order to generate an ensemble that shows both an average geometry and demonstrates the degree of conformational variability allowed by the geometrical constraints.

In order to compare the usefulness of different structural constraints, a backbone RMSD among the 40 structures of each ensemble was calculated. This shows the level of precision attained by the different NMR experiments, indicating their ability to define overall conformation in this peptide. The results are summarized in Figure 4.4 and show excellent agreement with the crystal structure. Since we were not able to perform a measurement that constrains the \(\Psi_{\text{Gly}}\) angle, the Gly carboxyl group was omitted from the RMSD calculations. The final NMR structure and the crystal structure agree to within an RMSD of 0.09 Å.

Conclusions

Precise \(^{13}\text{C}^{13}\text{C}, ^{13}\text{C}^{15}\text{N},\) and \(\psi\) torsion angles were measured in a uniformly \(^{13}\text{C}, ^{15}\text{N}\) labeled peptide using \(R^2\)W in a de novo manner, DANTE-REDOR and local field NCCN experiments. Using these sub-angstrom structural constraints we were able to determine a high quality structure of APG. The 0.02 Å backbone RMSD structure was sufficient to observe the same conformational heterogeneity of the proline ring that is seen in the x-ray structure at 100 K. The ability to discriminate the two conformers by using structural constraints with sub-angstrom precision and CNS could be applied to address biological questions that require high-resolution structural detail. Furthermore, the short
intramolecular contacts present in the tripeptide lattice studied here should not be present in larger proteins and should enable the extension of this de novo R²W strategy to distances up to 8 Å.

Finally, the NCCN torsion and many of the internuclear distances that were experimentally determined with SSNMR are indicative of trans-proline. Although proline is almost always in the trans isomer, the isomerization to the cis can play an important role in mitotic regulation [23], and we emphasize the strategies for structure determination presented here could be used to further investigate the biological function of proline isomerization.

**Experimental Methods**

**Sample Preparation**

Experiments were performed on a sample of uniformly $^{13}$C and $^{15}$N labeled Alanyl-Prolyl-Glycine (APG) that was diluted to 10% in the corresponding natural abundance tripeptide to attenuate intermolecular dipolar couplings. The tripeptide was synthesized by CS Bio (CA) with labeled amino acids from Cambridge Isotopics (Andover, MA) and natural abundance material from BaChem (Switzerland). A 9:1 natural abundance to labeled APG mixture was dissolved in a minimal amount of water (solubility was approximately 45 mg/ml). The aqueous solution was placed in a desiccator next to a container of excess ethylene glycol. After a week, crystals were collected, crushed and packed into a rotor.
Assignments

To assign the $^{13}$C resonances, a 2D $^{13}$C-$^{13}$C SPC-5 [24] spectrum was recorded spinning at $\omega_r/2\pi = 7.5$ kHz using TPPM decoupling[25]. $\gamma B_1$ for $^{13}$C was set to 37.5 kHz required by the recoupling condition. The mixing time was 1.07 ms corresponding to 16 rotor cycles, during which time a 120 kHz continuous wave $\gamma B_1$ field was applied on the proton channel for decoupling. 256 time points were recorded in the indirect dimension using 16 scans for each $t_1$ point. The overall measurement time was 7 hours. The direct and indirect dimensions were linearly predicted forward to 1024 points, zero-filled to 2048 points and Fourier transformed using NMRPIPE [26].
NCCN Local Field Correlations

The torsion angles, $\varphi_{\text{Ala}}$ and $\varphi_{\text{Pro}}$ were determined with the double-quantum heteronuclear local field experiment previously described[13]. SPC-5 [24] was used to create the $^{13}$C-$^{13}$C double quantum coherence. The homonuclear mixing time was 800 $\mu$s, corresponding to 8 rotor periods and the $^{13}$C carrier was set precisely between the two resonances targeted for recoupling. $\gamma B_1$ equal to 50 kHz was used for both $^{13}$C and $^{15}$N during homonuclear mixing and the REDOR [28] dephasing pulses. The spinning

![Dephasing curves for extraction of $\Psi$ torsion angle. An $N_{\text{Gly}}$CCN$_{\text{Pro}}$ $\Psi$ angle of 152° (blue) was extracted compared to 153° from the X-ray data and $N_{\text{Ala}}$CCN$_{\text{Gly}}$ $\Psi$ angle was found to be 162° (red) versus 157° from the X-ray data. SPINEVOLUTION[27] was used to fit the data.](image)

Figure 4.5. Dephasing curves for extraction of $\Psi$ torsion angle. An $N_{\text{Gly}}$CCN$_{\text{Pro}}$ $\Psi$ angle of 152° (blue) was extracted compared to 153° from the X-ray data and $N_{\text{Ala}}$CCN$_{\text{Gly}}$ $\Psi$ angle was found to be 162° (red) versus 157° from the x-ray data. SPINEVOLUTION[27] was used to fit the data.
frequency was 10 kHz and 110 kHz continuous wave $\gamma B_1$ decoupling field on protons was applied during SPC-5 mixing and REDOR periods; 136 kHz TPPM [25] decoupling was used during the acquisition period. 512 scans were taken per dephasing time, and a 3 s recycle delay was used between scans. Each dephasing point was normalized to the intensity of the corresponding resonances absent of REDOR pulses during the dephasing period. The resonances were integrated and the intensities fit to extract the torsion angle with SPINEVOLUTION[27].

**Rotational Resonance Width**

NMR spectra were recorded on a 360 MHz Spectrometer (courtesy of Dr. D. J. Ruben) with a commercial Chemagnetics triple resonance MAS probe equipped with a 4.0 mm Chemagnetics spinning module. Spinning frequencies were regulated with a Bruker MAS controller.

The crosspeak volumes were extracted by automated fitting to two-dimensional Gaussians using NMRPIPE [26]. All data points in each 2D-slice were normalized to the carbonyl intensities of reference experiments conducted at identical spinning frequencies with zero mixing time.

A Gaussian pulse of 25 $\mu$s duration was used, followed by a z-filter of 750 $\mu$s to select the carbonyl region of the spectrum. 32 $t_1$ points were recorded with an increment of 250 $\mu$s and 16 scans per point with a recycle delay of 3.1 s leading to a total experimental time of about 50 minutes per two dimensional slice. A series of experiments were performed as a function of the sample spinning frequency under a constant mixing time of 30 ms.
Figure 4.6. $R^2W$ spectra at 5 spinning frequencies. The $P\gamma$ crosspeak intensifies as the spinning frequency is swept the $R^2$ condition between the spin pair.

Figure 4.7. a) Contour plot of the root mean square deviation between experimental and simulated $R^2W$ profiles for $P_o$ to $P\gamma$ as a function of distance and relaxation. b) Magnetization transfer from Pro C' to Pro C'' in $R^2W$ as a function of spinning frequency. The experimental data is shown in blue, and the fit in green.
DANTE-REDOR

DANTE-REDOR measurements were carried out on a custom assembled NMR spectrometer with a Discovery console (Tecmag; Houston, TX), 14.1 T magnet (Magnex; Oxford, England), and a 39-channel matrix shim system (Resonance Research, Inc.; Billerica, MA), operating at a proton frequency of 600.377 MHz (150.987 MHz for $^{13}$C). A doubly tuned 4 mm magic angle spinning probe (Doty Scientific; Columbia, South Carolina) was employed.

The DANTE-REDOR pulse sequence is described in [10]. This sequence adapts the orientation information available in a specifically labelled sample to a uniformly labelled system. By employing a DANTE pulse train as the selective pulse in FS-REDOR, the orientation information in REDOR sidebands is retained. Thus at the end of the REDOR evolution time, the $^{13}$C spin-echo intensity is modulated only by dipolar couplings to the selected $^{15}$N and the resulting REDOR curve can be fit to the analytical expression for an isolated spin pair. The effects of proton couplings are attenuated with Small Phase Incremental Alternation (SPINAL-64) decoupling [25]. The proton decoupling field was 100 kHz, the $^{15}$N REDOR $\pi$-pulse width was 16 us and the DANTE $\pi$-pulse trains consisted of 33 2 $\mu$s rotor synchronized pulses.

Fourier transforms were applied with no apodization and zero filling to twice the acquisition time of 15.36 ms. Only zero order phase correction was used. Dephasing (S) and full-echo ($S_0$) peak areas were determined by integrating the center band and all observed sidebands. For distance determinations, the sum of all bands in the S and $S_0$ spectra were used. For extraction of orientation information, the dephasing of each individual band was compared.
Distances were determined by fitting the experimentally observed REDOR curve to the analytical expression,
\[ \frac{\Delta S}{S_0}(\tau) = \lambda \left[ 1 - \langle \cos(\omega_{CN} \tau) \rangle \right]. \]  
(4.1)

\( \Delta S/S_0 = 1 - S/S_0 \), where \( S \) and \( S_0 \) are the dipolar dephasing and reference intensities, respectively. The coupling \( \omega_{CN} \) is a function of the dipolar coupling constant, \( b_{CN} \), and \( \langle \; \rangle \) indicates the powder average over a uniform distribution of crystallite orientations. The scaling factor \( \lambda \) accounts for \(^{13}\text{C}\) spins without a neighboring \(^{15}\text{N}\) spin, which is a result of imperfect labelling and dilution of the labelled compound in natural abundance material. In the DANTE-REDOR experiment, \( \lambda \) also accounts for imperfect inversion of the \(^{15}\text{N}\) spin magnetization by the selective pulse and decay of coherence due to insufficient decoupling.

For each distance measurement, the weighted \( \chi^2 \), \( \chi^2_w \), was minimized to fit the data to:
\[ \chi^2_w = \sum_i w_i (s_{i,exp} - s_{i,calc})^2 \]  
(4.2)

In this expression, \( w_i \) is the inverse uncertainty in each point squared (\( \sigma^2 \)). Since the noise in each data point is approximately constant, \( w_i \) was easily determined by propagation of error. In the minimization of \( \chi^2_w \), the coupling constant, \( b_{CN} \), was varied freely and the amplitude scaling factor \( \lambda \) was varied within the narrow range of values determined for strong couplings, for which this parameter can be easily determined. The reported uncertainties are at the 95% confidence level according to the following procedure [29]. First, \( b_{CN} \) and \( \lambda \) are varied to determine the best-fit dipolar coupling,
\( \chi^2_{\nu, \text{min}} \). Next, several trial b_{CN} values are selected about the best fit, and the data are re-fit by optimizing \( \lambda \) within the previously defined range. The uncertainties are represented by \( \chi^2_{\nu} \) values that differ from \( \chi^2_{\nu, \text{min}} \) by \( F(v) \times \chi^2_{\nu, \text{min}} \), where \( F(v) \) is a constant that depends on the number of degrees of freedom, \( v \), and the confidence level. For the case of 95% confidence, and 2 degrees of freedom, \( F(v) = 19 \).

Each best-fit b_{CN} value was linearly scaled by dividing by 0.95 in order to account for thermal motion at 298 K. Internuclear C–N distances are related to the dipolar coupling constants by the equation:

\[
b_{CN} = -\frac{\mu_0 \gamma_C \gamma_N \hbar}{4\pi r^3_{CN}}
\]

(4.3)

The orientation of the nitrogen in the CSA principle axis system was determined by fitting the experimentally observed REDOR curves of each sideband to numerical simulations explained previously [31]. The weighted \( \chi^2 \), \( \chi^2_{\nu} \), was minimized to fit the data. \( \chi^2_{\nu} \propto \sum_{i,N} w(s_{\text{exp}} - s_{\text{sim}})^2 \), where the sum is taken over spectral bands \( N \), and mixing times \( i \). As before, \( w_i \) is the inverse uncertainty in each point squared (\( \sigma^2 \)). The uncertainties in the dipolar and azimuthal angles alpha and beta are represented by \( \chi^2_{\nu} \) values that differ from \( \chi^2_{\nu, \text{min}} \) by \( F(v) \times \chi^2_{\nu, \text{min}} \), where \( F(v) \) is a constant that depends on the number of degrees of freedom, \( v \), and the confidence level. The best-fit dipolar coupling is used in this determination, so this is really a three-parameter fit. However, since the REDOR dephasing is approximately linear in the beginning of the curve (before the first extremum in the REDOR curve, where we fit the data) a small error in the dipolar
Figure 4.8. The $\chi^2$ surface for azimuthal ($\alpha$) and polar ($\beta$) angles of the CSA-dipole orientation with each contour level 2 times the previous level. Labeled APG was diluted to 10% in natural abundance APG and data were collected at 14.1 Tesla with an MAS spinning frequency of 8929 Hz. The superimposed dashed line shows the allowed azimuthal and polar angles corresponding to a rotation about the terminal $\psi$ dihedral angle, with the best fit at $\alpha=31^\circ$, $\beta=90^\circ$.

coupling does not significantly alter the position of the best-fit orientation, and thus can be treated as a two parameter fit.

**Acknowledgments**

A.B.B. was supported through an NSF graduate research fellowship. We thank Marvin Bayro for supplying the pulse sequence for the NCCN experiment and Jozef Lewandowski and Matthew Eddy for helpful discussions, and Matthias Zeller for the
crystal structure. This research was supported by the National Institutes of Health (EB003151 and EB002026).

References


Chapter 5. Cryogenic Sample Exchange Probe for Dynamic Nuclear Polarization Magic Angle Spinning NMR


Abstract

We describe a cryogenic sample exchange system that dramatically improves the efficiency of magic angle spinning (MAS) dynamic nuclear polarization (DNP) experiments by reducing the time required to change samples and by improving long-term instrument stability. Changing samples in conventional cryogenic MAS DNP/NMR experiments involves warming the probe to room temperature, detaching all cryogenic, RF, and microwave connections, removing the probe from the magnet, replacing the sample, and reversing all the previous steps, with the entire cycle requiring a few hours. The sample exchange system described here — which relies on an eject pipe attached to the front of the MAS stator and a vacuum jacketed dewar with a bellowed hole — circumvents these procedures. To demonstrate the excellent sensitivity, resolution, and stability achieved with this quadruple resonance sample exchange probe, we have performed high precision distance measurements on the active site of the membrane protein
bacteriorhodopsin. We also include a spectrum of the tripeptide N-f-MLF-OH at 100 K which shows 30 Hz linewidths.

**Introduction**

Dynamic nuclear polarization (DNP) increases the sensitivity of NMR by transferring the large spin polarization from stable paramagnetic centers to the nuclear spin reservoir [1, 2]. In recent applications to biological solids, the enhanced polarization is generated by millimeter wave irradiation of the EPR transitions of biradical polarizing agents [3] and is uniformly dispersed to the system of interest via proton spin diffusion [4, 5]. During the polarization process, spin-lattice relaxation mechanisms compete with the polarization growth in the proton bath and hinder the saturation of the EPR transition. These $T_1$ processes are slower at lower temperatures and cryogenic operation (presently around 85 K) permits the polarization from the electrons to effectively complete a relayed transfer to such systems as membrane or amyloid proteins [6, 7] and peptide nanocrystals [8]. In addition, in the temperature regime discussed here, the spin polarization is proportional to the inverse of the sample temperature; thus, at 85 K the polarization is another factor of approximately three larger than at room temperature.

However, conventional low temperature magic angle spinning (MAS) NMR experiments generally suffer from poor experimental efficiency due to the time required to change samples. In particular, the simple task of switching from a standard sample used to adjust $B_0$ homogeneity, the magic angle, etc. to the sample of interest involves warming the probe to room temperature, detaching all cryogenic, radiofrequency (RF), and microwave connections, removing the probe from the magnet, and replacing the sample. Subsequently, the procedure is reversed, with the entire warming-cooling cycle
requiring a few hours. This process also increases mechanical wear and the risk of damage to the microwave waveguide and mirror system that delivers the millimeter wave radiation to the sample.

The sample exchange system described here permits the sample to be changed in a matter of minutes without disturbing the probe. It is driven by N\textsubscript{2} and consists of a custom designed eject pipe attached to the front of a Revolution NMR (Ft. Collins, CO) MAS stator that mates with a bellowed hole in a vacuum jacketed dewar. Thus, the rotor is ejected through the top of the dewar into a vacuum jacketed transfer tube running through the magnet bore. A teflon tube on top of the magnet is used to slow and receive the sample. The quadruple resonance (\textsuperscript{1}H, \textsuperscript{13}C, \textsuperscript{15}N and e\textsuperscript{-}) MAS probe described here is a fourth generation design, and the system (probe plus sample eject) has been operating routinely for over a year and has dramatically increased the efficiency of our experiments. It is used routinely with an existing 380 MHz / 250 GHz DNP spectrometer described elsewhere \cite{9, 10}.

This paper is divided into two sections. First, we describe the overall system architecture and the instrumentation required for achieving and maintaining cryogenic sample temperatures while thermally isolating the probe tuning elements. This includes a detailed description of the design of the cryogenic sample exchange system. The instrumentation section closes with a description of the quadruple resonance RF/microwave circuit based on the Schaefer-McKay air dielectric transmission line design.

In Section 3 we demonstrate the capabilities of the system by providing illustrative examples, which include a demonstration of stable DNP enhancements.
over multiple sample exchanges, a spectrum showing the exquisite resolution achievable for a peptide at cryogenic temperature, and a precise distance measurement between two selectively-labelled $^{13}\text{C}$ sites in the membrane protein bacteriorhodopsin.

**Instrumentation**

**System Architecture**

Figure 5.1 shows an overview of the system architecture used for cryogenic DNP/NMR. The major components consist of (left to right in the figure) the $\text{N}_2$ control system, the heat exchanger, the quadruple resonance MAS probe and finally (moving vertically) the sample chamber and eject pipe, the eject tube, and the receiving and

![Figure 5.1 Overview of the system architecture for cryogenic MAS experiments and sample exchange. Thick black lines indicate vacuum-jacketed transfer lines.](image-url)
inserting chambers.

For cryogenic MAS experiments, dry pressurized nitrogen gas is separated into the bearing and drive lines required to spin the sample. The bearing gas passes through a manually controlled pressure regulator, while a standard Bruker MAS unit controls the drive gas pressure. The two independent gas streams transition to evacuated transfer lines before entering a heat exchanger that cools the gases from room temperature to \(-80\) K. Both gas streams are then vacuum insulated from the point they leave the heat exchanger until they enter the sample chamber and MAS stator.

During MAS experiments the valves controlling the sample eject gas at the bottom of the probe and at the top of the eject tube are closed, thereby forcing the cold MAS gas to exit the sample chamber via the vacuum jacketed exhaust line. To exchange the sample the spinning is stopped, the exhaust valve is closed, and the two eject valves opened. Next, pressurized room temperature gas is connected to the exhaust line, forcing the rotor out of the stator, into the eject pipe and tube leading to the top of the magnet. As the rotor exits the magnet bore, it is guided into a receiving chamber by a teflon tube. To insert a sample, the rotor is placed into the eject tube on the top of the magnet and a small flow of gas gently lowers the rotor into the sample chamber and stator. We now provide a more complete description of these components.

**Heat Exchanger and Cryogen Regulation**

To maintain stable temperatures of \(-90\) K for extended periods of operation, a custom designed heat exchanger is required. The heat exchanger used in the present experiments is depicted in Figure 5.2 and is a refinement of a design we described
previously [11]. Room temperature $N_2$ gas enters and exits the heat exchanger through two sets of rigid vacuum jacketed transfer lines. Vacuum insulated lines are used on the input to the heat exchanger so that the overall cooling capacity is invariant to changes in the level of liquid nitrogen in the reservoir. The output transfer lines mate to 2.5 m flexible transfer lines that connect to the probe. The rigid transfer lines are fabricated from stainless steel and are individually silver soldered together, and also soldered to the inner heat exchanger can, as illustrated in Figure 5.2.

The heat exchange system is also designed to permit the liquid nitrogen reservoir to be refilled during operation [11] and to allow control of the pressure of the inner heat exchanger vessel (and thus liquid level) that provides the user direct control of the overall cooling capacity. This makes it possible to bring the temperature of the cryogen to just above its boiling point, while avoiding condensation that results in pressure pulses in the bearing and drive gas, spinning frequency instability, and ultimately rotor crashes.

For MAS experiments, high pressure (~7.5 bar) dry $N_2$ from an external dewar is warmed and passes through multiple ballasts to provide a stable nitrogen gas source. A Bruker MAS control unit functions in the same mode as in standard room temperature applications — monitoring the spinning frequency with optical fibers and maintaining $\leq$5 Hz spinning frequency stability up to ~6 kHz with tight P.I.D. control of the drive pressure. Bearing gas pressure is controlled manually, bypassing the Bruker unit, and is fed directly into the heat exchanger. A manual override controlling the main pressure supplied to the MAS control unit allows the user to stop spinning in a controlled manner in the case of loss of main pressure or power failure.
Manual control of the bearing gas is also implemented to adjust the gas flow, and thus overall cooling capacity, supplied to the probe. Typically, at $\omega_0/2\pi = 4$ kHz, the drive pressure is 600-900 millibar, and the bearing pressure is 700-1400 millibar. At 700 millibar bearing pressure we typically achieve temperatures of 100 K, whereas at 1400 millibar we can maintain ~85 K. We note that significant gains in the DNP enhancement are realized by reducing the temperature from 100 to 85 K, but defer a more detailed discussion of temperature dependence of DNP enhancement to a forthcoming manuscript.
Figure 5.3 CAD drawings of the probe instrumentation needed for cryogenic MAS. a) Evacuated bearing and drive transfer lines in the probe deliver the cryogens to the probe, while an exhaust transfer line brings the cold cryogens out of the probe without cooling the box containing the tuning elements. 50 W heaters at the termination of the flexible evacuated transfer lines allow for precise control of the temperature. The probe-box is purged with a low pressure flow of room temperature nitrogen gas to prevent condensation. b) Cut-out of probe. Multiple optical channels are required for in situ optical irradiation of the sample, temperature sensing, and monitoring of the MAS frequency. Modifications to the dewar, an angled eject pipe that mates to a commercially available Revolution NMR stator, a GORE-TEX seal, and other modifications are needed for the cryogenic sample exchange system.
Cryogenic MAS Strategies

Selected components of the experimental apparatus that connect to the output of the heat exchanger are shown in Figure 5.2. Salient features include two flexible transfer lines with integral 50 W heaters, three vacuum jacketed lines internal to the probe that deliver cryogens to the sample chamber and safely bring the cold gas out of the probe. We also show a non-magnetic, vacuum jacketed-dewar modified with a bellowed hole in the top providing a pathway for sample exchange, and a custom designed eject pipe to direct the rotor into and out of the stator.

The vacuum-jacketed dewar and the use of insulating material in the probe body reduces heat transfer from the probe box and magnet bore, which are maintained at ambient temperature, to the sample chamber. The use of cryogenic bearing and drive gases, which are required at high flows and pressures for MAS, supplies a significantly higher cooling power than a traditional dedicated variable temperature gas stream, and is more than sufficient to overcome the thermal losses through the probe-body, dewar, and transfer lines. Our design also maintains the entire sample chamber at cryogenic temperature, in contrast to traditional designs that cool only the rotor. Cooling the entire sample chamber (the Cu can and base in Figure 5.3b) minimizes temperature gradients across the sample. In implementations of MAS DNP/NMR, the cold spinning gas also effectively overcomes microwave, RF, and frictional heating, especially with an appropriate selection of rotor materials.

Rotors for DNP/NMR are machined from single crystals of sapphire (Inasco; Quakertown, PA). Sapphire has high thermal conductivity, which facilitates active cooling of the sample. It is also transparent to the microwaves needed for DNP and to
visible light for experiments involving optical irradiation of the sample, such as
calcification of photocycle intermediates of bR [6]. The wall thickness of the sapphire
rotor can be optimized to transmit the maximum amount of microwave power to the
sample [12], and 4 mm sapphire rotors are sufficiently strong to spin routinely at \( \omega/2\pi \) 
\( \leq 10 \) kHz. Details of the preparation of the drive-tip and spacers for cryogenic use are
shown below.

A fiber optic temperature sensor (Fiso Technologies; Quebec, Canada) placed
near the rotor (Figure 5.2b) provides an accurate reading of the sample temperature. This
optical sensor is better suited than a platinum resistance thermometer, because the wiring
of the latter often acts as an antenna and couples RF pickup to the sample coil.

As mentioned above, systems that combine room temperature bearing and drive
gas and a variable temperature gas stream directed at the sample can introduce a
temperature gradient across the sample. It is possible to minimize this gradient by use of
a long zirconia rotor but long rotors are difficult to fit into conventional 89 mm magnet
bores [13]. Furthermore, ejection of such long rotors requires rotation of the stator with
respect to the magnet bore; a process that is difficult to implement in a cryogenic probe
and compromises the adjustment of the magic angle.

**Transfer Lines**

During cryogenic operation, room temperature bearing and drive supplies of
nitrogen are cooled to \( \sim 80 \) K by the heat exchanger (Figure 2) [11] and delivered to the
MAS stator with minimal heat transfer from the environment. This is accomplished
using flexible stainless steel transfer lines (Precision Cryogenics; Indianapolis, IN) that
connect the heat exchanger to the probe, and non-magnetic vacuum-jacketed lines in the
probe. 50 W heaters and temperature sensors (Lakeshore Cryogenics; Westerville, OH) are installed near the ends of the transfer lines to control the bearing and drive gas temperatures to ±0.5 K, a stability that is required to obtain stable DNP enhancements.

The flexible evacuated transfer lines from the heat exchanger are joined to the probe transfer lines with stainless steel, vacuum-jacketed bayonet fittings (Precision Cryogenics; Indianapolis, IN). A small cryogen gas space between the male and female sections of the joint is sealed by a silicon O-ring at the warm-end joint [14]. These bayonets maintain excellent thermal isolation between the cryogen and environment.

Within the probe, a set of rigid vacuum-jacketed transfer lines constructed of stainless steel, aluminum and fiberglass (see Figure 4a) provide well-insulated transfer of the bearing and drive cryogens to the sample chamber. These transfer lines are a significant improvement over vacuum-jacketed lines made of glass, which are fragile, and also are commonly connected to the cryogen supply using a glass ball and socket joint that leads to substantial heat transfer. The bottom section of the inner tube is made of stainless steel that is silver-soldered to the bayonet and transitions to aluminum a few inches after the 90° turn into the probe-body. Stainless steel is not used near the stator because it often becomes slightly magnetic after repeated temperature cycling [15]. This is most evident in degradation of the homogeneity required for high-resolution MAS experiments.

While the inner tube of the transfer line is maintained at cryogenic temperatures, the outer tube is thermally coupled to the probe box (ambient temperature) and connected to the sample chamber at the top of the line which is maintained at cryogenic temperature. Thus a nonmagnetic material with a low thermal conductivity must be used
for the outer tube of the transfer line. 577CR fiberglass (Spaulding; Rochester, NH) is a good thermal isolator and is constructed of layers of fiberglass sheets of vacuum tight material, resulting in a transfer line suitable for vacuum applications. We use 577CR fiberglass in the transfer lines and also in the probe-dewar.

**Probe Dewar**

A schematic of the non-magnetic dewar used to thermally isolate the cryogenic probe interior from the ambient temperature magnet bore is shown in Figure 4b. The dewar also prevents the bore of the superconducting magnet from dropping to temperatures where the O-ring sealing the magnet cryostat fails. As an added safety precaution, a low flow of room temperature purge nitrogen gas flows into the magnet bore to ensure a dry environment free of condensation. Finally a bellowed hole in the top of the dewar provides a path for the rotor to enter and exit the MAS stator.

The dewar has an outside diameter of 127 mm and accommodates the 93 mm diameter copper can of the sample chamber. The outer vessel is thin-wall aluminum (Future Metals; Tamarac, FL) and the inner vessel is 577CR. Both vessels are joined with epoxy to an aluminum base, which has 4 semicircular holes needed to attach the dewar to the shim stack on the bottom of the magnet bore. The holes allow for 15 degrees of angular adjustment of the dewar with respect to the magnet bore, leeway that is required for proper alignment of the sample eject system. These mounting holes and details of mating the dewar to the box and magnet are shown in Figure 4b.
Fig. 1. Thermal isolation strategies a) A vacuum-jacketed transfer line used to deliver the bearing and drive cryogens and transport the exhaust gas out of the probe. b) Non-magnetic probe dewar with a bellowed transfer pathway to accommodate the sample ejection pipe (from the bottom) and transfer tube (from the top).
The aluminum base of the dewar mates to the probe-box via an O-ring to establish the air-tight seal necessary to prevent frost and condensation during extended periods of use. Copper fingerstock between the aluminum base of the dewar and the probe establishes an electrical ground that reduces RF pickup. Both the O-ring and the fingerstock are designed such that the dewar-to-probe connection is vertically adjustable. In this manner, the vertical position of the probe can be properly aligned with the stationary horizontal waveguide [16] that couples the probe to the microwave output from the gyrotron. In addition to forming an air-tight seal to the probe, the dewar must also connect securely to the sample eject pipe.

Changing the sample from above the magnet requires a vacuum jacketed transfer tube that passes through the top of the dewar. However, connecting the inner and outer vessels of the dewar with a thermally conductive material would compromise the thermal isolation of the dewar. The fiberglass tube shown in Figure 4b provides a channel for the sample transfer tube, while at the same time acting as a thermal break to prevent heat transfer between the vessels of the dewar. Beryllium copper bellows connect the fiberglass thermal break to the dewar’s outer vessel and accommodate the vertical shrinkage of the inner vessel at lower temperatures (Figure 4b). Beryllium copper was chosen because it is non-magnetic, yet still malleable enough to expand and contract as needed during temperature cycling.

**Cryogenic Sample Exchange**
Figure 5.6 Sample ejection schematics a) Cut-away of probe showing the rotor (purple). A burst of nitrogen gas into the exhaust port creates a flow of gas out of the probe and into the vacuum-jacketed sample exchange tube, carrying the rotor from the stator (1) into the angled sample eject pipe (1 and 2) and finally into the vertical exchange tube (4). For sample insertion, the rotor is lowered on a cushion of gas from the top of magnet and follows the reverse path. b) Angled sample eject pipe. The rotor exits the stator and is guided by the bottom surface of the eject pipe to make the turn from 54.7° to 0° with respect to the magnet bore axis in the limited space provided. The top of the eject pipe is a cone which fits inside the larger cone of the vertical eject tube to allow for adjustment of the magic angle. c) Vertical sample exchange tube. A threaded aluminum attachment allows for a secure coupling into the probe-dewar and proper alignment of tube with respect to the cone on the sample eject pipe. A custom designed valve is closed during operation to prevent frost formation, and is remotely opened during sample exchange. For ejection the rotor travels into a ½” teflon tube which coils around to slowly and safely slow the rotor.
The sample exchange system, including the eject pipe and dewar, is designed to integrate into the 130 mm bore of the 380 MHz magnet. Within that space, the rotor must turn from 54.7° relative to the magnetic field as it leaves the stator, to 0° with respect to magnetic field to travel out of the magnet, in a system that allows adjustment of the magic angle. A custom designed angled eject pipe (Figure 5.5b) (Accelerate Global Sourcing; Austin, TX) bolted to the face of a 4 mm Revolution NMR stator mechanically guides the rotor into and out of the stator. A cone on the eject pipe fits into a larger cone on the eject tube to allow adjustment of the magic angle. Various seals on the sample chamber, and the dewar are needed for sample exchange and thermal isolation (Figure 5.5a).

During the sample exchange process, the rotor is slowed to a stop, and both the bearing and drive gas valves are closed. A custom pneumatic valve in the sample exchange tube at the top of the magnet is opened, and a burst of high pressure, room temperature N₂ gas entering the sample chamber through the exhaust transfer line increases the pressure in the sample chamber and forces the rotor out of the stator and into the vertical sample transfer line (Figure 5.5a). A GORE-TEX seal around the eject pipe (Figure 5.3b) is needed to direct the gas in the sample chamber to flow out of the stator, through the eject pipe, and into the transfer tube. To prevent damage it is imperative that the rotor decelerates slowly once it is clear of the magnet. This is accomplished by directing the rotor into a ½” Teflon tube (Figure 5.5c) that slows the rotor over a ~2 m length; the small coefficient of friction between the rotor and the tube reduces the force on the rotor during deceleration by extending the time it takes the rotor to come to a stop.
To insert the rotor, the valve is opened at the top of the transfer tube, and a small flow of room temperature nitrogen gas from the exhaust line serves as a cushion to ease the rotor into the stator. Although room temperature gas is used for ejection and insertion, the heat capacity of the sample chamber is sufficiently large that the brief flow of the warmer gas does not raise the temperature of the chamber significantly. The rotor can be spun up to 7 kHz while re-establishing a temperature of 90 K in the sample chamber in about 10 minutes.

**Quadruple Resonance RF/microwave circuit**

The probe couples four frequencies — 38, 96, and 380 MHz and 250 GHz — to the sample utilizing an air dielectric transmission line pioneered by Schaefer and McKay [17]. A schematic of the circuit is shown in Figure 5.6, and photographs are available in the Appendix.

Millimeter wave power from the 250 GHz gyrotron is carried in a Gaussian-like mode by a corrugated waveguide and is coupled quasi-optically in the probe-box to a smaller corrugated waveguide that leads to the sample chamber [16]. The circuit, involving a spherical concave mirror and a flat mirror, focuses the larger beam to a smaller one with a beam shape and beam waist that are approximately tuned to the dimensions of the smaller waveguide. The DNP enhancement is sensitive to alignment of the mirrors in the box, and the mirror mounts are both adjustable to allow optimization of the angle of reflection and the horizontal placement of the mounts in the box. Figure 5.6b depicts the mirrors and the trajectory of the microwaves into the vertical corrugated waveguide resulting in low insertion loss [16]. The microwaves travel up the waveguide and then reflect off a miter mirror oriented at 35.7° with respect to B₀. They then travel
Fig. 2. 250 GHz microwave channel and rotor details. a) Enlarged view of the probe with selected components shown. A small groove in the vespel drive-tip is filled with a cryogenic epoxy, Hysol EA 9361 (Loctite) to prevent the drive-tip from coming loose at cryogenic temperatures. The epoxy is also used to seal the top of the rotor. The top Kel-F spacer is threaded, providing a simple and safe way to empty and fill the rotor; the epoxy can be removed with a standard epoxy-stripper, while a 0-80 threaded tool threads into the top spacer, providing a grip to pull the spacer out of the rotor. The microwave power is coupled from the $\text{HE}_{11}$ mode of the corrugated waveguide to the sample by launching the microwaves from the waveguide in the form of a free space propagating Gaussian beam. b) A system of mirrors inside the probe-box focuses the beam delivered by the horizontal corrugated waveguide from the gyrotron into the vertical waveguide leading to the sample. The red lines trace the trajectory of the microwaves.
through another short segment of corrugated waveguide and are launched into the sample cavity in a Gaussian mode. Details of the sample cavity, including the rotor, are shown in Figure 5.6a.

The air dielectric transmission line between the sample coil and the box [17] thermally isolates the tuning elements from the cryogenic temperatures in the sample chamber. The bottom section of the outer conductor of the main transmission line (T_{main}) is thin-walled stainless steel (non-magnetic alloy 321), chosen because it is structurally strong enough to support the probe and has a low thermal conductivity. However, stainless steel has poor electrical properties, so the surface is plated with 10 μm silver and 1 μm gold to prevent oxidation (Precious Metals Plating Company, Santa Ana CA). The silver plating is approximately three times thicker than the skin depth at 380 MHz, resulting in excellent electrical performance of the transmission line [18]. The top section of the inner conductor is copper (Figure 5.6b) which mates to the silver/gold plated stainless section ~10 cm above the box.

The box is fabricated from $\frac{1}{4}$" aluminum and includes modifications to improve the RF shielding because the $^{13}$C frequency of 96.4 MHz lies in the middle of the FM band, which can lead to significant RF pickup. The RF components in the box, including the 6 variable matching and tuning capacitors (Polyflon; Norwalk, NY) and the $1/2"$ $^{13}$C, $^{15}$N transmission line (T_{CN}), are enclosed in a copper box made up of $1/16"$ copper screwed onto the aluminum scaffold (see Figure 5.6b) and a horizontal copper shield. The copper shielding reduces the RF pickup.
Poor isolation between the RF channels results in power and signal loss between the spectrometer and the sample coil on excitation and reception. Our isolation strategies consist of connecting the \(^{13}\text{C}, \^{15}\text{N}\) transmission line at the inherent \((3/4) \lambda \^{1}\text{H}\) node of the main transmission line that occurs inside the box, resulting in isolations of -20 dB from \(^{1}\text{H}\) to \(^{13}\text{C}\) and -19 dB from \(^{1}\text{H}\) to \(^{15}\text{N}\). To achieve -50 dB isolation from \(^{15}\text{N}\) to \(^{13}\text{C}\), capacitors near the coil adjust the \(^{15}\text{N}\) node to the point on the \(^{13}\text{C}\) and \(^{15}\text{N}\) line where \(^{13}\text{C}\) diverges from \(^{15}\text{N}\). The capacitor holder between the coil and ground (denoted \(C_{\text{Sample}}\) in Figure 5.7) can accommodate two non-magnetic fixed capacitors (American Technical Ceramics; Huntington Station, NY), which are held in place with BeCu finger stock because of the mismatch of the thermal expansion of the ceramic capacitors and the copper connections. A parallel LC trap between \(^{13}\text{C}\) and \(^{15}\text{N}\) (\(L_{\text{Filter}}\) and \(C_{\text{Filter}}\) in Figure 5.7) blocks 95 MHz power (at the \(^{13}\text{C}\) frequency) and passes 38 MHz, resulting in -21 dB isolation from \(^{13}\text{C}\) to \(^{15}\text{N}\). We also achieve an isolation of -14 dB from \(^{13}\text{C}\) to \(^{1}\text{H}\) and -18 dB from \(^{15}\text{N}\) to \(^{1}\text{H}\). The isolations between the three RF channels are summarized in Table 6.1.

Table 5.1. Isolations between three RF channels.

<table>
<thead>
<tr>
<th>Power in (dB)</th>
<th>(^{13}\text{C})</th>
<th>(^{15}\text{N})</th>
<th>(^{1}\text{H})</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^{13}\text{C})</td>
<td>(X)</td>
<td>-50</td>
<td>-20</td>
</tr>
<tr>
<td>(^{15}\text{N})</td>
<td>-21</td>
<td>(X)</td>
<td>-19</td>
</tr>
<tr>
<td>(^{1}\text{H})</td>
<td>-14</td>
<td>-18</td>
<td>(X)</td>
</tr>
</tbody>
</table>
Figure 5.7. a) RF circuit diagram. The sample coil and capacitor at the probe (for $^{15}$N isolation) are connected to the box via a transmission line ($T_{\text{main}}$). b) Computer assisted design rendition of the RF circuit elements. Photographs of the actual RF circuit are shown in the Appendix.
Results and Discussion

The probe and cryogenic sample exchange system have been integrated into a 250 GHz/380 MHz DNP/NMR spectrometer and have provided robust performance over an extended period. Here we provide illustrative examples of the type of experiments that are possible with this cryogenic MAS system.

Instrumental Stability

The entire instrument (gyrotron, probe, heat exchanger, etc.) has operated continuously for a two-month period without detrimental frost formation. Over 40 sample insertions and ejections were performed over a six-month period without damage to the sapphire or zirconia rotors. During this period we did not have a single rotor that failed to eject, demonstrating the robust nature of this ejection strategy and design.

Because of the instrument stability we are able to optimize parameters such as the rotor wall-thickness or the composition of the polarizing matrix in order to improve DNP enhancements. Figure 5.8a shows that the enhancement of ~100 is stable to ±4% between sample changes which is a considerable improvement over similar studies on a probe without a sample ejection system [12].

$^{13}$C MAS Spectra of N-f-MLF-OH at 100 K

The cryogenic sample eject permits the optimization of the magic angle, shim coils, and other parameters needed to perform contemporary high-resolution cryogenic SSNMR experiments because the required standard samples (KBr, etc.) can be inserted and ejected without the need to temperature cycle and disassemble the system.
Figure 5.8 Spectra acquired with the cryogenic sample exchange equipped DNP probe. Asterisks identify spinning sidebands. 

a) Stack-plot showing stable enhancements of 1 M $^{13}$C-urea between sample exchanges. The matrix consists of, by volume, 60% d$_8$-glycerol, 30% H$_2$O, 10% D$_2$O, and 15 mM TOTAPOL [19]. The lower three spectra were collected without MW radiation, and the upper three with 250 GHz microwave irradiation, enabling ~100-fold DNP enhancement. The spinning frequency was 4000 +/- 5 Hz and the temperature in the probe was 85 K for all three trials.

b) $^{13}$C spectrum of natural abundance N-formyl-Met-Leu-Phe-OH ($\gamma$-MLF-OH) recorded at 100 K. The linewidth at half-height of three resonances is indicated. $\omega_p/2\pi = 6000$ Hz. 83 kHz $^1$H TPPM [20] decoupling was applied for the 50.8 ms of acquisition. No line-broadening or linear prediction was used. The recycle delay was 3.5 seconds, 2048 transients were averaged. N-$\gamma$-MLF-OH was obtained from Bachem (King of Prussia, PA).
A natural abundance $^{13}$C spectrum of the polypeptide, N-formyl-Met-Leu-Phe-OH (N-f-MLF-OH) recorded at 100 K without DNP is shown in Figure 5.8b. [21]. The resolution available at 100 K with this instrumentation is demonstrated by the 30 Hz linewidth of the methionine-methyl peak. Furthermore, the data show resolution of signals from the $^{13}$C’s in the phenyl ring as its flipping rate entered the slow exchange regime near 100 K. The linewidths in the aromatic region and of the central carbonyl in Figure 5.8b (~51 Hz) demonstrate our ability to properly set the magic angle and shim at low temperature. The excellent resolution apparent in Figure 6.8b suggests that, with proper attention to sample preparation protocols, high resolution spectra of peptides and proteins at low temperature should be attainable.

**Distance Measurements in the Active Site of Bacteriorhodopsin**

Bacteriorhodopsin (bR) is a 26 kDa light-driven ion pump that establishes an ion gradient across the cellular membrane of *Halobacterium salinarium*. In the active site of bR, a retinal co-factor is covalently bound to the protein via a Schiff base linkage to Lysine 216. At thermal equilibrium, bR exists as a 60:40 mixture of bR$_{568}$ and bR$_{555}$. The functional bR$_{568}$ has a 13-trans, 15-anti retinylidene chromophore and bR$_{555}$ has a 13-cis, 15-syn chromophore (Figure 5.9).

Detailed knowledge of the conformation around the C15=N$_{\zeta}$ bond at each stage of the bR photocycle is crucial to understanding the ion translocation mechanism. For example, characterizing the twist in the retinal polyene in the predischarge L state is important in understanding the pump's vectoriality.
Figure 5.9 Chromophore in the active site of bacteriorhodopsin with a covalent Schiff base linkage to Lys216. The retinylidene assumes a 13-cis, 15-syn conformation in bR555 and 13-trans, 15-anti in bR568. These conformers are named by the absorption maximum in the UV-VIS spectrum; 568 nm for bR568 and 555 nm in bR555.

Preliminary SSNMR studies suggest that double bond twist in L is localized on the C15=N bond [6]. Here we use labeled $^{13}$C-14-retinal and $^{13}$Ce-Lys (see Figure 5.9) in order to perform a standard radio frequency driven recoupling (RFDR) [22] experiment. Accordingly, we determine the distance between the labeled sites, and thus the extent of isomerization around the C15=N bond. Although this experiment was attempted some time ago in the absence of DNP [23], only one mixing time was recorded due to low sensitivity and instrument stability (the single 2D correlation spectrum reported took ~5 days to record). With DNP at 90 K, where the effective enhancement is ~90, it required only 7.4 hours to record a high quality 2D spectrum (see Figure 5.10) from 15 mg of protein even when the intensities of interest are divided into a roughly 60:40 ratio, corresponding to the populations of the two different intermediates. Consequently, we
Figure 5.10. RFDR spectrum of $^{13}$C-retinal, $^{13}$Ce-Lys]-bacteriorhodopsin: 15 mg of protein in 60 vol% d$_8$-glycerol, 40 vol% aqueous 0.3 M Guanadine HCl (pH = 10). $\omega_r/2\pi$ = 8000 Hz, temperature is 93 K at the stator, 32 indirect T$_1$ points, spectrum recorded in 7.4 hours.

were able to acquire spectra with seven different mixing times, providing reliable, quantitative data even when small amounts of sample are available.

The recoupling build-up profiles yield distances of 3.11±0.22 Å and 3.90±0.08 Å between C14 of retinal and -Ce of Lys216 in bR$_{555}$ and bR$_{568}$, respectively, corresponding well to the distances of purely geometrical cis and trans conformations (3.1 Å and 3.9 Å). The data were fit using the least-squares algorithm in SPINEVOLUTION, an NMR simulation package [24]. The random error associated with the measurements was also calculated using the sum of least squares function in the same software package; the contour plots of which are shown in Figure 5.10b,c.
Conclusion and Outlook

We have designed and constructed a cryogenic, sample exchange, MAS probe and successfully integrated it into a DNP/NMR spectrometer. The reliability of the system demonstrates the robustness of the approach. The resulting system dramatically improves experimental efficiency by eliminating the long periods of time previously required for warming and cooling the probe to change samples. Finally, while a cryogenic sample exchange system is advantageous for experiments performed at ~85 K with nitrogen cryogens, this technology should prove even more valuable at lower temperatures accessible with helium since the heat capacity of He is lower and the corresponding warming/cooling cycles would be longer.

DNP has been shown effective at 5 and 9 Tesla, but its promise resides in scaling of gyrotron technology and recent polarization schemes to still higher fields [25, 26] corresponding to 700-900 MHz. Movement of necessarily large and metallic probes in such high magnetic fields generates eddy currents that decay over several hours. Since
they are generated each time the probe is removed, a sample exchange system becomes even more desirable at higher magnetic fields.

Finally, in comparison to traditional NMR, DNP is well-suited for studies of reaction intermediates. In particular, the cryogenic temperatures needed for DNP can also serve to trap reaction intermediates, and provide the increase in sensitivity required to effectively study mixtures of states. Maintaining a cryogenic temperature in the sample chamber during sample insertion permits experiments on flash-frozen samples. Although we have only generated reaction intermediates of bacteriorhodopsin in situ [6], it should be possible to flash freeze samples ex situ and without warming insert them into the probe via the cryogenic sample exchange system described here.

For these reasons we expect the instrumentation for sample exchange at low temperatures described in this paper to play a substantial role in applications of DNP to structural studies of biological solids.

Acknowledgments

This research was supported by the NIBIB through grants EB-002804, EB-001960, EB-001035, EB-002026, and EB-003151. A.B.B. was supported through an NSF graduate research fellowship and Y.M. acknowledges partial financial support from the Naito Foundation. We thank Paul P. Woskov, Guo-Xing Miao, Jagadeesh S. Moodera, Loren Andreas, Albert Smith, and Thorsten Maly for their invaluable assistance and stimulating conversations during the course of this research.

Appendix

Photographs of Instrument
Figure 5.A1 a) Photograph of the probe. b) The inside of the probe-box showing the RF circuit. c) Top of the probe-box showing the seals to the probe-dewar. d) Mirrors on the bottom plate of the probe-box for coupling microwaves from the horizontal waveguide to the vertical waveguide e) Sample insertion chamber at the top of the magnet f) Deceleration tube that gently stops the rotor during the ejection process.
References


Chapter 6. Resolution and Polarization Distribution in Cryogenic DNP/MAS Experiments


Abstract

This contribution addresses four potential misconceptions associated with high-resolution dynamic nuclear polarization/magic angle spinning (DNP/MAS) experiments. First, spectral resolution is not generally compromised at the cryogenic temperatures at which DNP experiments are performed. As we demonstrate at a modest field of 9 Tesla (380 MHz $^1$H), 1 ppm linewidths are observed in DNP/MAS spectra of membrane protein in its native lipid bilayer, and <0.4 ppm linewidths are reported in a crystalline peptide at 85 K. Second, we address the concerns about paramagnetic broadening in DNP/MAS spectra of proteins by demonstrating that the exogenous radical polarizing agents utilized for DNP are distributed in the sample in such a manner as to avoid paramagnetic broadening and thus maintain full spectral resolution. Third, the enhanced polarization is not localized around the polarizing agent, but rather is effectively and uniformly dispersed throughout the sample, even in the case of membrane proteins. Fourth, the distribution of polarization from the electron spins mediated via spin diffusion between $^1$H-$^1$H strongly dipolar coupled spins is so rapid that shorter magnetization recovery periods between signal averaging transients can be utilized in DNP/MAS experiments than in typical experiments performed at ambient temperature.
**Introduction**

Dynamic nuclear polarization (DNP) increases the polarization in the bulk nuclear spin bath by 2-3 orders of magnitude,[1-3] and thus results in a significant gain in the sensitivity of NMR experiments. Concurrently, the excellent resolution available with contemporary magic angle spinning (MAS) instrumentation and methods is not compromised by DNP, establishing DNP/MAS as a sensitive and high-resolution spectroscopic technique of great utility for structural characterization of membrane proteins in lipid layers[2, 4], amyloid fibrils[5], and crystalline peptides and proteins.[6] The enhanced polarization can be exploited synergistically with recent advances in solid-state NMR methodology that has been made possible assignments of membrane proteins[7] and high-quality structure determination of amyloid fibrils[8] and microcrystalline protein.[9]

Generally, unpaired electrons in stable, soluable, exogenously introduced free radicals are the source of the high electron Boltzman polarization for DNP experiments.[10] In particular, biradicals are used as polarizing agents at high field because they can take full advantage of the cross effect (CE), an electron-nuclear polarization transfer mechanism that performs well at high frequencies.[11] These polarizing agents can be used at sufficiently low concentration to be statistically separated from the spins of interest, and are therefore not in direct dipolar contact with the bulk nuclei detected in MAS NMR experiments. Nevertheless, they still perform as effective sources of polarization. On the other hand high concentrations of radicals can be used when they are sterically excluded from the sites of interest.[6] In either case, the absense of direct electron-nuclear dipolar couplings between the
unpaired electron spins and the detected nuclear spins has important implications.

First, an efficient transfer scheme is required to distribute the high polarization from the shell of directly electron-coupled nuclear spins to the bulk of the sample. In the case of protonated biological samples, the ~100 M concentration of 1H spins with their high gyromagetic ratio provide a sufficiently strongly coupled spin bath to uniformly distribute polarization throughout the medium, even if the sample is a heterogenous mixture comprised of cryoprotectant and membrane protein embedded in native lipid bilayers.[12] Proton spin diffusion distributes the polarization originating from the electrons to the entirety of the sample with a timescale of hundreds of milliseconds, thereby permitting short magnetization recovery delays between signal averaging transients.

Also, because the 1H spin diffusion and subsequent cross polarization (CP) polarize nuclei that are well separated from the biradicals, spectral resolution is not compromised by the presence of the paramagnetic polarizing agents. Due to the relatively low gyromagnetic ratio of the 15N and 13C, the nuclei most commonly detected in biomolecular MAS experiments, the paramagnetic electron spin must be relatively close (~10 Å) to induce pseudo-contact shifts and line broadening. In fact, Nadaud and colleagues recently showed that even when a nitroxide label is covalently bound to a small globular protein, only spins in relatively close proximity are affected by the spin label.[13] In the case of most DNP experiments, the biradicals are simply present in the matrix, not covalently bound to the protein of interest, and thus not in close spatially proximity to the detected spins.

However, even with a very efficient spin-diffusion process, the temperature
must still be lowered to ≤100 K to lengthen the $^1\text{H} T_1$ relaxation time sufficiently for relayed transfer of enhanced magnetization throughout the sample and also extend the relaxation properties of the electron spins participating in the initial polarization transfer. The resolution of MAS spectra at these temperatures is a point of concern in the solid state NMR community. However, although the resolution of some spectra degrade at low temperatures, either due to improper cryoprotection or trapped conformational heterogeneity, we demonstrate here that high-resolution spectra of a membrane protein and crystalline peptides can be acquired at temperatures below 100 K. We also show spectra of a peptide in which some of the resonances heterogeneously broaden at lower temperature, while other resonances remain narrow.

**Experimental**

**Membrane Preparation**

$[14-{^{13}}\text{C}]$retinal, $[\epsilon^{13}\text{C}]$lysine-labeled bacteriorhodopsin (bR) was obtained as indicated by Griffiths et al.[14] $[\xi^{-15}\text{N}]$lysine-labeled bR was obtained as described by Argade et al.[15] Uniformly $^{13}\text{C},^{15}\text{N}$-labeled bR was obtained as set out by Bajaj et al.[16] The purple membranes were isolated according to the method of Oesterhelt and Stoeckenius[17], washed in 0.3 M guanidinium hydrochloride at pH 10, until the supernatant had the same pH, and then washed further with 60 vol% d$_8$-glycerol, 40 vol% aqueous 0.3 M Guanadine HCl (pH = 10), which acts as a cryoprotectant. The concentration of the nonperturbing biradical polarizing agent, TOTAPOL[18] was 15-20 mM in the $[14-{^{13}}\text{C}]$retinal, $[\epsilon^{13}\text{C}]$lysine-labeled and uniformly $^{13}\text{C},^{15}\text{N}$-labeled samples, and 50 mM in the $[14] [\xi^{-15}\text{N}]$lysine-labeled sample. Membranes were
collected in a pellet by ultracentrifugation (5 h at 323,000 g) and packed into a 4-mm outside diameter, single-crystal sapphire rotor that is transparent at both optical and microwave wavelengths.

**Peptide Preparation**

N-formyl-Met-Leu-Phe-OH (N-f-MLF-OH) was obtained from Bachem (King of Prussia, PA). Uniformly $^{13}$C, $^{15}$N-labelled N-f-MLF-OH was synthesized by CS Bio Inc. (Menlo Park, CA) and diluted with natural abundance compound. In each case, the peptide was recrystallized from isopropanol. Small, needlelike crystals were obtained after dissolution in warm solvent and subsequent drying.

Uniformly $^{13}$C, $^{15}$N labeled Alanyl-prolyl-glycine (APG) was also diluted to 10% with the corresponding natural abundance tripeptide. The tripeptide was synthesized by CS Bio (Menlo Park, CA) with labeled amino acids from Cambridge Isotopes (Andover, MA) and BaChem (Switzerland). APG was recrystallized from water. The peptide, dissolved in a minimal amount of water (the solubility was approximately 45 mg/ml), produced crystals within a week of being placed in a desiccator next to a container of excess ethylene glycol.

All peptide crystals were crushed and packed in 4 mm zirconia rotors. The drive-tips on all of the rotors were bonded to the rotor sleeve with a low-temperature epoxy as described by Barnes et al.[19]

**Instrumentation**

All spectra were collected on a custom-built 250 GHz, 380 MHz, 95 MHz, 38 MHz ($e^{-}, ^1H, ^{13}C, ^{15}N$) frequency spectrometer (courtesy of D. J. Ruben). The quadruple resonance MAS probe is equipped with a cryogenic sample exchange
system, microwave transmission circuit, and a fiber-optic for in situ optical illumination of the sample described by Barnes et al.[19] Details of the 250 GHz gyrotron, corrugated microwave transmission line, and heat exchanger can be found elsewhere.[19-21]

**Accumulation of bR photointermediates**

Accumulation of bR photointermediates was performed in situ, via optical irradiation from a fiber-optic while the rotor was spinning in the MAS module. In this manner, access to the full temperature range of the probe can be utilized to both accumulate photointermediates at the appropriate temperature and cryogenically trap them at ~90 K for data acquisition in the dark. At such low temperatures the lifetimes of the photointermediates can be extended indefinitely. For example, experiments were performed on the M₀ intermediate for over a month without the protein relaxing to intermediates further along the photocycle.

bR₅₆₈ was accumulated by irradiating dark-adapted bR (a thermally equilibrated mixture of bR₅₅₅ and bR₅₆₈) at 273 K with 1 W of 532 nm irradiation for 90 minutes. The M₀ photocycle intermediate was accumulated by irradiating light adapted bR at 210 K with 1 W of 532 nm light for 120 minutes.

**Results and Discussion**

**High resolution spectra of the active site of a membrane protein**

Figure 6.1 shows well-resolved correlation spectra of bR embedded in its native lipid bilayer. We emphasize that we acquired these spectra with samples containing 15-20 mM TOTAPOL (i.e., 30-40 mM electrons) and that they do not show signs of
paramagnetic broadening. The high signal-to-noise ratio available with the boost in nuclear polarization from DNP enable otherwise inaccessible quantitative measurements.[19] Here we discuss the narrow linewidths and excellent site-resolution seen in the spectra. We emphasize two strategies, selective labelling and utilizing unique chemical shifts, employed to acquire the well-resolved spectra of this large (26 kD) membrane protein at a modest field of 9 Tesla, as shown in Figure 6.1.

Figure 6.1a and Figure 6.1b show resolved $^{13}\text{C}-^{13}\text{C}$ correlation spectra of $[14^{13}\text{C}]$ labelled retinal covalently bound to $[e^{13}\text{C}]-\text{lysine}$ in bacteriorhodopsin.[14] Strategically labelling sites of interest is a well-established technique to reduce spectral crowding and acquire high-quality distance constraints, although we also note that this strategy reduces the amount of structural information that can be extracted from a given sample. Nonetheless, crosspeaks arising from the two states present in the dark-adapted protein are clearly resolved and exhibit narrow resonances. The slightly larger linewidth of the bR$_{555}$ resonances is a manifestation of the previously characterized heterogeneity of bR$_{555}$.[22] The bR$_{568}$ cross peak has a linewidth of 1.2 ppm in the C14 direct dimension (Figure 6.1a) and 1 ppm (95 Hz) in the Ce direct dimension (Figure 6.1b). These are the narrowest resonances of a membrane protein from DNP/MAS experiments reported thus far in the literature. Any static disorder frozen out at these cryogenic temperatures does not degrade the resolution of the spectra. However, we expect optimization of the lipid environment and cryoprotectant as well as extension of DNP to higher magnetic fields to lead to still higher resolution.
Figure 6.1. High resolution DNP spectra of bR. a) RFDR[23] correlation spectrum of 14 mg of [14-13C]retinal, [ε-13C]lysine-labeled bR in the dark-adapted state with 1D direct 13C dimension slices overlaid on the 2D contours. 12.5 days worth of acquisition time from mixing times between 8 and 28 ms were averaged together. The DNP enhancement is ~40, relative to the signal recorded in the absence of microwaves. \( \omega/2\pi = 8000 \) Hz, temperature is 93 K. The resulting signal-to-noise negated the need for any line-broadening. The bR_{555} correlation in the upper left has a linewidth of 1.2 ppm (115 Hz) b) Expansion of the lower right portion of the spectrum in (a) demonstrating the narrow linewidths achievable with MAS DNP. Whereas the bR_{555} correlation is slightly broader due to the presence of two conformations of bR_{555}, the bR_{568} resonance is extremely narrow with a linewidth of 1.0 ppm (95 Hz). c) Carbon-nitrogen 2D spectrum of uniformly 13C, 15N-labeled bR_{568} [16] \( \omega/2\pi = 6000 \) Hz. After an indirect chemical shift evolution on the resolved Schiff base (165 ppm in the 15N dimension), the magnetization is transferred along the retinal chain all the way to C11, and also in the other direction directly to Cε. The carbon resonances show relatively narrow linewidths (1.2-2.2 ppm).

The spectrum in Figure 6.1c utilizes the single site resolution available in the spectrum of uniformly 13C, 15N-labelled bR. The Schiff base situated in the active site of the protein has a very unique chemical environment that manifests as a 15N chemical shift well-resolved from the other amide and side-chain nitrogen resonances. Therefore, experiments that first evolve an indirect 15N chemical shift evolution will
yield single site resolution from the Schiff base and result in resolved, high-quality spectra. Reducing the spectral crowding in the $^{13}\text{C}$ dimension by limiting the observed resonances to spins in close proximity to a single resolved nitrogen resonance yields excellent resolution and the capability to simultaneously assign multiple carbon resonances in the active site of the protein. This strategy of transferring polarization from a site which is resolved in the indirect dimension, either by existing in a unique chemical environment, like the Schiff base in bR, or by selective isotopic labelling, yields resolved spectra of high molecular weight systems even at relatively low magnetic fields (9 T).

**High resolution MAS spectra of peptides from 75 to 273 Kelvin**

The absence of appropriate instrumentation capable of acquiring high-resolution spectra at cryogenic temperatures has contributed to the misconception that MAS spectra are always broader at cryogenic temperatures. The recent development of a cryogenic sample exchange DNP probe demonstrated the resolution achievable at 90 K (0.3 ppm, 30 Hz).[19] Here, we elaborate further on the high resolution available in MAS spectra between 75 K and 273 K. We focus on crystalline tripeptides at low temperature, because they do not contain bulk water that necessitates the use of a cryoprotectant, such as glycerol, to maintain long-range homogeneity at low temperatures.

**N-formyl-Met-Leu-Phe-OH (N-f-MLF-OH)**

Figure 6.2 shows high-resolution $^{13}\text{C}$ MAS spectra of crystalline natural abundance N-f-MLF-OH recorded from 273 to 75 K and $^{15}\text{N}$ spectra of uniformly $^{13}\text{C}$, $^{15}\text{N}$-labelled peptide at 80 K. All of the resonances are narrow (<0.6 ppm, 60 Hz)
over the entire temperature range. An expansion of the aromatic region is shown in Figure 6.3b. The top spectrum at 273 K shows only the Fζ resonance because the 2-fold flipping of the phenylalanine ring interferes with the $^1$H decoupling field, broadening the δ,δ’ and ε,ε’ aromatic resonances beyond the detection limit.[24] However, the dynamics slow down at 190 K revealing resonances arising from two coexisting forms of the peptide. Without proper optimization of the homogeneity and the magic angle, this spectral crowding leads to an apparent broadening. However, the spectrum in Figure 6.3a at 190 K clearly shows that nearly all of the single aromatic resonances are resolved from one another. Each single resonance is not by itself appreciably broader than resonances at room temperature.

An expansion of the Cα region is shown in Figure 6.3b revealing two backbone conformations present at 75 K. The two coexistent forms are manifest as a doubling of the resonances, while each of the lines comprising the doublet however remains narrow. We attribute this to a small change in torsion angles rather than a globally different structure because the Me resonance is still extremely sharp <30 Hz at 75 K, indicating a lack of heterogeneous broadening due to cryogenically freezing the peptide. The two low temperature conformations are also apparent in the asymmetric lineshapes of the $^{15}$N resonances (Figure 6.3c). In a similar ratio to the two conformations seen in the Cα region of the $^{13}$C spectrum, the two conformations manifest as a single $^{15}$N resonance with a shoulder; the dominant resonances however remain narrow (only 39 Hz for the amide of Met). We emphasize that the $^{15}$N spectrum was acquired on a uniformly $^{13}$C,$^{15}$N-labelled sample, thus the $^{15}$N-$^{13}$C J-coupling contributes to the linewidths in the spectrum.
Figure 6.2. Temperature dependent high-resolution MAS spectra of N-f-MLF-OH. The spinning frequency is between 4000 and 5500 Hz, with +/- 5 Hz stability. a) Temperature dependent $^{13}$C spectra from 273 K to 75 K showing excellent resolution is maintained throughout the 200 K temperature range.
Figure 6.3. Temperature dependent high-resolution MAS spectra of N-f-MLF-OH. The spinning frequency is between 4000 and 5500 Hz, with +/- 5 Hz stability. A) Expansion of the aromatic regions at 273, 190, and 82 K showing resolved resonances, even with coexisting conformations at 190 K. b) Expansion of the alpha carbon region showing the resolved resonances of the two conformations present at 75 K, each different from the resonances of the single conformation observed at 273 K. c) $^{15}$N spectrum of [U-$^{13}$C,$^{15}$N]-N-f-MLF-OH. The shoulder on the resonances is a manifestation of the two conformations present at 75 K. Nonetheless excellent resolution (linewidth of 39 Hz) is still maintained at 75 K.

**Alanyl-proyl-glycine (APG)**

To demonstrate that the resolution seen in the N-f-MLF-OH spectra are not unique to a single system, we also show comparable resolution in cryogenic MAS spectra of another tripeptide, APG. APG is different from N-f-MLF-OH in two important aspects. First, the crystal lattice contains one water molecule per unit cell that we show does not lead to significant broadening of resonances at low temperatures. Secondly, the proline ring samples two puckered conformations, a
structural feature of five-membered rings that has been well characterized in the literature[25, 26] and has also been observed in both the X-ray and solid state NMR structures of APG.[27] We emphasize that in contrast to the $^{13}$C spectra of natural abundance N-f-MLF-OH in Figure 6.2, the $^{13}$C spectra in Figure 6.4 are taken of uniformly labelled $^{13}$C,$^{15}$N-labelled APG.

Examination of the linewidths at 293 K and 75 K in Figure 6.4a reveals that most of the $^{13}$C lines remain narrow (broadening <17 Hz) at 75 K, including the Pα, Gα, and Aβ resonances. In fact, a $^{13}$C-$^{13}$C J-coupling is resolved on the Gα resonance in the in the $^{15}$N-$^{13}$C correlation spectrum (Figure 6.4b). The three sites that do show broadening are on the proline ring; the Pβ and Pγ resonances (Figure 6.4a) and the Pδ

Figure 6.4 High-resolution spectra of APG showing only low temperature heterogeneous broadening for only the 3 carbon resonances that sample two puckered conformations, $\omega/2\pi = 4831$ Hz. a) $^{13}$C spectra of APG at 293 and 75 K. b) The carbon aliphatic region of a $^{15}$N-$^{13}$C correlation spectrum with 1D direct $^{13}$C dimension slices overlaid on the 2D contours. c) X-Ray and solid state NMR structures of APG from Barnes et al.[27]
resonance (Figure 6.4b) broaden by \( \sim 40 \) Hz. We attribute this to the conformational heterogeneity of the five-membered ring (Figure 6.4c) which is currently under investigation.

**Efficient and uniform polarization distribution via proton spin diffusion**

The \(^{15}\text{N}\) 1D DNP CPMAS spectrum of light-adapted \([\text{U-}^{13}\text{C}, ^{15}\text{N}]\)-bR is shown in Figure 6.5. The buried Schiff base at 165 ppm is well resolved from the amide, arginine and other lysine sidechain nitrogen resonances. To address whether the enhanced polarization originating from the electron spins and DNP is uniformly distributed across the membrane, and the protein embedded therein, we integrated the intensity of the four separated lineshapes in the \(^{15}\text{N}\) spectrum in Figure 6.5. If the polarization is uniformly distributed, the integral of each resonance should be proportional to the number of contributing nuclei. As shown in Table 6.1, the relative intensities of the resonances correspond very well to this expectation, providing clear evidence that the polarization propagates to the buried active site as thoroughly as to the rest of the protein embedded in its native membrane. Proton spin diffusion thus effectively transfers the DNP enhanced polarization throughout the heterogeneous sample of cyroprotectant and membrane protein in lipid bilayers.
Figure 6.5 1D $^{15}$N spectrum of U-$[^{13}C,^{15}N]$-bR showing the $^{15}$N Schiff base resonance adjacent to the peptide backbone signal, $\omega_r/2\pi = 6000$ Hz.

Table 6.1 Relative experimental intensities (peak areas) of a DNP enhanced $^{15}$N spectrum of bR vs. number of $^{15}$N sites present.

<table>
<thead>
<tr>
<th>Peak Assignment</th>
<th>Peak Area $\pm$</th>
<th>Number of Sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schiff Base</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Amides</td>
<td>$240 \pm 13$</td>
<td>248</td>
</tr>
<tr>
<td>Arginines</td>
<td>$25 \pm 2$</td>
<td>21</td>
</tr>
<tr>
<td>Lysines</td>
<td>$6 \pm 1$</td>
<td>6</td>
</tr>
</tbody>
</table>
Accelerated Data Acquisition

In the original expression for the sensitivity enhancement from the classical CP experiment, the ratio of the spin-lattice relaxation times of the abundant and rare spins ($T_{1S}/T_{11}$) plays an important role.[28] This term represents the gain in time savings from utilizing the shorter spin-lattice relaxation time of $^1H$ magnetization versus $^{13}C$ or $^{15}N$. This is important as the experimental gain in sensitivity between a Bloch decay and CP experiment is typically only a factor of ~2.5 versus the theoretical maximum of 4. Thus much of the improvement in sensitivity from using CP actually comes from the reduced time needed to recover longitudinal magnetization between signal averaging transients. The situation is similar in DNP experiments, although the polarization enhancements are of course much larger.

The radical concentration, initial electron to nuclear polarization transfer rate, and proton spin diffusion efficiency govern the rate of longitudinal build up of magnetization during the polarization period between DNP/MAS signal averaging transients. This time constant differs from classical NMR experiments in which the rate of recovery of longitudinal magnetization is dependent on the spectral density function evaluated at the nuclear Larmor frequency. Optimal repetition rates of DNP/MAS experiments at cryogenic temperature are thus much higher than for samples without paramagnetic dopants. This has recently been exploited at ambient temperatures to reduce the proton $T_1$, which leads to much improved repetition rates and sensitivity.[29] Here we show here that similar improvements in data acquisition rates are seen by increasing the concentration of polarizing agents in DNP
experiments.

As shown in Figure 6.6a, increasing the biradical concentration from 20 mM to 50 mM reduces the longitudinal proton magnetization build-up time constant ($T_{1DNP}$) by a factor of two, thus doubling the rate of signal averaging. Relatively short recycle delays of 1.6 s ($1.26*T_{1DNP}$) for experiments on a sample of $[^{15}\text{N}]$lysine labeled bR were used to exploit the short proton $T_1$. Due to the cryogenic temperatures, extensive cooling power from the spinning gases, and the use of thermally conductive sapphire rotors, the sample is not at risk of detrimental heating that can arise from high RF powers and short delays between transients at ambient temperatures.

Figure 6.6b shows spectra of the $M_0$ photocycle intermediate after only 1.5 days of signal averaging. There is sufficient signal-to-noise to complete a Herzfeld-Berger analysis[30] and extract the chemical shift anisotropy parameters, which are given in the figure caption. Even the +2 sideband with only a small fraction of the intensity of the centerband has sufficient signal-to-noise to be fitted and used in the chemical shift analysis.

The resolution in the $^{15}\text{N}$ resonance in the active site is not broadened even when the electron concentration is increased to 100 mM. The linewidth of the Schiff base resonance is the same in samples containing 15 and 50 mM TOTAPOL. The biradical is excluded from the center of the protein by steric hindrance and paramagnetic electrons are not close enough to the active site to result in pseudo-contact shifts and broadening.
Figure 6.6 Accelerated DNP a) difference in longitudinal relaxation time, $T_{1\text{DNP}}$, under microwave irradiation between 20 and 50 mM TOTAPOL. The magnetization of the bulk protons is measured via CP to the carbonyls of [U-$^{15}$N,$^{13}$C]-bR. b) DNP spectrum of [$\epsilon^{15}$N]lysine-labeled bR trapped in the Mo photocycle intermediate. 81,920 Transients, 1.6 s recycle delay, 36.4 hr acquisition time, $\omega/2\pi = 5000$ Hz, $T=89$ K. The isotropic shift is 318.3 ppm, the span is 620 ppm, the skew is $-0.034$, and the tensor elements are: $\delta_{11}=618$, $\delta_{22}=340$, and $\delta_{33}=-2.5$ ppm.
Conclusions

Excellent resolution and sensitivity are available in cryogenic MAS DNP spectra of membrane proteins and crystalline peptides. With proper cryoprotection, similar linewidths to those reported here should be achievable on crystalline proteins and other integral membrane proteins.

Acknowledgements

This research was supported by the National Institutes of Health through grants EB002804, EB003151 and EB002026 to RGG, EB001035 to JH, and grants EB001965 and EB004866 to RJT. ABB and LBA were partially supported by graduate research fellowships from the National Science Foundation. BC was funded by the Deutsche Forschungsgemeinschaft (research fellowship CO 802/1-1). Stimulating conversations with T. Maly and M. Bayro are gratefully acknowledged.

References


Chapter 7. Microwave Field Distribution in a Magic Angle Spinning Dynamic Nuclear Polarization NMR Probe


Abstract

We present a calculation of the microwave field distribution in a magic angle spinning (MAS) probe utilized in dynamic nuclear polarization (DNP) experiments. The microwave magnetic field ($B_{1s}$) profile was obtained from simulations performed with the High Frequency Structure Simulator (HFSS) software suite, using a model that includes the launching antenna, the outer Kel-F stator housing coated with Ag, the RF coil, and the 4 mm diameter sapphire rotor containing the sample. The predicted average $B_{1s}$ field is $13\mu T/W^{1/2}$, where $S$ denotes the electron spin. For a routinely achievable input power of $5 \text{ W}$ the corresponding value is $\gamma_sB_{1s} = 0.84 \text{ MHz}$. The calculations provide insights into the coupling of the microwave power to the sample, including reflections from the RF coil and diffraction of the power transmitted through the coil. The variation of enhancement with rotor wall thickness was also successfully simulated. A second, simplified calculation was performed using a single pass model based on Gaussian beam propagation and Fresnel diffraction. This model provided additional physical insight and was in good agreement with the full HFSS simulation. These calculations indicate approaches to increasing the coupling of the microwave power to
the sample, including the use of a converging lens and fine adjustment of the spacing of the windings of the RF coil. The present results should prove useful in optimizing the coupling of microwave power to the sample in future DNP experiments. Finally, the results of the simulation were used to predict the cross effect DNP enhancement (e) vs. $\omega_{1S}/2\pi$ for a sample of 1 M $^{13}$C-urea dissolved in a 60:40 glycerol/water mixture containing the polarizing agent TOTAPOL; very good agreement was obtained between theory and experiment.

**Introduction**

Magic angle spinning (MAS) nuclear magnetic resonance (NMR) is an effective method to extract biologically relevant structural constraints with sub-angstrom precision from a variety of different systems such as membrane proteins embedded in native lipid bilayers [1-8], antibiotics bound to bacterial cell walls in whole cells [9] and amyloid fibrils [10-14]. All three of these are examples of systems that are not accessible by either X-ray crystallography or solution NMR spectroscopy, the standard tools of structural

![Figure 7.1. Microwave irradiation into the stator housing (a) for an ideal Gaussian beam emitted from the end of an 8 mm corrugated waveguide and radiated to a distance of 12 mm from the waveguide end, corresponding to the location of the sample, compared to (b) the measured output. (c) A cross section of the stator and DNP MAS probe showing the coil, sample, waveguide, and drive-cup and bearings of the spinning assembly.](image-url)
biology. However, NMR detection of biomolecules in the solid state is inherently insensitive due to at least three factors. First, nuclear magnetic moments are very small, a property that yields long lived relaxation times and high-resolution spectra, but also results in weak Faraday induction and thus poor coupling to the RF coil. Second, investigating large macromolecular complexes in biologically relevant environments such as lipid bilayers or intact cells results in a low concentration of sites. Finally, detection in MAS experiments is typically performed on nuclei with a low gyromagnetic ratio ($^{13}$C and $^{15}$N) rather than the more sensitive $^1$H detection prevalent in solution NMR. Dynamic nuclear polarization (DNP) is a powerful technique used to overcome these sensitivity issues in MAS experiments [15-19].

DNP dramatically improves the sensitivity of NMR experiments by transferring the large polarization of electron spins to bulk nuclei, a process which requires excitation of electron paramagnetic resonance (EPR) transitions with high-intensity millimeter wave radiation [20, 21]. Sensitivity enhancements of 50-300 on membrane proteins, amyloid fibrils, and micro-crystalline peptides [18, 19, 22] are routine and are having a profound impact on biomolecular structural studies. Such results are due to the availability of stable, high-power gyrotrons as microwave sources [15, 23, 24], technical advances in cryogenic MAS [25-27] and the design of more effective polarizing agents [28-30].

DNP enhancements are dependent on a number of factors, and among the most important is the intensity of the microwave $B_{1s}$ field throughout the sample, where $B_{1s}$ denotes the component of the microwave magnetic field that is orthogonal to the Zeeman field $B_0$ and oscillating in resonance with the electron spins. In MAS DNP experiments, the $B_{1s}$ field is generated by millimeter waves produced from an external source coupled
to the sample via waveguides and/or quasioptics. However, due to the physical constraints imposed by the MAS probe, the design of an efficient mechanism for coupling the microwave power into the sample has proved challenging. These challenges include a high dielectric loss tangent of the sample, a cylindrical sapphire rotor, a large RF coil, the highly overmoded nature of the sample and the surrounding Kel-F stator.

Furthermore, the rotor is often filled with sample extending the entire length of the RF solenoid to increase the signal detected by the RF coil. This situation is in contrast to EPR/ENDOR resonators in which the sample occupies a small fraction of the volume where the $B_{1s}$ field is both uniform and intense [31]. For MAS DNP it is important to have a $B_{1s}$ field uniformly irradiating the sample in order to optimize the signal enhancements. Thus, the design and fabrication of a resonator with dimensions large compared to the microwave wavelength and containing a lossy dielectric sample is the paramount challenge in achieving large signal enhancements in high frequency MAS DNP experiments.

This paper presents two models used to analyze and predict the performance of microwave coupling to the sample in DNP experiments. The first approach utilizes the High Frequency Structure Simulator (HFSS) code [32] to model the electromagnetic fields within the MAS stator. The distribution of the microwave field in the sample is used to calculate the signal enhancement for a given input power. The second and approximate method is a single pass model based on Gaussian beam propagation and Fresnel diffraction that allows for quick inspection of the $B_{1s}$ field throughout the sample. Using this second method we are able to develop physical intuition about the impact that different components have on microwave coupling into the sample. However, the model
includes some approximations that simplify the probe geometry in order to make an analytical solution possible. We also discuss implications of the results of these models for the optimization of microwave coupling and an understanding of the $\omega_{1S}$ dependence of the DNP enhancement.

**Physical Structure and Dielectric Constants**

We characterized the microwave power distribution within the stator of a custom-built quadruple resonance ($^{15}$N, $^{13}$C, $^1$H, e$^-$) cryogenic sample-exchange DNP probe recently developed in our laboratory [25]. The microwave source for these experiments is a 250 GHz gyrotron oscillator developed at MIT [24, 33], capable of producing 15 W with a Gaussian beam output. The cross section of Gaussian beam is defined as having an intensity profile given by $e^{-2x^2/w_x^2}e^{-2y^2/w_y^2}$, where $w_x$ and $w_y$ are the beam waists in the x
and y direction, respectively. The microwave power is coupled from the gyrotron to the stator through corrugated waveguides and quasioptics, as described previously [25, 34]. After optimizing the alignment of the system, the microwave beam entering the stator was imaged 12 mm from the waveguide aperture, corresponding to the location of the sample, using a Spiricon Pyrocam III pyroelectric detector to verify the Gaussian beam content. Figure 7.1 shows excellent agreement between the pyroelectric camera image and an ideal Gaussian beam being radiated from an 8 mm corrugated waveguide into free space. The radiated beam is 93% Gaussian with a waist of 3.1 mm in the rotor short axis direction and 3.4 mm in the rotor long axis direction, determined by finding the best fit for a 2D Gaussian. The white box in Figure 7.1(a) delineates the sample region. The optimization of overlap between the sample and the Gaussian beam using a cylindrical converging lens is discussed in a later section.

**HFSS Model**

The probe geometry as modeled in HFSS is shown in Figure 7.2. The MAS stator module houses a cylindrical sapphire rotor with an outside diameter of 4 mm, oriented at 54.7° with respect to B₀. Rotors with an inside diameter (ID) ranging from 2.1 to 2.8 mm were tested, with the optimal results obtained for an ID of 2.4 mm. A rotor with this ID accommodates ~56 µl of the cryo-protected sample of interest. A cylindrical Kel-F stator with a radius of 13.5 mm and a height of 12.45 mm encloses the entire RF coil and sample assembly. The leads of the RF coil enter from the side of the stator and form a 6.5 turn solenoid wrapped from 0.8 mm diameter (20 gauge) silver-plated copper wire. A corrugated 8 mm cylindrical waveguide launches microwave power into the stator towards the sample (Figure 7.1c and 7.2).
Figure 7.3. Normalized magnitude of $B_{1S}$, the transverse component of the microwave magnetic field, for the (a) vertical cross section of the probe, (b) the horizontal cross section of the probe and (c) the rotor and sample cross section. The magnitude $\left(\sqrt{B_{1S}^2 + B_{1S}^2}\right)$ provides the maximum value of $B_{1S}$ allowing for the observation of the standing wave patterns present in the input and the horizontal direction of the sample, as well as the interference fringes from the RF coil in the vertical direction of the sample.

The dielectric constant of the sample must be known in order to accurately model the microwave coupling. Our standard sample consists of 1 M $^{13}$C-urea and 10 mM TOTAPOL [28, 29] dissolved in a cryo-protecting matrix consisting of $d_8$-glycerol/D$_2$O/H$_2$O (60/30/10)% by volume. The dielectric constant of this matrix at 77 K was measured at 140 GHz using a vector network analyzer; the real part was $3.5\pm0.1$ with a loss tangent of $0.005\pm0.0005$. The dielectric constant was determined by measuring transmission through a thin planar sample of the matrix from 90-147 GHz and fitting the
Fabry-Perot resonances to determine the real and imaginary part of the dielectric constant; the loss tangent is defined as \( \tan \delta = \varepsilon_i / \varepsilon_r \). Based on typical properties of similar materials such as ice at low temperatures, we estimate that the real part of the dielectric constant will vary only slightly with frequency, while the imaginary part will increase approximately linearly [35, 36]. To obtain the dielectric constant at 250 GHz, we scaled the 140 GHz values, keeping the real part constant (3.5) and scaling the loss tangent linearly with frequency (0.009). The general conclusions of this study are not critically dependent on the exact values of these parameters, which will likely vary with sample content.

The precise \( B_{1S} \) field distribution was obtained using a full 3D HFSS model of the stator, as shown in Figure 2. A cylindrical Kel-F stator that is not fully illustrated in Fig. 2, but was included in the model, encloses the RF coil and rotor. The stator was partially coated with Ag [25] in order to increase the confinement of the microwave radiation. The coating's effectiveness is limited because power escapes through holes required for the rotor bearing and drive gas and the high voltage leads of the RF coil. The magnitude of \( B_{1S} \), the transverse component of the magnetic field, can be seen in Figure 7.3. The microwave magnetic field in the sample is linearly polarized. The linearly polarized microwave field can be decomposed into two circularly polarized components, only one of which contributes to the excitation of electron spins. Figure 7.3 plots the normalized magnitude of the magnetic field, therefore the distribution is the same regardless of whether the field is linearly or circularly polarized. Figure 7.3a is a cross section of the center of the probe along
Figure 7.4. Axial profile at (a) $r = 0.6$ mm and (b) radial profile at $z = 0$ mm.
the vertical axis of the sample, Figure 7.3b is a cross section of the center of the probe perpendicular to the vertical axis of the rotor, and Figure 7.3c is an expanded image of the sapphire rotor and sample region for the same cross section shown in Figure 7.3a. Several items of interest appear in the HFSS model. First, a standing wave develops at the input port of the probe due to the large reflection from the RF coil. Second, the diffractive nature of the waves transmitted through the windings of the RF coil, which have a wire diameter on the order of a wavelength, manifest as local maxima and minima along the vertical axis of the rotor. Third, reflections, especially in the silver coated Kel-F stator, create some moding in the radial direction of the stator. Finally, the amplitude of the field decays towards the vertical extremes of the sample attenuating the signal enhancements produced in those regions.

The HFSS simulations provide the electric and magnetic field throughout the stator. Using the known orientation of the sample, it is straightforward to calculate the $B_{1S}$ value from the magnetic field. The software suite allows the user to perform simulations with progressively smaller mesh size to confirm that the solution becomes independent of grid size. The simulations and the $B_{1S}$ value converged with a mesh element volume of $\sim(100\mu m)^3$ yielding the spatial, time average $B_{1S}$ field of $13\mu T/W^{1/2}$. For a routinely achievable input power of 5 W the corresponding $\gamma_s B_{1S} = 0.84$ MHz. The same calculation repeated without the silver coating of the stator yields 0.70 MHz for 5 W of input power. These values of $B_{1S}$ are obtained by first averaging the physically important quantity, which is $B_{1S}^2$, over the volume of the sample and then taking a square root.
Axial and radial distributions of $\gamma S_{15}$ for an input power of 5 W can be seen in Figure 7.4. Once again, the diffractive effect of the RF coil is evident in the vertical direction, and moding is seen in the radial direction. Figures 7.3 and 7.4 illustrate the extremely inhomogeneous irradiation that the sample receives from the Gaussian beam launched by the overmoded corrugated waveguide. In the sample, the $\gamma S_{15}$ values range from 0.1 MHz to 4 MHz. This non-uniform distribution of $\gamma S_{15}$ impacts the effectiveness of the probe, especially in the case of large volume samples. Calculating enhancements for a given $\gamma S_{15}$ distribution in the sample taken from the HFSS model will be discussed in Section 7.4.

An additional advantage of the HFSS model is the ability to track power flow through the stator. The thermal energy deposited in the sample must be considered because of the inverse dependence of enhancement on temperature [25]. The HFSS model indicates that the incident power is distributed as 5% deposited into the sample, 35% reflected back through the input port and 60% radiated from the ends of the rotor or deposited ohmically in the stator and RF coil.

**Dependence of $\varepsilon$ on $\omega_{15}$**

The HFSS simulation, together with a knowledge of the DNP mechanism, permits us to examine the dependence of the DNP signal enhancement ($\varepsilon$) on the microwave field strength, $\omega_{15} = \gamma S_{15}$.

When TEMPO based radicals are used as polarizing agents, then the primary high field DNP mechanism is the cross effect (CE) which involves two electrons and
a nuclear spin. Specifically, the EPR spectrum of TEMPO is dominated by the g-anisotropy and satisfies the inequality $\Delta > \omega_{01} > \delta$, where $\Delta$ is the inhomogeneous breadth of the spectrum, $\omega_{01}$ is the nuclear Larmor frequency, and $\delta$ is the homogeneous EPR linewidth. Thus, at high fields the Larmor frequency separation of two electrons $S_1$ and $S_2$ can be matched to the nuclear Larmor frequency, $\omega_{0S1} - \omega_{0S2} = \omega_{01}$. Microwave irradiation flips $S_1$ that is dipolar coupled to $S_2$ and the difference in energy goes into polarizing the nuclear reservoir. Kessenikh, et al. [37-39] first described the CE in the limit of infinite $\omega_{1S}$. Subsequently, Hwang and Hill [40, 41] extended the theory to account for finite $B_{1S}$'s, but did not allow for leakage. Leakage was introduced by Wollan [42, 43] by modifying the rate equations of Hwang and Hill to examine the case of a well-resolved CE, two narrow EPR lines at $\omega_{0S1}$ and $\omega_{0S2}$ separated by exactly $\omega_{01}$. These results were then extended to the case of an inhomogeneously broadened EPR spectrum with $N$ unresolved spin packets that leads to an expression for the steady state enhancement. This treatment is applicable to the experimental situation presented by biradical polarizing agents such as BTnE [28, 44], TOTAPOL [29] and bTbk [30] where the e-e dipole couplings are $\sim 20-30$ MHz. Recently, the CE has been treated quantum mechanically with a particular emphasis on understanding and optimizing the enhancements available with biradicals [45].

Using classical rate equations, Wollan derived the following expression for the steady state enhancement, $E_{SS}$, in a CE experiment.
\[ E_{ss} = 1 + E_i \left( \frac{S^0}{1 + S^0} \right) \left( \frac{S_{CE}}{1 + S_{ss}X} \right) \left( \frac{3 [G(\omega - \omega_{os})]^2}{G(\omega_{os})G(\omega_{os} \pm \omega_{ol})} \right) \times \xi \left[ G(\omega_{os} - \omega_{ol}) - G(\omega_{os} + \omega_{ol}) \right] \]

(7.1)

Here the \( G(\omega)'s \) are the normalized EPR lineshape functions with \( \omega_{os} \) the center of the lineshape, \( (\omega - \omega_{os}) \) denoting the frequency of microwave irradiation, \( (\omega_{os} \pm \omega_{ol}) \) the solid effect frequencies, and \( \xi \) is the width of the spin packet, assumed to be rectangular, in the inhomogeneously broadened lineshape. The difference in the last factor represents the difference between spin packet populations. \( S_{CE} = W_{CE}^{HIB} T_n' \) is the CE saturation parameter where \( W_{CE}^{HIB} \) is the transition probability for the inhomogeneously broadened (IHB) line and \( T_n' \) accounts for all modes of spin lattice relaxation other than that due to the CE. We take \( X = \left( W_{CE}^{HIB} T_n CE \right)^{-1} = 1 \), and refer the reader to Wollan’s paper [42] for a discussion of this point. Thus, for a given irradiation frequency in the EPR spectrum, the last three factors on the RHS of Eqn. (1) are constant. Furthermore, we identify \( E_{ss} \) with \( \varepsilon \), assume that \( \varepsilon >> 1 \), and incorporate the constants into \( \varepsilon_{\text{max}} \) to obtain the simplified expression

\[ \varepsilon = \varepsilon_{\text{max}} \left( \frac{S^0}{1 + S^0} \right) \]

(7.2)

Again following Wollan, \( S_0 = W_0 T_1 S \), \( W_0 = \pi \omega_{1S}^2 g(\omega) \), and we approximate \( g(\omega) = T_{2S} / \pi \) which yields

\[ \varepsilon = \varepsilon_{\text{max}} \left( \frac{\omega_{1S}^2 T_{1S} T_{2S}}{1 + \omega_{1S}^2 T_{1S} T_{2S}} \right) \]

(7.3)
Figure 7.5. DNP enhancement vs. $\omega_1/2\pi$ (bottom) and power (top) for a fully packed rotor of 1 M $^{13}$C-urea and 10 mM TOTAPOL dissolved in $d_8$-glycerol/D$_2$O/H$_2$O (60/30/10% by volume) [29, 44].

Since the microwave field, $\omega_{1S}$, is inhomogeneous, as demonstrated in Figures 3 and 4, we integrate the expression over the field dependence for the sample volume

$$
\varepsilon = \frac{1}{V} \varepsilon_{\text{max}} \int \frac{\alpha \omega_{1S}^2}{1 + \alpha \omega_{1S}^2} dV
$$

(7.4)

where now $\alpha = (T_{1S}T_{2S})/2$. The factor of 1/2 converts from the time averaged linearly polarized $\omega_{1S}^2$ to the time averaged circularly polarized component that interacts with the electrons. $T_{1S}$ for a Nitroxide radical is expected to range from 10-400 $\mu$s in the 90 Kelvin temperature regime, and $T_{2S}$ is a strong function of radical concentration being in the range of 10 to 200 ns for the 20 mM electron concentration in our sample. The magnitude of the product $T_{1S}T_{2S}\omega_{1S}^2<< 1$ to obtain the quadratic dependence observed at low $\omega_{1S}^2$. It then saturates at high $\omega_{1S}^2$. Figure
7.5 is a plot of the experimental enhancement vs. \( \omega/2\pi \) at 250 GHz for the sample and probe described in Figures 7.1 and 7.2 and the field distribution shown in Figures 7.3 and 7.4. An excellent fit to the experimental data is obtained for the parameters \( \varepsilon_{\text{max}} = 230 \), \( T_{1S}T_{2S} = 8 \times 10^{-12} \text{ s}^2 \). We emphasize that only by employing a volumetric integral that explicitly accounts for the large \( \gamma_S B_{1S} \) fluctuations were we able to fit the enhancement curve with a \( T_{1S}T_{2S} \) factor that corresponds to published experimental values [46]. Calculating the value of \( \gamma_S B_{1S} \) at the level of a mesh element volume defined by the HFSS model is crucial to understanding the DNP enhancement data and, indeed, to modeling the enhancement more generally obtained from the cross effect [42].

**Rotor Wall Thickness**

Minor modifications to the geometry of the rotor and stator can result in significant changes to the coupling of the microwave beam into the sample. For

![Graph showing the relationship between Rotor Wall Thickness and Signal Enhancement](image)

**Figure. 7.6.** \(^{13}\text{C}\) signal enhancement for a center-packed (5 mm length) sample as a function of rotor wall thickness.
example, to optimize the rotor wall thickness, we have experimentally determined the DNP enhancement using rotors of varying wall thickness, and performed HFSS simulations in order to obtain an understanding of the influence of the rotor geometry on the microwave field strength in the sample.

The outer radius of the rotor is constrained to 4 mm by the bearings that support the rotor during sample rotation, but the inner radius can be varied to maximize the coupling of microwave power to the sample. The sample diameter, equal to 4 mm minus twice the rotor wall thickness, was varied from 2.1 to 2.8 mm. Using Eqn. (7.4), we determined the expected enhancement from the $\gamma_s B_{1s}$ field distributions corresponding to wall thicknesses ranging from 0.6 to 1 mm, with the results shown in Figure 7.6. The predicted enhancements are in reasonable agreement with the experimental values determined for the four wall-thicknesses shown. This supports our approach to calculating the enhancement for a given field distribution and concurrently sets a precedent for further optimization of the DNP enhancement through geometrical considerations. As shown in Figure 7.6, a sample diameter of 2.4 mm optimizes the enhancement. Coincidently, this is the wall thickness used in commercial 4 mm rotors (Revolution NMR Ft. Collins, CO).

Local maxima in the enhancement were observed near a rotor wall thickness of 0.6, 0.8 and 1 mm corresponding to peaks in transmission through the sapphire rotor wall. The general downward trend with decreasing rotor thickness is due to the increased sample volume with correspondingly lower overall average $\gamma_s B_{1s}$ values for constant input power.

**Single Pass Model**
A single pass model was also used as a simple approximation for microwave irradiation of the sample. The input corrugated waveguide launches a Gaussian beam into the probe that propagates through the RF coil and sapphire rotor to the sample (Figure 7.2). The metallic coil acts as a grating causing the incident beam to be both transmitted, with diffraction into the sapphire rotor, as well as reflected. The transmitted radiation then passes through the sapphire rotor that acts as a cylindrical converging lens and focuses the beam as it propagates into the sample.

Using Gaussian beam propagation and Fresnel diffraction, one can model this system with some approximations. The main assumption is the absence of multiple reflections, i.e. a single pass model. This assumption can be justified by the lack of coherence in the reflections caused by the highly overmoded, lossy and complex geometry of the sample region. A second approximation models the RF coil as a parallel array of cylindrical wires, also called a wire array or wire grating. The dominant feature of the present arrangement of the RF coil is the spacing to wavelength ratio [47, 48]. We note that resonances that can occur for certain wire diameter to spacing ratios require a 3D treatment as will be discussed in Section 7.7.

The advantage of the single pass model is its reduced computational requirements and a physically intuitive understanding of how components such as the RF coil and sapphire rotor behave. Using this model, the highly overmoded volume can be analyzed rapidly, and it is straightforward to add or modify simple dielectric or metallic structures to explore the change in behavior. The physical understanding of the role that the various components play in the probe geometry is achieved by modeling the components individually.
To calculate the average value of $\gamma_S B_{1S}$ in the sample, a 5 W Gaussian beam with an initial waist of 2.6 mm was propagated from the corrugated waveguide to the surface of the RF coil. In the paraxial limit, the propagation of the Gaussian beam through vacuum is solely governed by initial beam waist. The power transmission through the RF coil was determined using published data [48]. Using Fresnel diffraction, the beam is radiated past the coil. The Gaussian beam incident on the RF coil provides the illumination pattern that is the source term in the Fresnel diffraction integral. The transmission through the sapphire-sample boundary is calculated based on the incident angle. The microwave radiation no longer has a Gaussian distribution along the rotor long axis due to the diffractive effects of the wire array. However, in the plane perpendicular to the rotor long axis, the distribution is still Gaussian and this plane contains the curvature of the rotor that will focus the beam.

After performing these calculations, the single pass model yielded the highly non-uniform $\gamma_S B_{1S}$ distribution along the central axis of the sample shown in Figure 7.7. We calculated an average value of $\gamma_S B_{1S} = 0.7$ MHz for an input of 5 W in the sample, in good agreement with the calculated value of 0.835 MHz from the more rigorous HFSS simulations.

Considering these results, for a small sample volume located in the center of the rotor, the average $\gamma_S B_{1S}$ value would be much higher than in a large sample volume that covers the full extent of the rotor long axis. However, in order to maximize the signal produced by the sample one would use a large sample volume. This is of great concern because a sample under test sees a wide range of $\gamma_S B_{1S}$ values leading to lower overall
enhancements.

Furthermore, this model indicates that only 77% of the radiated Gaussian beam power impinges on the sample; the RF coil allows for only 60% transmission [48]; and the calculated reflection coefficient for the sapphire-sample boundary averaged over the incident angle produces only 63% power transmission. These effects result in a large fraction of the power being wasted. Some possibilities to address this will be mentioned below.

**Optimization of Coupling**

In order to optimize coupling of the microwave power to the sample, we optimized the rotor wall thickness and coated the inner surface of the Kel-F stator with silver [9]. One further improvement would be the use of a lens to focus the incident
microwave power, avoiding power lost around the sample. We present here the design of a Teflon lens that is a step towards this goal.

In the first step of the analysis, we use the single pass model and ignore the effect of the RF coil on the coupling of the microwaves to the sample. The microwaves are launched from an antenna towards the sample. In our present configuration, the antenna is the end of an 8 mm diameter circular, corrugated waveguide. This antenna forms a circular Gaussian beam with a beam waist of 2.6 mm at the launching position. The sample is located about 12 mm from the launching antenna. The microwave beam waist increases slightly, from 2.6 mm to about 3.1 mm, in propagating from the launching antenna to the sample. The sample length is larger than the microwave beam size, but the 2.4 mm sample diameter is smaller than the beam size. Fortunately, the sapphire rotor acts as a cylindrical converging lens that assists in coupling power into the sample. Nevertheless, we find that the sample only intercepts 77% of the microwave beam. This is largely because the Gaussian beam launched from the circular waveguide does not overlap optimally with the rectangular cross section of the sample. Therefore, a first improvement in coupling is to devise a cylindrically focusing lens that focuses the beam along the axis perpendicular to the long-axis of the rotor (Figure 7.8). This can be done with a specially shaped launching antenna or with a lens coupled to the existing antenna. Since an inexpensive lens can be made of a low loss, low dielectric constant material, it seems a simpler choice. Teflon was chosen because it has negligible absorption of microwaves at 250 GHz and it is easy to manufacture. Based on the single pass model the estimated increase in $B_{1S}$, which goes as the square root of power, is about 13%. A Teflon lens with a radius of curvature of 5 mm was designed to maximize coupling by
reducing the horizontal waist of the beam as seen in Figure 7.8a for the theoretical case and Figure 7.8b for the experimental case, as determined from the pyroelectric camera image. The radiated beam in Figure 7.8b is 90% Gaussian with a beam waist of 3.1 mm along the rotor long axis and 1.9 mm along the rotor diameter. HFSS simulations indicate that the average value of $\gamma_s B_{1S}$ should increase to 0.91 MHz from 0.835 MHz for an input power of 5 W, an increase of 8.6% in good agreement with the single pass model.

To further improve coupling, we consider the transmission through the RF coil, which is located between the waveguide and the sample. The transmission depends on the electric field polarization, which, in our case, is perpendicular to the wires. Using HFSS the transmission of a Gaussian beam through the coil was modeled for three geometries: a set of parallel metallic circular wires, a wire wrapped as a solenoid and the same solenoid wrapped around the sample filled rotor. In all cases the wire had a diameter $a$ and center-to-center spacing $d$. The power transmission results are shown in Figure 9. The transmission through the wire array, for wires that have no ohmic loss, is a function of the parameters $(a, d)$ and the wavelength, $\lambda$, as shown in Figure 9. Using $d/\lambda = 1.33$ and $a/d = 0.5$ as in the current setup, HFSS simulations indicate the transmission, $t$, is 0.6. As shown in Figure 9, all three models produce similar results for $d/\lambda = 1.33$. The transmission can be improved, but we are limited by the curvature of the solenoid and the presence of the rotor. If the RF coil were optimally spaced at $d/\lambda = 1$ a transmission value of 0.84 would be possible. This would further increase the $B_{1S}$ value by about 21%. We should also note that decreasing the $a/d$ ratio uniformly improves the transmission of power through the RF coil [47, 48]. This has important implications for successfully extending DNP to higher fields and smaller rotor sizes, as it implies the microwave
power could diffract efficiently through a tightly wound solenoid such as those currently used in high MAS frequency (>40 kHz) stators.

Combining these two improvements, namely the use of a lens and the proper spacing of the rotor windings, we can substantially increase the average $B_{1s}$ value. HFSS simulations including the cylindrically converging Teflon lens and improved wire spacing of $d/\lambda = 1$ and $a/d = 0.5$ showed an increase in the average value of $\gamma_s B_{1s}$ to 1.03 MHz from 0.835 MHz for an input power of 5 W, an increase of 23% in reasonable agreement with the single pass model.
Fig. 7.8. Ideal focused Gaussian beam and (b) the measured beam from a cylindrical dielectric focusing lens.
Fig. 7.9. Power transmission for a Gaussian beam radiated onto three models of the RF pickup coil with wire diameter \( a \), spacing \( d \) and wavelength \( \lambda \). The current spacing of \( d/\lambda = 1.33 \) is marked with a dashed line. All simulations were performed for an \( a/d = 0.5 \).

**Discussion and Conclusions**

In the present experiments, the \( B_{1S} \) value achieved in the sample is sufficient to reach satisfactory enhancement values with the available input power of 5 Watts. However, a more uniform distribution and improved coupling could produce greater enhancements. The current stator/coil arrangement produces a \( B_{1S} \) value 13.3 \( \mu \)T/W\(^{1/2} \). In comparison, EPR and ENDOR resonators are able to achieve much higher average \( B_{1S} \) values per W\(^{1/2} \) and improved homogeneity. Specifically, by using low order metallic
resonant structures and sample volumes that occupy a small fraction of the resonator, $B_{1S}$ values on the order of 1 mT/W$^{1/2}$ have been reported [49-51]. However, to optimize S/N in MAS DNP experiments, large sample volumes are required. Furthermore, metallic resonators or rotating metallic components are not permissible in the stator as they would produce eddy currents and levitation effects.

In addition to analyzing the current geometry of the probe, other input configurations were considered. Some time ago the use of axial coupling into the sample [7,36,37] whereby the microwaves are launched along the axis was investigated. Besides the added physical difficulty of coupling into the sample from along the axis, the relatively high loss tangent will cause the 12.45 mm long sample to see a highly non-uniform illumination, due to 67% of the power being absorbed as the wave propagates down the axis of the sample. Furthermore, with such a large sample volume and high loss tangent, the Q-factor will remain low even with the addition of reflective end caps. However, the utility of axial coupling could be optimized by full electromagnetic simulations to understand the determinants of the $B_{1S}$.

Using two methods we have analyzed the 250 GHz $B_{1S}$ field distribution throughout the sample in a 250 GHz / 380 MHz NMR DNP probe. The Gaussian beam propagation and Fresnel diffraction model proved effective in describing the effect of the Gaussian beam, RF coil, dielectric loss tangent and wall thickness on the coupling of microwave power into the sample. Full 3D simulations including reflections were performed using HFSS. For the Ag coated stator the simulations yielded an average $\gamma_0B_{1S}$ in the sample of 0.835 MHz for 5 W of input power into the stator. HFSS simulations were used to calculate the enhancement of the NMR signal as a function of rotor wall
thickness using the full distribution of the $B_{1S}$ field in the sample. An optimized sample diameter of 2.4 mm (wall thickness of 0.8 mm) was found to maximize the DNP enhancement. Further improvements to the probe were characterized including silver coating the inside of the stator, adding a cylindrical lens, and optimizing the RF coil spacing.

**Acknowledgements**

This research was supported by the National Institutes of Health through grants EB002804, EB003151, EB002026, EB001960, EB001035 and EB004866. A.B.B. was partially supported by graduate research fellowship from the National Science Foundation. We thank Albert Smith for helpful discussions.

**References**


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Chapter 8. Dynamic Nuclear Polarization Utilizing a Novel High Power 250 GHz Gyrotron with a 3 GHz Bandwidth

Abstract

Gyrotron oscillators can greatly improve sensitivity in dynamic nuclear polarization (DNP) solid-state nuclear magnetic resonance spectroscopy. However, relatively modest continuous wave power levels and tuning bandwidths have limited their performance as THz sources for DNP. A novel tunable 250 GHz gyrotron oscillator with >10 W output power over a 3 GHz band and >35 W peak power has been constructed and integrated into a DNP spectrometer, resulting in much improved DNP enhancements.

Introduction

Dynamic nuclear polarization (DNP) increases the sensitivity of nuclear magnetic resonance (NMR) experiments by transferring the relatively large polarization of electron spins to nuclear spins[1, 2]. The theoretical limit of increased polarization (enhancement) is 657 in the case of DNP of \(^1\)H nuclear spins. Experimentally, DNP enhancements vary from 20 to 400[3, 4], resulting in accelerations in signal averaging time by factors of 400 to 160,000. The performance of the DNP process depends on many parameters including the strength of the THz field at the sample.[5] As relatively lower (15-40) enhancements are achieved on biologically relevant samples [6-8] there is considerable room for improvement in DNP as it applies to investigating problems of biomedical importance.

Obtaining stronger THz fields across the sample can improve DNP enhancements as[5];
\[ \varepsilon = \varepsilon_{\text{max}} \left( \frac{\alpha B_1^2}{1 + \alpha B_1^2} \right) \]  

(8.1)

where \( B_1 \) is a component of the THz field originating from the millimeter wave source, and \( \alpha \) depends on properties of the electron spin. Geometrical restrictions on the sample holder such as the large volume of lossy sample (~60 µl) and components of the cryogenic magic angle spinning apparatus required for high-resolution NMR spectroscopy greatly hinder the design of a high quality factor resonator surrounding the sample. Thus, the most straightforward strategy to increase the strength of the \( B_1 \) field at the sample (and thus the DNP enhancements) is to increase the output power of the THz source, in this case a gyrotron oscillator. In addition to higher continuous wave output powers, extending the bandwidth of DNP gyrotrons[9] is advantageous while developing polarizing agents (the sources of the unpaired electron spins and ultimately the higher polarization and sensitivity) and polarization transfer mechanisms that rely on different THz frequency matching conditions[10].
Design

Figure 8.1. Schematic of the 250 GHz tunable gyrotron oscillator
A schematic of the gyrotron is shown in Figure 8.1, and the operating parameters are given in Table 8.1. The electron beam is created from a magnetron injection gun labeled at the bottom of Figure 8.1, the design of which has been discussed elsewhere.[9, 11] The annular beam is emitted from the gun with a radius of 5.38 mm and is adiabatically compressed by the magnetic field gradient to a radius of 1.02 mm in the interaction mode of the cavity. The electron beam interacts with the \( \text{TE}_{5,2,q} \) mode into the interaction cavity. This interaction leads to bunching of the electrons in phase space allowing them to coherently deposit energy into the operating mode. A mode converter and window allow for efficient coupling of the \( \sim 250 \) GHz radiation out of the tube. The electron beam then expands as it leaves the interaction cavity and is safely collected in a water-cooled collector labeled at the top of Figure 8.1.

**Interaction cavity**

Although the first harmonic of the cyclotron resonance condition interacts more efficiently than the second harmonic tunable oscillators recently built[11, 12], care was still taken to maximize the efficiency of the interaction of the beam with the cavity mode due to increased DNP enhancement with higher microwave powers. The rotating \( \text{TE}_{5,2,q} \)

<table>
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<tr>
<th>Operating mode ( \text{TE}_{m,n,q} )</th>
<th>( \text{TE}_{5,2,q} )</th>
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<tbody>
<tr>
<td>Frequency</td>
<td>248.2 GHz</td>
</tr>
<tr>
<td>Tuning range</td>
<td>2.9 GHz</td>
</tr>
<tr>
<td>Cavity magnetic field ( B )</td>
<td>9.03-9.29 T</td>
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<td>Cyclotron harmonic</td>
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<td>Beam Voltage ( V_b )</td>
<td>8-13.5 kV</td>
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<tr>
<td>Beam current ( I_b )</td>
<td>50-180 mA</td>
</tr>
<tr>
<td>Output power</td>
<td>( &gt;10 ) W over band, ( &gt;35 ) W peak</td>
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<tr>
<td>Compression</td>
<td>28</td>
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</table>
mode was selected because of its efficient interaction with the ~1 mm radius electron beam and isolation from competing modes. For example, the nearest mode is the TE$_{8,1}$ at 253 GHz. Even with the full 4 GHz of separation (see Figure 8.2) between the two modes, mode competition with the TE$_{8,1}$ is still what ultimately limited the bandwidth of the oscillator.

The cavity profile was optimized to support hybridized axial modes by extending the length of the cavity, compared to the previous 250 GHz gyrotron, to 23 mm (19g, where g is the free-space wavelength). As is usual for designs of oscillator cavities, the up-taper was designed to minimize wave reflections and mode conversion for the operating mode as it exits the cylindrical resonator. However, whereas previous designs have employed several steps in the radius after the interaction cavity to vary the taper angle, this design incorporates one single straight up-taper. This allows the cavity and uptaper to be machined (Midwest Precision Tool and Die, South Dakota) rather than electroforming.

**Internal Mode Converter**

A helically cut Vlasov launcher radiates the rotating TE$_{5,2}$ mode on to an off-axis parabolic mirror forming a beam consisting of a main lobe and small side lobe. The radiation then reflects off of two concave mirrors that focuses the beam into a Gaussian profile that is directed out of the tube through a sapphire window.
Figure 8.2. Mode map and starting currents of modes surrounding the operational mode of the interaction cavity, $\text{TE}_{5,2,q}$

Figure 8.3. Cavity profile. The intensity of the electric field of the first 3 axial modes are shown in blue, red, and green, respectively. The transverse $\text{TE}_{5,2}$ mode is also shown on the right with the beam radius overlapped in white.
Figure 8.4 Internal mode converter and window. a) schematic of cross section showing the path of the microwaves in yellow. b) solid rendition schematic showing how the mirrors and launcher are bolted to the stainless steel mount. c) photograph of the viewport and stainless steel housing prior to assembly d) photograph of the copper helical cut Vlasov launcher and three mirrors of the mode converter

**Results**

At the same time that the gyrotron THz source should have a substantial bandwidth, it is of paramount importance that a significant power level be maintained over the entire band. For example, at the high frequency edge of the 3 GHz band of the gyrotron oscillator presented here, 10 W of output power (see Figure 8.5) resulted in an enhancement of 54 on a membrane protein sample of bacteriorhodopsin. This value is 35% higher than previous enhancements of 40 achieved on the same sample[13], but with a narrow-banded gyrotron capable of producing much lower power levels.
Figure 8.5. Demonstration of the power levels and bandwidth capability of this gyrotron oscillator. 10 W is achieved over almost the entire band of 3 GHz. Beam currents range from 30 – 190 mA, cathode potentials from 8 – 13 kV, and magnetic fields from 9.01 – 9.26 T.

Figure 8.6. Output beam 1.5 meters away from the gyrotron output window in a) linear scale and b) dB contour levels. The beam has >94% Gaussian content demonstrating not only the excellent performance of the TE_{5,2} to Gaussian mode converter but also the ability to maintain high purity of the HE_{11} mode in the corrugated waveguide over an extended distance away from the gyrotron.
In order to efficiently couple the THz radiation from the gyrotron to the sample, the power distribution in the interaction cavity is converted to a Gaussian profile by means of a helically cut Vlasov launcher and three mirrors. The output beam quality is shown in Figure 8.6.

![Graph](image)

Figure 8.7. Positive and negative cross effect enhancements on a sample of bacteriorhodopsin demonstrating 35% higher enhancements than with previous instrumentation and power levels.
Conclusions

The high-power wide-bandwidth gyrotron oscillator described here has resulted in significant gains in DNP enhancements. Further experiments exploiting the high power levels for both the cross effect and the solid effect polarization mechanisms should yield higher enhancements that have been previously demonstrated at fields > 9 T.

Acknowledgments

I thank Emilio A. Nanni for running MAGY simulations of the cavity and assisting with experiments, and Ivan Mastovsky and Ronald DeRocher for assisting with the mechanical design, fabrication and gun activation. I thank Dr. Jagadishwar R. Sirigiri for selecting the operational mode, designing the cavity profile, specifying the cathode to cavity and cavity to collector placement, and designing the surfaces and placement of the Vlasov launcher and mode converter mirrors. Professor Robert G. Griffin and Dr. Richard J. Temkin guided and supported this research.

References


Biographical Sketch

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POSITION TITLE
Postdoctoral researcher

eRA COMMONS USER NAME (credential, e.g., agency login)
ALEXANDER.BARNES

EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)

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<td>2004-2011</td>
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</tr>
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A. Positions and Honors.

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<tr>
<td>Research Technician</td>
<td>01/04</td>
<td>07/04</td>
<td>SSNMR and materials science</td>
<td>Universität Münster</td>
<td>Prof. Hellmut Eckert</td>
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Academic and Professional Honors:
Stephen Penrose Scholarship, Whitman College, 1999-2003
Outstanding Achievement in Physical Chemistry, Whitman College, 2002
Summer Research Program in Solid-state Chemistry, 2002-2003
B.A. With Honors in Chemistry, Whitman College, 2003
National Science Foundation Graduate Research Fellowship, 2005-2008

Memberships in professional societies:
American Chemical Society
Biophysical Society
Institute of Electronic and Electrical Engineers
American Association for the Advancement of Science
Phi Beta Kappa

B. Publications (in chronological order).

Refereed Research papers:


Manuscripts in preparation:


Abstracts:

Instrumentation for High-frequency DNP and Cryogenic MAS; Applications to Membrane Proteins and Peptides, July 2010. Rocky Mountain NMR Conferences. (selected oral presentation)

Instrumentation for DNP and Cryogenic MAS, January 2010. Winter School on Biomolecular Solid State NMR. (oral presentation)

High-Frequency Dynamic Nuclear Polarization, November 2009. Physics symposium at the University of Idaho, Moscow. (invited talk)


Solid State MAS DNP Probe Development; Cryogenic Sample Exchange, August 2007. Dynamic Nuclear Polarization Symposium. (oral presentation)

Experimental NMR Conference (ENC), Daytona, FL, April, 2007. Precise Homonuclear and Heteronuclear Distances and Torsion Angles for High Resolution Structure Determination of Uniformly $^{13}$C and $^{15}$N Labeled Peptides by Solid State NMR (poster)


Reviews:


Nanni, E. A., Barnes, A.B., Griffin, R.G., Temkin, R.J. 2011. THz Dynamic Nuclear Polarization NMR. Accepted for publication in the inaugural issue of IEEE THz.