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Dynamic nuclear polarization-enhanced ^1H – ^{13}C double resonance NMR in static samples below 20 K

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Abstract

We demonstrate the feasibility of one-dimensional and two-dimensional ^1H – ^{13}C double resonance NMR experiments with dynamic nuclear polarization (DNP) at 9.4 T and temperatures below 20 K, including both ^1H – ^{13}C cross-polarization and ^1H decoupling, and discuss the effects of polarizing agent type, polarizing agent concentration, temperature, and solvent deuteration. We describe a two-channel low-temperature DNP/NMR probe, capable of carrying the radio-frequency power load required for ^1H – ^{13}C cross-polarization and high-power proton decoupling. Experiments at 8 K and 16 K reveal a significant T_2 relaxation of ^{13}C , induced by electron spin flips. Carr–Purcell experiments and numerical simulations of Carr–Purcell dephasing curves allow us to determine the effective correlation time of electron flips under our experimental conditions. The dependence of the DNP signal enhancement on electron spin concentration shows a maximum near 80 mM. Although no significant difference in the absolute DNP enhancements for triradical (DOTOPA-TEMPO) and biradical (TOTAPOL) dopants was found, the triradical produced greater DNP build-up rates, which are advantageous for DNP experiments. Additionally the feasibility of structural measurements on ^{13}C -labeled biomolecules was demonstrated with a two-dimensional ^{13}C – ^{13}C exchange spectrum of selectively ^{13}C -labeled β -amyloid fibrils.

Keywords

Dynamic nuclear polarization; Double resonance probe; Polarizing agent; Transverse relaxation

1. Introduction

Dynamic nuclear polarization (DNP) is a process of transferring large equilibrium electron spin polarization to nuclei. The maximum theoretical enhancement is given by the ratio of electron and nuclear gyromagnetic ratios (γ_e/γ_n), which for protons is a factor of 660 [1]. The resulting nuclear magnetic resonance (NMR) signal enhancements may be used to reduce dramatically the time required for accumulating NMR spectra or to reduce sample size requirements. Initially, DNP in solids was mainly used for polarizing nuclear targets in high-energy particle physics, but later it was extended to NMR studies of amorphous polymers [2–4]. Currently, high-field solid state NMR experiments with signals enhanced by DNP are of particular interest because of their application to studies of a wide range of systems where the sample is available only in low concentrations or low quantities, including membrane proteins [5–9], amyloid peptides [10–12], oriented samples [13], viral

DNA [14] and surfaces of inorganic materials [15,16]. Such experiments are typically carried out at temperatures in the 80–100 K range under magic-angle spinning (MAS), and require a high-power high-frequency microwave source such as a gyrotron [17,18] (power levels > 5 W) or extended interaction klystron [19]. However, at lower temperatures, even a solid state source with an output power of tens of milliwatts can provide significant enhancements [20–22]. Recently Tycko and coworkers have shown the feasibility of solid state NMR measurements at 6–30 K, using a DNP system equipped with a 264 GHz source producing 30 mW of microwave power [20,21]. Besides, small power sources are routinely used in dissolution DNP setups [23–26].

In DNP experiments, it is important to determine experimental conditions that provide the largest signals without compromising the quality of the NMR spectra. Therefore, the type and concentration of paramagnetic dopant should be optimized. Previously it was shown that the cross-effect DNP mechanism, arising from mixing of spin states in a system of two coupled unpaired electrons and one or more nuclei, provides considerable enhancements with nitroxide radicals as polarizing agents at high fields (> 5 T) at 10 K [27]. This finding led to the design of a water soluble TOTAPOL [28,29] biradical, consisting of two nitroxide radicals tethered by a short flexible linker. The short distance between nitroxide moieties in TOTAPOL increases the efficiency of the cross-effect mechanism (relative to monoradical dopants). Later, the triradical DOTOPA-TEMPO was reported to be an effective dopant for solid state DNP [20]. Both TOTAPOL and DOTOPA-TEMPO were shown by Thurber et al. to produce larger DNP enhancements of ^1H spin polarizations and/or more rapid build-up of ^1H polarizations than 4-amino-TEMPO, under conditions similar to those of the experiments described below [20].

Another important aspect of optimizing the DNP experiment is to minimize perturbations of the NMR spectra by couplings to the paramagnetic dopant. It was reported previously that TOTAPOL has a minimal effect on the ^{13}C NMR linewidth at 80 K under MAS, at electron spin concentrations around 40 mM [16,30]. In those experiments, MAS attenuates electron–nuclear dipole–dipole interactions and reduces spin diffusion rates, which exist in static samples. Therefore, the situation may be different at lower temperatures and without MAS.

In this work, we report the design of a DNP/NMR probe for static samples capable of ^1H – ^{13}C cross-polarization (CP) and high-power proton decoupling (100 kHz ^1H radio-frequency fields for up to 30 ms). We investigate the optimal conditions for performing the DNP experiment, such as the type and concentration of the paramagnetic dopant and the temperature. We report effects of these parameters on transverse relaxation and DNP build-up rates. We show that transverse relaxation of ^{13}C nuclei is quite pronounced in static samples and is governed by electron flip–flop transitions, which can be suppressed by Carr–Purcell pulse sequences. Numerical simulations of Carr–Purcell dephasing curves allow us to estimate the electron flip–flop rate. Finally, we demonstrate the feasibility of structural studies of biomolecules with a two-dimensional (2D) exchange experiment on a selectively ^{13}C -labeled sample of 40-residue β -amyloid (Ab_{1-40}) fibrils.

2. Methods

2.1. Low temperature DNP/NMR probe

Our two-channel low-temperature DNP/NMR probe is a modification of the single-channel probe for static samples that was described previously, along with the quasioptical microwave (MW) setup [20]. Fig. 1a shows the modified continuous flow Janis Super-Tran cryostat (Janis Research Co. ST-200-NMR), which has a “cold finger in vacuum” design. The cryostat includes an outer vacuum can and a probe insert consisting of an instrumentation skirt and a stainless steel guide tube ending with a copper block. The

instrumentation skirt is equipped with sealed feedthroughs for radio-frequency (RF) lines and tuning rods, and an evacuation port. A helium transfer line inserts into the guide tube and delivers coolant to the copper block at the bottom of the probe. Thermal insulation is provided by evacuating the space between the probe and the outer can, using a turbomolecular pump (Varian Inc., TV301 NAV). The temperature controller (Lakeshore Inc., model 340) reads the temperature from a Cernox sensor attached to the copper block and provides current to the heater installed above the copper block. Electrical lines for temperature control are equipped with shunt capacitors to ground to filter out RF noise, which otherwise interferes with NMR measurements. The tuning elements are mounted on a radiation shield anchor, while the sample support is in indirect contact with the cold copper block as shown in Fig. 1b. All of these elements are encased by a radiation shield (not shown in Fig. 1) to reduce thermal losses. The outer can has a high density polyethylene (HDPE) window at the bottom, which allows us to deliver MW irradiation to the sample.

Fig. 1b shows the head of the probe, including two RF channels tunable around the ^{13}C (~ 100.8 MHz) and ^1H (~ 400.88 MHz) NMR frequencies in a 9.39 T field. The 8-turn solenoidal RF coil (4 mm coil diameter, 0.25 mm diameter copper wire, ~ 1 mm spacing between turns) is thermally anchored to the copper cold finger block through sapphire pieces. The sample is placed into a PTFE tube (3.8 mm OD, 3.1 mm ID) and sealed from both ends with sapphire rods (Swiss Jewel Co.). It is held between two copper plungers that provide thermal contact between the sapphire rods (and hence the sample) and the copper block (see Fig. 1c). The typical sample volume of 40 μl is limited mainly by the area effectively irradiated by the MW. In terms of RF efficiency and sensitivity, the probe is comparable to commercial 3.2 mm MAS probes at room temperature; at low temperatures, the RF circuit Q-factor becomes somewhat higher and the efficiency of the ^{13}C RF channel improves from 67 kHz^2/Watt at room temperature to about 100 kHz^2/Watt at 8 K. Commercial variable capacitors (Polyflon Co.) are used for tuning and matching of the ^{13}C channel and matching of the ^1H channel. The diameter of pistons within these capacitors is reduced to avoid binding due to thermal contraction at low temperatures. A transmission line with variable electrical length is used to tune the ^1H channel, by adjusting the position of the PTFE rod inside the line [31].

When the probe temperature is above 30 K, its RF circuitry undergoes serious electrical discharge arcing, apparently due to imperfect vacuum within the cryostat. Nevertheless, at temperatures below 30 K, cryopumping takes place and arcing disappears after running a dummy experiment, employing ^1H - ^{13}C cross polarization and strong proton decoupling. The dummy experiment is initially accompanied by strong arcing, which disappears after several minutes. We attribute this behavior to the presence of residual gas adsorbed on or condensed around the surfaces of RF circuit elements. Applying high power RF pulses apparently heats the circuitry somewhat and may cause the release of the adsorbed gas. Interestingly, similar effects were already reported for an NMR probe in a cold finger cryostat [32]. After settling, the probe is able to carry up to 30 ms of proton decoupling pulses at 100 kHz RF amplitude without arcing. This RF strength is more than sufficient for decoupling ^1H from carbonyl ^{13}C as shown in Fig. S1.

For DNP, MW irradiation is transmitted through the HDPE window at the bottom of the cryostat and enters a tapered corrugated waveguide (Thomas Keating, Ltd.; largest ID 12.5 mm, smallest ID 3.0 mm), followed by a short piece of a tubular brass waveguide (3.5 mm ID). The waveguides are attached to the copper block using a PTFE support. A cylindrical PTFE lens at the exit of the waveguide is designed to focus the outgoing beam in one dimension [33]. The focus distance was calculated using standard geometrical optics formulae, even though their applicability for sizes on the order of a wavelength is marginal.

Experimental measurements confirm an increase in DNP enhancements by about 20% when the lens is included.

To determine the real temperature of the sample, we used the temperature dependence of ^{79}Br spin–lattice relaxation times [34]. A test sample consists of two compartments separated by a thin PTFE wall. The first compartment contains 30 mM of DOTOPA-TEMPO in 60% w/w glycerol/water solution, while the second compartment contains finely ground KBr powder. The temperatures derived from T_1 relaxation times of ^{79}Br were in excellent agreement with readings from the Cernox thermocouple attached to the copper cold finger. Continuous MW irradiation and RF irradiation, simulating a typical heat load in real experiments, do not affect the sample temperature as seen through ^{79}Br relaxation. Despite the fact that the tapered waveguide projects into the space between the radiation shield and outer can of the cryostat, the consumption of liquid helium for cooling is unaffected. At 8 K, the cryostat consumes about 1.5–2 l/h of liquid helium.

After a sample was mounted in place for low-temperature NMR measurements, the entire probehead was immersed in liquid nitrogen for about 1 min (outside the NMR magnet) to freeze the sample and to cool the copper block and RF circuitry. The probe was then placed in the outer can, transferred into the NMR magnet, and attached to the pumping system. After several minutes of pumping, the pressure inside the cryostat became low enough for cooling with liquid helium. During the entire process, the temperature of the copper block was monitored and did not exceed 130–140 K. With this procedure, the sample could be prepared and maintained in a glassy state.

2.2. NMR measurements

NMR measurements were carried out using a Varian Infinity spectrometer console. ^1H NMR signals were attenuated before entering the receiver in order to avoid saturating the preamplifier. Furthermore, the DNP-enhanced ^1H NMR signals at low temperatures are so strong that they create voltages capable of opening the diodes that protect the preamplifier, and thus the signal gets clipped. The ratio of the voltage at the input of the preamplifier to the digitized NMR signal was determined. Using this conversion ratio, we estimated the ^1H NMR signal amplitude at which the signal is clipped to be ~ 1 V, which is consistent with the threshold voltage for opening the diodes. Therefore the read-out pulse in some ^1H NMR measurements was set to have a nominal flip angle of 15° , in order to prevent opening of these diodes. ^{13}C NMR signals in our experiments were much smaller and therefore were measured in a usual manner without such precautions.

Using a calibrated noise source, we measured the noise figure of the ^{13}C receiver to be 1.6 db, which corresponds approximately to a noise temperature of 130 K. The level of noise produced by the RF circuitry, as measured at room temperature, was the same as the noise level produced by a $50\ \Omega$ resistor, however it did not change upon cooling. This happens because even upon cooling down to 8 K some RF circuitry elements are still maintained at somewhat higher temperature. Additionally, external sources contribute to an average noise level and this contribution is not removed by cooling.

The MW source frequency was set to 264.0 GHz, since this provides maximal DNP enhancements for both TOTAPOL and DOTOPA-TEMPO dopants at 9.39 T [20]. For ^{13}C T_2 measurements, we employed the pulse sequence shown in Fig. 2a, where the line intensity obtained by Fourier transformation of the echo was recorded as a function of τ_{echo} .

For various samples, the decay can be approximated by stretched exponentials $\exp(-a\tau_{\text{echo}}^x)$, where x varies between 1.1 and 1.3. However, for practical purposes we used a single exponential fit for all T_2 measurements. The Freeman–Hill variant of the Carr–Purcell

sequence (Carr–Purcell–Freeman–Hill, or CPFH), used in this work, employs a (0, 180) alternation of the π pulse phases. The pulse sequence is shown in Fig. 2b [35]. In these experiments, the echo intensity was recorded as a function of the number of CPFH blocks for various τ_{CPFH} values. Single exponentials were used for fitting the decay rates in these CPFH experiments. The 2D ^{13}C – ^{13}C exchange pulse sequence, shown in Fig. 2c, is a typical exchange experiment with Hahn echo detection [36]. All pulse sequences also include independent (0, 180) phase cycles of the first ^1H $\pi/2$ pulse and the last ^{13}C π echo pulse.

^{13}C chemical shifts were referenced using a spectrum of a static sample of carboxyl-labeled L-alanine ($1\text{-}^{13}\text{C}$ -Ala) powder at room temperature. Chemical shift anisotropy tensor components were determined from the carboxyl ^{13}C powder pattern and were used to calculate the isotropic chemical shift, which was taken to be 177.95 ppm relative to tetramethylsilane.

2.3. Sample preparation

A typical sample for DNP contains 60% w/w of ^{13}C -depleted (^{12}C -99.95%) glycerol- d_8 (Cambridge Isotopes Inc.) as a glass forming agent. This deuterated glycerol was the only ^{13}C -depleted version available at the time of experiments. ^{13}C depletion was needed to minimize interference from natural-abundance ^{13}C signals from the frozen solvent. Syntheses of TOTAPOL and DOTOPA-TEMPO are described elsewhere [20,28]. Samples containing DOTOPA-TEMPO were prepared in a 0.2 M acetate buffer at pH 3.0; TOTAPOL was dissolved in 0.25 M phosphate buffer at pH 7.2. Samples for optimization of DNP conditions contained 20 mM of $1\text{-}^{13}\text{C}$ -Ala. A DNP-enhanced ^{13}C spectrum of such a typical sample, obtained with ^1H - ^{13}C CP and on-resonance proton decoupling is shown in Fig. 3. The spectrum has contributions from the labeled carbonyl site and the residual ^{13}C of glycerol, which can be approximated with a 48 ppm wide Gaussian line at 70 ppm. Although there is some overlap between the two features, still a substantial portion of carboxyl lineshape is free from any other contributions.

Solid phase synthesis was used to produce the $\text{A}\beta_{1-40}$ peptide, labeled at the backbone carbonyl sites of Val18 and Phe19 and the methyl site of Ala30. Monomers were dissolved in 10 mM pH 7.4 phosphate buffer at 200 μM concentration, and seeds (5% by mass relative to $\text{A}\beta_{1-40}$ monomers) produced by sonication from other fibrils were added to initiate the growth. After 4 h of growth, the fibrils were pelleted by centrifugation for 2 h at $\sim 56,000 \times g$. The wet pellet was resuspended in an aqueous solution containing 13.2 mM of DOTOPA, and pelleted down. Finally, 60% (by weight) glycerol- d_8 was added, and the pellet was resuspended and pelleted one more time. The resulting pellet contains ~ 6.6 mM DOTOPA, assuming that the pellet density before adding glycerol was $\sim 1 \text{ g/cm}^3$.

3. Results and discussion

3.1. Experimental ^{13}C T_2 relaxation measurements

The DNP-enhanced ^{13}C CP spectrum of $1\text{-}^{13}\text{C}$ -Ala in Fig. 3 lacks sharp features, thus pointing to significant broadening from T_2 relaxation or disorder. The broadening due to static hyperfine interactions of ^{13}C nucleus with electrons amounts to 560 Hz at [*nitroxide*] = 40 mM [37]. To determine the source of the homogeneous part of the broadening, we carried out a systematic study of the effective carbonyl ^{13}C T_2 using the two-pulse echo sequence in Fig. 2a. In Fig. 4a, the transverse relaxation rates $1/T_2$ under continuous wave 100 kHz on-resonance proton decoupling, under 100 kHz Lee–Goldburg (LG) proton irradiation [38], and without proton irradiation are plotted as a function of the electron spin concentration [*nitroxide*]. For all three types of measurements, there is a strong dependence

on [*nitroxide*], but no difference between DOTOPA-TEMPO and TOTAPOL was found. This suggests a significant T_2 mechanism for ^{13}C nuclei caused by electron spin flips.

The transverse ^{13}C relaxation rates without ^1H decoupling are significantly higher than those with ^1H decoupling due to the presence of ^1H – ^{13}C couplings and ^1H – ^1H spin diffusion. Effects of ^1H – ^{13}C couplings can be suppressed by LG irradiation. Transverse relaxation rates obtained in such an experiment are significantly lower than those without any proton irradiation. However, they are still somewhat greater than those under on-resonance decoupling. A possible reason is that inhomogeneity of the proton RF field leads to incomplete homonuclear decoupling of protons.

Electron spin flips can be caused by the interaction of electron spins with the lattice (T_{1e} process) or by dipole–dipole interactions among electron spins (T_{2e} process). T_{1e} relaxation is expected to be dominated by a direct spin-phonon process, which has a weak but

significant temperature dependence for temperatures $h\nu \gtrsim kT$ [39,40]: $\frac{1}{T_2} \sim cth\left(\frac{h\nu}{2kT}\right)$, where ν is the electron paramagnetic resonance (EPR) frequency. Raising the temperature from 8 K to 16 K should decrease T_{1e} by a factor of 1.8. At the same time, T_{2e} is generally temperature independent, except that at temperatures well below Zeeman temperature ($T_Z \sim 12$ K at 9.4 T) electron spin diffusion starts to freeze out [41]. However we do not reach this regime in our experiment. Results in Fig. 4a show that such a temperature change from 16 K to 8 K does not produce any noticeable change in $1/T_2$, which suggests that electron spin flips driven by magnetic dipole–dipole interactions among electrons are the still the main mechanism for transverse ^{13}C relaxation in our conditions.

The rate of electron spin flips can be determined using the CPFH pulse sequence in Fig. 2b.

Fig. 4b shows the effective transverse relaxation time T_2' as a function of τ_{CPFH} . Since the concentration of ^{13}C -labeled alanine is 20 mM, the average dipolar coupling between ^{13}C nuclei is ~ 0.09 Hz, which is too small for the dipolar evolution to interfere in our this measurement. Also, application of MW irradiation does not perturb a lot of electron spins. A typical hole width burnt by the MW on the timescale of T_{1e} will be estimated below as ~ 11 MHz. Since this is much smaller than 1 GHz linewidth of nitroxide radical, the effects of MW irradiation on T_2 can be ignored. For large τ_{CPFH} , T_2' is very similar to the T_2 measured with a two-pulse echo experiment. As τ_{CPFH} becomes less than the time scale for local field fluctuations that we attribute to electron spin flips, T_2' increases significantly. Data in Fig. 4b suggest that this time scale is on the order of 100 fs. In order to determine more precisely the correlation time of local field fluctuations, we have simulated the dependence of T_2' on τ_{CPFH} as described in the next section.

3.2. Simulations of transverse ^{13}C relaxation under a Carr–Purcell sequence

As shown above, the dominant mechanism for transverse ^{13}C relaxation (after on-resonance proton irradiation removes contributions of ^1H – ^{13}C couplings) arises from electron spin flips, which create fluctuating magnetic fields at the nuclear sites. After preparation of transverse spin polarization, we assume that each ^{13}C site accumulates a random phase given by

$$\varphi(t) = \int_0^t \omega(t') dt' \quad (1)$$

where $\omega(t)$ is driven by random fluctuations of the local magnetic field caused by electron spin flips. The decay in time of the total transverse magnetization is then given by an average over all nuclear sites:

$$M \sim \langle \exp[i\varphi(t)] \rangle = \langle \exp\left[\int_0^t \omega(t') dt'\right] \rangle \quad (2)$$

In our experiments, we observe only the spins beyond a spin diffusion barrier for protons. In evaluating Eq. (2), we therefore discard cases when hyperfine couplings of ^{13}C becomes larger than 25 kHz. In previous publications, the expression in Eq. (2) was evaluated analytically for several limiting cases [42,43] which did not include $R\tau \sim 1$, where R is the electron spin flip rate and τ is the observation interval. Later, a general solution for FID, two-pulse and three-pulse echo experiments was obtained in a closed form involving special functions [44]; the latter however is not easily adapted to give a solution for a Carr–Purcell experiment. A numerical simulation does not require simplifying approximations and is therefore useful for treatment of the intermediate regime $R\tau \sim 1$. Furthermore, such a calculation allows us to include easily the effects of ^{13}C π pulses during the CPFH sequence. In related work, a numerical Monte Carlo simulation was used previously to model the optical free-induction decay for Pr^{3+} impurities in LaF, where transverse relaxation arises from local field fluctuation caused by resonant flip–flops of ^{19}F spins [45].

In order to simulate the evolution of transverse ^{13}C magnetization in the presence of electron spin flips with correlation time τ_c , we first generate the randomly fluctuating field for a particular ^{13}C site as follows: (i) Random coordinates are assigned to a certain number of electron spins. The coordinates are produced inside a cube with a size chosen to match the real concentrations of electron spins in our samples. Once the coordinates are known, the dipole–dipole interactions between the ^{13}C site in the center of the cube and all electron spins are calculated; (ii) For each electron spin, a random trajectory of spin projections $\{q_i\}$ is produced for a series of time steps with length δt . To do this, a sequence of random numbers $\{p_i\}$ uniformly distributed in the interval (0, 1) is created and processed to produce $\{q_i\}$ according to the rule

$$q_i = \begin{cases} q_{i-1}, & p_i \leq \exp(-\delta t/\tau_c) \\ -q_{i-1}, & p_i > \exp(-\delta t/\tau_c) \end{cases} \quad (3)$$

Initial values of the electron spin projection q_1 are chosen to be either 1/2 or –1/2 according to a Boltzmann distribution. Eq. (3) is valid in the limit that $\delta t \ll \tau_c$; (iii) Effects of the temperature-dependent electron spin polarization [46] are included by introducing an extra decision step, where a random number r is used to decide, based on a Boltzmann factor, whether the spin projection should indeed be changed. For example, if $q_{i-1} = 1/2$, $p_i >$

$\exp(-\delta t/\tau_c)$ and $r > \frac{e^\beta}{2(e^\beta + e^{-\beta})}$, where $\beta = \frac{h\nu}{2kT}$, then $q_i = -1/2$. Otherwise, $q_i = 1/2$.

The net fluctuating field at the ^{13}C site is calculated by summing the contributions from all electron spins, and the result is used to obtain the transverse magnetization as in Eq. (2), after averaging over 3000 repetitions of the calculation. Effects of ^{13}C π pulses (assumed to be instantaneous) are included by changing the accumulated phase according to $\varphi \rightarrow \pi - \varphi$ after each pulse. For typical electron spin concentrations and τ_c values used in our simulations, the number of electron spins required for convergent results can indeed be rather small. Typically 1 or 2 electrons were enough to reproduce most of the dephasing behavior, which means that the major contribution to dephasing arises from fluctuations of the nearest neighboring electron spin. However, for the sake of rigor, simulations described below included 27 electron spins.

Simulations of T_2' as a function τ_{CPFH} for various correlation times τ_c are shown in Fig. 5. The general agreement with experimental measurements is satisfactory, although the

simulations ignore effects of fluctuations from sources other than electron spin flips and the effects of finite pulses. The latter would appear due to the fact that relaxation during the RF pulse is different than during a free evolution period. Therefore, for very short τ_{CPFH} , the RF pulses occupy a large fraction of the overall pulse sequence length, and the apparent T_2' might deviate from the real one. The electron spin correlation time estimated from our simulations falls into a range of 100–300 μs . Previously, T_{1e} was inferred to be roughly 2 ms at 35 K from the effects of field modulation on the DNP enhancements [20]. Farrar et al. have measured T_{1e} at 5 T and 12 K [27]; extrapolation using the equation for the direct process gives ~ 2.5 ms at the same temperature [40]. Our measurements were performed at 8 K, where T_{1e} is expected to be ~ 8 ms. The value of τ_c obtained from our CPFH measurements is an order of magnitude shorter, consistent with the idea that the electron spin flips that affect the observed ^{13}C T_2' values arise from electron spin diffusion rather than T_{1e} relaxation.

Using the value for τ_c estimated above, the spectral diffusion constant may be estimated as well. During the T_{1e} , a particular electron spin hops $\sim T_{1e}/\tau_c$ times due to electron spin diffusion. With an average dipole–dipole interaction of $D_{ee} \sim 1.3$ MHz at $[\text{nitroxide}] = 40$ mM, a particular spin state would deviate from its initial frequency by

$\sqrt{\langle \Delta\omega^2 \rangle} \sim D_{ee} \sqrt{\frac{T_{1e}}{\tau_c}} \sim 11.6$ MHz. This is an estimate for the width of the “hole” burned by microwaves. The spectral diffusion coefficient is therefore

$$D \sim \frac{\langle \Delta\omega^2 \rangle}{T_{1e}} \sim 1.7 * 10^{16} \frac{\text{Hz}^2}{\text{s}}$$

This coefficient is an order of magnitude smaller than the one reported earlier [20]. The difference probably arises due to a contribution of electron–nuclear interactions to overall spectral diffusion.

Simulations and experiments are not expected to agree exactly, in part because the experimental measurements are affected by the non-zero π pulse lengths when τ_{CPFH} becomes small. In this limit, T_2' is expected to be determined by low-frequency fluctuations of local fields, because of the RF phase alternation in the CPFH sequence [47]. Furthermore, in a real sample, a distribution of electron spin correlation times may affect the dependence of T_2' on τ_{CPFH} .

3.3. DNP enhancements and build-up rates

Fig. 6a shows the DNP enhancements of ^1H NMR signals, plotted as a function of $[\text{nitroxide}]$. DNP enhancements with DOTOPA-TEMPO are somewhat bigger than those with TOTAPOL, which is probably due to a larger number of electron spin pairs having appropriate g-tensor orientations for the cross-effect mechanism. The DNP enhancement grows with $[\text{nitroxide}]$. At the highest $[\text{nitroxide}]$, a slight decrease is observed, which was previously explained by a smaller amount of detected nuclei [48,49]. Assuming that the radius of a spin diffusion barrier for protons is about 9 Å, the nuclei within this radius constitute $\sim 20\%$ of all protons for $[\text{nitroxide}] = 100$ mM. However, in our work, we could not reliably detect a reduction in ^1H NMR signal without MW irradiation with increasing $[\text{nitroxide}]$. Also, we report DNP enhancements relative to the ^1H NMR signals without MW irradiation in the same sample, rather than relative to an external standard. A possible reason for the lower DNP enhancements at higher dopant concentration is that faster electron spin diffusion spreads the MW-induced saturation of electron spin polarization over

the inhomogeneously broadened EPR line, thereby reducing the differences in electron spin polarization that are required in the cross-effect mechanism.

^1H T_1 relaxation times and DNP build-up times measured using both ^1H and cross-polarized ^{13}C NMR signals are shown in Fig. 6b. The ^1H T_1 relaxation times and the DNP build-up times are similar. At least two qualitative explanations for the approximate equality of T_1 relaxation times and DNP build-up times can be considered: (i) Qualitatively, the evolution of nuclear polarization in DNP can be regarded as a competition of two processes and will be determined by two rates, namely the rate of nuclear spin polarization production due to MW irradiation and the rate of polarization loss through nuclear T_1 relaxation towards equilibrium. The fastest of the two processes will determine the observed rate. In this view, MW irradiation directly saturates a forbidden EPR line of three-spin system [49,50], and when saturation is not very strong, the nuclear T_1 relaxation rate dominates over the rate of polarization production due to MW irradiation. In our samples, the relevant nuclear T_1 relaxation rate must depend on hyperfine interactions and electron–electron dipole–dipole interactions, because the ^1H T_1 in the absence of paramagnetic dopants is much greater than the observed DNP build-up times at the temperatures of our measurements and the ^1H T_1 in the presence of dopants depends on the choice of dopant (i.e., TOTAPOL vs. DOTOPA-TEMPO); (ii) Alternatively, DNP enhancements can be viewed as the result of a two-step cross-effect process [27,51]. Within electron–electron–nucleus triads in which one electron spin is nearly resonant with the MW irradiation, MW irradiation first drives an allowed electron spin transition, creating an electron spin polarization difference. This process is fast and does not directly affect the nuclear polarization. Then, for triads in which a three-spin transition conserves energy, three-spin flips drive the nuclear polarization towards an enhanced, non-equilibrium value. On the other hand, for triads in which three-spin transitions conserve energy but neither electron spin is resonant with the MW irradiation, three-spin flips drive the nuclear polarization towards the unenhanced, equilibrium value. Strictly speaking, the apparent nuclear T_1 relaxation rate is a rate of energy exchange between nuclear Zeeman and electron Zeeman-difference reservoirs [51] and it exists regardless of the presence or absence of MW irradiation. Nuclei that are not directly involved in a triad with energy-conserving three-spin transitions communicate with the other nuclei through ^1H – ^1H spin diffusion, which tends to average the nuclear polarizations generated within each triad. In this scenario, DNP enhancement and nuclear T_1 relaxation are essentially the same process, driven by the same types of three-spin flips. Therefore, the observed DNP build-up time is always nearly equal to the apparent nuclear T_1 relaxation time.

An important distinction between the two explanations above is that, in the first explanation, three-spin transitions driven by microwave irradiation generate DNP enhancements, while nuclear T_1 relaxation does not depend on three-spin transitions. This explanation is related to the viewpoints presented by Hu et al. [49] and Vega and coworkers [50]. In the second explanation, three-spin transitions produce both DNP and T_1 relaxation, simultaneously.

The similarity of DNP build-up times measured through ^1H NMR and cross-polarized ^{13}C NMR confirms that there is no significant difference between the build-up times for bulk protons and for the protons that are coupled to the carboxyl ^{13}C labels of 1- ^{13}C -Ala.

The rates observed using DOTOPA-TEMPO are significantly greater than those using TOTAPOL for the same value of [nitroxide]. This fact enables more rapid acquisition of NMR spectra when DOTOPA-TEMPO is used. While ^{13}C T_2 effects are similar, shorter recycle times and somewhat bigger enhancements (see Fig. 6a) make DOTOPA-TEMPO a more efficient polarizing agent than TOTAPOL under our experimental conditions.

3.4. Effects of deuteration

All experiments described so far were carried out on samples that were partially deuterated due to the use of glycerol- d_8 as the glass forming agent. To study the effects of deuteration, we have proceeded with measurements where H_2O was partially substituted by D_2O (Cambridge Isotopes Inc.). Fig. 7a shows that the ^{13}C $1/T_2$ rate with proton decoupling grows very slightly with increasing deuteration, while $1/T_2$ without decoupling decreases. This may mean that spin diffusion among deuterons may start to contribute to ^{13}C transverse relaxation.

The DNP build-up times and 1H T_1 relaxation times for deuterated samples are shown in Fig. 7b. These times decrease with increasing deuteration. When the proton concentration is low, each paramagnetic dopant molecule has to induce the relaxation or DNP enhancement of a smaller number of protons, which means that the observed DNP build-up times and T_1 relaxation times become shorter. Previous studies of proton relaxation times under MAS at 80 K have shown somewhat greater T_1 values at lower proton concentrations, which may be related to slower spin diffusion to the paramagnetic centers [52]. The deuteration level also apparently changes the 1H DNP enhancement, although the trend is difficult to interpret. At the highest deuteration level, 1H NMR signals arising from sources outside the sample may interfere with our measurements of DNP enhancements. To summarize, the greater DNP build-up rates at moderate deuteration levels may be advantageous, unless ^{13}C T_2 values become too short and spoil the spectral resolution.

3.5. DNP-enhanced NMR of ^{13}C -labeled -amyloid fibrils

In order to demonstrate the applicability of our double-resonance DNP system in structural studies of biomolecular systems, we carried out a 2D ^{13}C - ^{13}C exchange experiment on a sample of pelleted $A\beta_{1-40}$ fibrils, with 6.6 mM of DOTOPA-TEMPO. The $A\beta_{1-40}$ peptide was selectively ^{13}C -labeled at carbonyl sites of Val18 and Phe19, as well as the methyl site of Ala30. The 1D ^{13}C CP spectrum of this sample, shown in Fig. 8a, is a superposition of powder pattern lineshapes arising from the methyl label (sharper feature centered at ~25 ppm) and the carbonyl labels (broader feature extending from ~100 ppm to ~240 ppm). At 8 K, the ^{13}C DNP signal enhancement factor is about 18. Since the concentration of $A\beta_{1-40}$ molecules is ~20 mM (3.5 mg of $A\beta_{1-40}$) and each molecule contains three ^{13}C labels, residual natural-abundance ^{13}C NMR signals from ^{13}C -depleted glycerol are negligible. The 2D exchange spectrum, recorded with $\tau_{exch} = 2.5$ s in a total of 4 h, is shown in Fig. 8b. The 1D slice at 130 ppm in Fig. 8c illustrates the signal-to-noise ratio of the 2D spectrum. Exchange occurs not only between the two carbonyl labels (3.4 Å internuclear distance, according to structural models for $A\beta_{1-40}$ fibrils [53]) but also between the carbonyl and methyl labels (~10 Å internuclear distance). The shape of the 2D carbonyl-carbonyl exchange pattern encodes information about backbone ϕ and ψ torsion angles of Phe19 [54–56]. By simulating the shape of the 2D exchange pattern, it is in principle possible to obtain this important structural information. A more systematic study of the application of DNP-enhanced ^{13}C NMR to $A\beta_{1-40}$ fibrils will be reported separately.

4. Concluding remarks

Studies of ^{13}C T_2 effects described above show that, at helium temperatures in static samples, the transverse relaxation is rather pronounced and is related to the electron spin flip-flop transitions. Quite importantly, the correlation time of the electron spin flips falls in a range where applying a Carr–Purcell sequence allows the effective ^{13}C T_2 time to be increased considerably. For instance in experiments requiring evolution of dipolar couplings [55,57], employing CPMG would average out fluctuations automatically. In a similar

manner, MAS can average out the effects of electron spin flips and provide rather long ^{13}C T_2 times, thus enabling DNP experiments under low-temperature MAS [48].

The overall ^{13}C NMR sensitivity improvement in our experiments is provided both by the Boltzmann factor due to low temperature (factor of ~ 37) and the DNP enhancement (factor of 20–30), giving together a factor of 740–1100 increase in signal strength compared to the same measurements at room temperature. It is clearly feasible to obtain 1D and 2D ^{13}C NMR spectra of non-spinning samples in 40 μl frozen solutions containing 1–10 mM of labeled substance. It is important to mention that our MW setup operates well below the saturation of EPR transitions [20] and therefore larger enhancements are in principle attainable by using a more powerful source. The approach shown in this work is applicable to many other samples which are available in limited amounts. While most studies in biomolecular solid state NMR are carried out under MAS, there are still a number of important examples where static samples are studied [57–59]. Possible applications includes studies of oriented samples such as peptides and proteins incorporated into phospholipid bilayers [60–63]. Provided that the relatively narrow NMR linewidths observed in spectra of well-oriented membrane proteins near room temperature can be maintained in the frozen state, the DNP/NMR setup described above should permit structural measurements on samples containing 10–100 nmol of ^{13}C -labeled protein molecules.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Design of low-temperature DNP/NMR probe. (a) Photograph of the probe insert (right) and the outer vacuum can (left). Microwaves are transmitted through an HDPE window in the bottom of the vacuum can. The probe insert includes the instrumentation skirt (1), which contains electrical feedthroughs, sealed fittings for the RF tuning rods (2), the evacuation port (3) and the guide tube (4) for the liquid helium transfer line. (b) Photograph of the probehead used for low-temperature NMR measurements with DNP. The entire probehead is enclosed within a radiation shield (shown as dotted lines) during experiments. Tuning and matching capacitors (5) and transmission line (6) for tuning of the ^1H channel are mounted on the radiation shield anchor (7); the RF coil leads are thermally anchored to sapphire blocks (8). The sample is mounted using two copper plungers, providing good thermal contact with copper block (9), the temperature of which is monitored using a Cernox sensor (10). Microwaves are delivered using a tapered corrugated waveguide (11). A cylindrical PTFE lens (12) is used to focus the beam in the direction perpendicular to the sample axis. (c) A typical sample (middle) with copper plungers (top and bottom) for mounting in place.

Fig. 2.

Pulse sequences used in this work. (a) Hahn echo sequence used for measuring the ^{13}C T_2 relaxation time, where the echo intensity is recorded as a function of τ_{echo} ; (b) Carr-Purcell-Freeman-Hill sequence, where the echo intensity is recorded as a function of n , the number of CPFH blocks; (c) 2D ^{13}C - ^{13}C exchange experiment with ^{13}C chemical shift evolution in the t_1 and t_2 dimensions. All sequences employ independent (0, 180) phase cycles of the first ^1H $\pi/2$ pulse and the last π pulse on ^{13}C .

Fig. 3.
 ^{13}C CP spectrum of 20 mM 1- ^{13}C -Ala, 6.6 mM DOTOPA-TEMPO, in 60% w/w ^{13}C -depleted glycerol- d_8 /water (solid) under MW irradiation at 264 GHz (1 scan). Signal from residual natural-abundance ^{13}C of glycerol is approximated by a Gaussian line at 70 ppm (dashed). Remaining signals belong to 1- ^{13}C -Ala (dotted).

Fig. 4.

(a) The $1/T_2$ transverse relaxation rates of the alanine carboxyl ^{13}C site as a function of the electron spin concentration [nitroxide]. Triangles show the $1/T_2$ rates without ^1H decoupling for TOTAPOL at 8 K, DOTOPA-TEMPO at 8 K, and DOTOPA-TEMPO at 16 K ($\blacktriangle$, $\triangle$, and $\blacktriangle$, respectively); Squares show the $1/T_2$ rates under CW 100 kHz ^1H decoupling for TOTAPOL at 8 K, DOTOPA-TEMPO at 8 K and DOTOPA-TEMPO at 16 K ($\blacksquare$, $\square$, and $\blacksquare$, respectively); Circles show the $1/T_2$ rates under 100 kHz ^1H Lee–Goldburg irradiation for TOTAPOL at 8 K, DOTOPA-TEMPO at 8 K and DOTOPA-TEMPO at 16 K ($\bullet$, $\circ$, and $\bullet$, respectively). To avoid congestion, error bars are shown only for DOTOPA-TEMPO at 8 K. (b) Effective transverse relaxation time T_2' in the Carr–Purcell–Freeman–Hill experiment vs. the length of CPFH block for 13.3 mM DOTOPA-TEMPO at 8 K ($\blacksquare$), and 6.6 mM DOTOPA-TEMPO at 8 K ($\square$).

Fig. 5.

Numerical simulations of CPFH curves and comparison with experiments. (a) Results at 6.6 mM of DOTOPA-TEMPO, or $[\text{nitroxide}] = 20 \text{ mM}$, assuming electron spin-flip correlation times $\tau_c = 1000 \mu\text{s}$ (■), $300 \mu\text{s}$ (●), $200 \mu\text{s}$ (◆), $100 \mu\text{s}$ (▲), and $30 \mu\text{s}$ (▼). Experimental values are shown with hollow squares; (b) 13.3 mM of DOTOPA-TEMPO, or $[\text{nitroxide}] = 40 \text{ mM}$, assuming electron spin-flip correlation times $\tau_c = 1000 \mu\text{s}$ (■), $300 \mu\text{s}$ (●), and $100 \mu\text{s}$ (▲). Experimental values are shown with hollow squares.

Fig. 6.

(a) DNP enhancements of ^1H NMR signals for DOTOPA-TEMPO at 16 K (◆), DOTOPA-TEMPO at 8 K (■), and TOTAPOL at 8 K (▲). (b) DNP-enhanced ^1H NMR build-up, ^1H T_1 , and cross-polarized ^{13}C NMR build-up times. Squares show DNP-enhanced ^1H NMR build-up times with DOTOPA-TEMPO at 8 K, DOTOPA-TEMPO at 16 K and TOTAPOL at 8 K (■, ⊕, and □, respectively). Circles show the ^1H T_1 relaxation time with DOTOPA-TEMPO at 8 K, DOTOPA-TEMPO at 16 K and TOTAPOL at 8 K (●, ⊕, and ○, respectively). Triangles show the DNP-enhanced ^{13}C NMR build-up times with DOTOPA-TEMPO at 8 K, DOTOPA-TEMPO at 16 K and TOTAPOL at 8 K (▲, ⊕, and ▼, respectively). Error bars are shown only for DOTOPA-TEMPO at 8 K.

Fig. 7.

Effects of deuteration on relaxation rates and DNP enhancements in a frozen solution containing 40 mM of TOTAPOL. (a) The ^{13}C $1/T_2$ relaxation rate under 100 kHz CW ^1H decoupling (■), under 100 kHz Lee-Goldburg ^1H irradiation (▲), and without decoupling (●). (b) The ^1H DNP build-up time (■), ^1H T_1 relaxation time (●), cross-polarized ^{13}C DNP build-up time (▲). (c) ^1H signal ^{13}C DNP enhancements.

Fig. 8. DNP-enhanced ^{13}C NMR of $\text{A}\beta_{1-40}$, selectively labeled as described in the text. (a) ^{13}C CP spectrum with (solid line) and without (dotted line) MW irradiation, 4 scans. (b) 2D ^{13}C - ^{13}C exchange spectrum with $\tau_{\text{exch}} = 2.5$ s, 4 h total measurement time. (c) 1D slice from the 2D exchange spectrum, taken at 130 ppm (dashed line in the 2D spectrum)