Dynamic Nuclear Polarization enhanced NMR at 187 GHz/284 MHz using an Extended Interaction Klystron amplifier

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A Dynamic Nuclear Polarisation (DNP) enhanced solid-state Magic Angle Spinning (MAS) NMR spectrometer which uses a 187 GHz (corresponding to 1H NMR frequency of 284 MHz) Extended Interaction Klystron (EIK) amplifier as the microwave source is briefly described. Its performance is demonstrated for a biomolecule (bacteriorhodopsin), a pharmaceutical, and surface functionalised silica. The EIK is very compact and easily incorporated into an existing spectrometer. The bandwidth of the amplifier is sufficient that it obviates the need for a sweepable magnetic field, once set, for all commonly used radicals.

The variable power (CW or pulsed) output from the EIK is transmitted to the DNP-NMR probe using a quasi-optic system with a high power isolator and a corrugated waveguide which feeds the microwaves into the DNP-NMR probe. Curved mirrors inside the probe project the microwaves down the axis of the MAS rotor, giving a very efficient system such that maximum DNP enhancement is achieved with less than 3 W output from the microwave source. The DNP-NMR probe operates with a sample temperature down to 90 K whilst spinning at 8 kHz. Significant enhancements, in excess of 100 for bacteriorhodopsin in purple membrane (bR in PM), are shown along with spectra which are enhanced by ≈25 with respect to room temperature, for both the pharmaceutical furosemide and surface functionalised silica. These enhancements allow hitherto prohibitively time consuming experiments to be undertaken. The power at which the DNP enhancement in bR in PM saturates does not change significantly between 90 K and 170 K even though the enhancement drops by a factor of ≈11. As the DNP build up time decreases by a factor 3 over this temperature range, the reduction in $T_1$ is presumably a significant contribution to the drop in enhancement.

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The DNP NMR spectrometer is similar to that previously reported by Pike et al. [8] but with a tuneable 187 GHz Extended Interaction Klystron (EIK) amplifier as the microwave source, which obviates the need for the magnetic field to be varied once initially set, and a 7.05 T Oxford NMR magnet operating at 6.66 T (1H frequency 283.7 MHz) with a Varian Infinity Plus console. The NMR probe is a modified triple channel MAS Doty DI-4 with extended VT capability which is able to spin stably at 8 kHz at 90 K for extended periods. The microwave system consists of a VDI Tx219 phase locked amplifier/multiplier chain having a maximum output of 71 mW feeding, via an isolator and a variable attenuator, a 187 GHz CPI EIK amplifier which has a maximum gain of 23.7 dB. The multiplier is driven either by an internal 11.700 GHz source or externally by an Agilent Technologies E8257C signal generator. The amplifier has a power output of up to 9 W with a 4 dB bandwidth of 0.41 GHz centred on 187.09 GHz. The microwaves are transmitted to the NMR probe via a quasi-optic transmission system similar to that described earlier [8] but optimised for 187 GHz. A ferrite rotator and polarising grids (Thomas Keating Ltd) act as a high power isolator to protect the EIK from any reflected power due to inadvertent poor coupling to the quasi-optic transmission system, the probe, or the sample itself. After the microwaves exit from the corrugated waveguide they pass through a PTFE window in the cryostat and are focussed by two mirrors onto the middle of the rotor cap where the beam waist (defined as 1/e amplitude) diameter is approximately 1.2 mm (as described by Pike et al. [8]). The loss in the transmission system is estimated to be approximately 4 dB. The beam passes through the rotor cap and a Teflon spacer, both of which are hollow for most of their lengths, before reaching the sample. The beam waist at the top of the sample is expected to be comparable to the diameter of the rotor and, in addition, the silicon nitride rotor is likely to aid confinement of the microwave beam further improving the efficiency of the system. As in our earlier work [8], silver foil of thickness 0.5 μm was attached to the top surface of the bottom spacer to increase the enhancement and to improve the uniformity of enhancement throughout the sample.

3. Samples

3.1. Urea with TOTAPOL or AMUPOL

The same sample (2 M 13C labelled urea (99%, Cambridge Isotope Laboratories Inc.) in glycerol-d8, D2O and H2O (60:30:10 by volume) with 40 mM TOTAPOL) as in Pike et al. [8] was used for initial set-up. Most experiments were carried out with a lower concentration (10 mM TOTAPOL or 10 mM AMUPOL) of radical.

3.2. Bacteriorhodopsin in Purple Membrane (bR in PM)

3H-labelled (70%) and uniformly 13C, 15N-labelled bacteriorhodopsin (bR) was expressed in Halobacterium salinarum strain S9 using rich labelled media and purified as purple membrane (PM) leaflets as described previously [9]. Isotopic labelling was achieved by the use of appropriately labelled celtone media made up in 70% D2O. After purification, 1.5 mg of labelled bR in PM was resuspended in 75 μl of 20 mM sodium citrate buffer at pH 6.0 containing 15 mM AMUPOL [10] and 0.01% w/v Na2S2O3, made up with glycerol-d8, D2O, H2O at a ratio of 6:3:1. This sample was thoroughly mixed before being pipetted directly into a 4 mm rotor for DNP measurements.

3.3. Furosemide

Furosemide (100 mg) (supplied by AstraZeneca plc) was added to a pestle and mortar and ground for approximately 10 min until a talc like consistency was achieved. 60 μl TEKPOL [11] solution (16 mM in 1.3 dibromobutane (98% Alfa Aesar)) was then added and ground for a further 5 min keeping the mixture moist by adding extra dibromobutane as required. The furosemide-TEKPOL mixture (101 mg) was then packed into a 4 mm rotor.

3.4. 3-Aminopropyl functionalised silica

Functionalised silica (Davisil G636, 60 Å pore size) was mixed with an excess of 5 mM TOTAPOL in D2O:H2O (90:10 v:v) solution and roughly mixed. This mixture was left for approximately 24 h at room temperature and was then centrifuged (12100g, 5 min) and excess TOTAPOL solution removed. The remaining damp powder was packed into a 4 mm rotor. Grinding the silica beforehand made no detectable difference to the enhancement achieved.

4. NMR

For all DNP experiments, microwave irradiation was applied during the recycle delay before the pulse sequence. CP experiments were performed with an 80% to 100% ramp [12] on the 1H channel and optimised contact times of between 0.6 ms and 1.5 ms were used. The 1H decoupling sequence used for all CP experiments was SPINAL-64 [13] with nutation frequency v1 = 100 kHz except for the 13C CP MAS of the furosemide sample where v1 = 80 kHz. The 13C spectra were referenced to the carbonyl peak at 177.8 ppm of l-alanine as an external reference with respect to TMS at 0 ppm. The 15N spectra were referenced using a known peak in the bR sample and is relative to liquid NH3 at –50 °C. The temperature was calibrated using the 119Sn shift of Sm2Sn2O7 as reported by Kemp et al. [14] from room temperature down to 80 K, and all experiments were carried out at 90 K and spinning at 8 kHz, unless otherwise stated.

Here enhancement, ε, is defined as ε = I/I0, where I and I0 are the spectral intensities of the enhanced and unenhanced spectra respectively under identical conditions.

4.1. 1H–13C HETCOR

A 1H v1, nutation frequency of 100 kHz was used for the 90° pulse, PMLG [15] decoupling during mixing and TPPM [16] decoupling during acquisition. The contact time was 1 ms with a ramped 1H v1 from 36 kHz to 40 kHz, and a constant 13C v1 ≈ 52.5 kHz. Microwave irradiation was applied during the 10 s recycle delay. 16 transients were co-added for each of the 64 t1 slices with a spectral width in F1 of 5.36 kHz giving a total experimental time of 2 h 52 min. States-TPPI was used for sign discrimination in the indirect dimension.

4.2. 15N–13C Double CP (DCP) HETCOR

The experiment is similar to that described by Baldus et al. [17] with the addition of DNP enhancement. Microwave irradiation is
applied for 1.5 s to transfer the electron polarisation to the hydrogen bath. A 7.1 µs 90° nitrogen preparation pulse is followed by 1H to 15N CP, a t1 period, 15N to 13C CP and then acquisition on 13C. The 1H to 15N CP sequence is a (t1 = 45 kHz) 1H 90° pulse followed by a 0.6 ms (v1 = 40 kHz) pulse on 1H together with a tan pulse of central amplitude of 35 kHz on 15N. For 15N to 13C CP, the contact time was 10 ms with v1 = 40 kHz on 15N whilst the 13C v1 is ramped from 56 kHz to 43 kHz. TPPM 1H decoupling of 100 kHz is applied during both the t1 period and acquisition with 100 kHz CW decoupling during the 15N to 13C CP transfer. 32 acquisitions per slice were acquired for a total of 98 slices and a t1 increment of 282 µs. The signal had decayed by 40 slices (11.28 ms) giving an experimental time (with 40 slices) of 32 min or 78 min for the complete experiment. TPPI was used for sign discrimination in the indirect dimension.

5. Results

In any DNP set up it is first necessary to determine the optimum frequency for DNP since the enhancement varies as a function of irradiation position within the EPR spectrum. This was done using the 2 M urea with 40 mM TOTAPOL sample from Pike et al. [8]. Normally the NMR magnetic field is swept to find the optimum frequency, but in our case the field is fixed and the frequency varied. The frequency dependence of signal enhancement for this sample is shown in Fig. 1. The maximum enhancement of approximately +80 is at 187.0 GHz. The shape roughly follows the first derivative of the ESR lineshape, is asymmetric with the maximum negative enhancement being approximately 75% of the positive value, and the separation between the maximum positive and negative enhancements is 0.14 GHz. The 4 dB points of the EIK (minimum of 3.5 W between 186.88 GHz and 187.29 GHz) cover both the positive and negative peaks of the enhancement curve and also give sufficient range to adjust the frequency for any similar radical. For the experimental data presented subsequently the frequency corresponding to the positive maximum enhancement was employed. Of the radicals tested so far TOTAPOL, AMUPOL and TEKPOL give maximum enhancements at very similar frequencies, and we did not find any changes necessary, however future radicals are likely to require some adjustments in irradiation frequency depending on the g value and the nature of the radical.

Fig. 2 shows the 13C enhancement achieved for the partially deuterated bacteriorhodopsin sample at 90 K using 15 mM AMUPOL as the radical. Both the 12C and 15N (shown in Fig. S1I) enhancements are approximately 120, although the 15N enhancement is somewhat less certain due to the poor signal to noise ratio in the unenhanced spectrum, even after approximately one hour of averaging. Notably, the microwave power is not the limiting factor in the performance of the spectrometer since the enhancement saturates at a source power level of approximately 3 W (Fig. 3a) corresponding to, at most, 1.5 W at the rotor top. This is in contrast to systems which couple the microwave power to the sample through the rotor walls where the enhancement at 80 K did not saturate at their maximum power of 12.5 W, see Ni et al. [5]. Furthermore the enhancement does not reduce with increased power indicating that sample heating is minimal even with 9 W of microwave source power (Fig. 3a). As an additional check for sample heating, a split KBr powder and water/glycerol sample was made and the minimal changes in 79Br shift [19] showed that any change in temperature with increased microwave power was less than 5 K.

There have been a number of simulations of the enhancement due to the Cross Effect for example by Thurber and Tycko [20], Mentink-Vigier et al. [21,22], Mance et al. [23]. In the simulations by Thurber and Tycko [20], it was found that, after initially increasing rapidly with the microwave nutation frequency, v1e, the nuclear spin polarisation becomes approximately constant at nutation frequencies of between 0.5 MHz and 1.0 MHz as the electron spin lattice relaxation time, T1e, changes from 2 ms to 0.2 ms. The very recent simulation of Mentink-Vigier et al. [22] also found that, for a T1e of 1 ms, the enhancement was only weakly dependent on rf amplitude for nutation frequencies above ~1.0 MHz indicating that v1e in our system reaches values in this range for an EIK output power of 2 W to 3 W. The resolution of the br spectrum is significantly worse than at room temperature at 800 MHz for a sample with no radical, particularly in the aliphatic region. As the broadening of the lines is due to the molecular motion being much reduced at 90 K, as well as possibly the presence of the paramagnetic radical, the temperature dependence of the enhancement was measured (Fig. 3b). Although the enhancement decreases quite rapidly with increasing temperature it is still greater than 10 at 170 K. Also the build-up time, T1R (shown in Fig. S12) becomes significantly shorter at higher temperatures, decreasing by a factor of 3 from ≈1.5 s at 90 K to ≈0.5 s at 170 K which enables data to be acquired more rapidly.

The effect of microwave power on enhancement was also determined at 170 K and somewhat surprisingly the behaviour was very similar to that at 90 K, i.e., the power needed for maximum

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Fig. 1. 1H DNP enhancement (13C Detected) for 2 M Urea in glycerol-d2, D2O and H2O with 40 mM TOTAPOL as a function of irradiation frequency at 6.66 T.
enhancement remained the same (shown in Fig. SI3) even though the enhancement had dropped by a factor \(\frac{1}{11}\). Since \(T_1\) decreased by a factor \(\frac{1}{3}\) in going from 90 K to 170 K, it would seem that a significant contribution to the reduction in enhancement in this case is the reduction in \(T_1\). The nuclear spins have a range of distances from the polarising agent and a shorter \(T_1\) will reduce the time for the polarisation to diffuse through the sample and thus the effective sample volume.

With the increased sensitivity for the BR sample, a 2D DCP HET-COR experiment could be run at 90 K in less than 1 h (on an undeuterated sample where the enhancement in the 1D spectrum was approximately 50) compared with 30 h at room temperature and with the sensitivity advantage of a higher field (800 MHz) (shown in Fig. SI4). However, as found elsewhere (see Koers et al. [24] for a recent discussion) the resolution is much worse than at room temperature. Nevertheless the rapid acquisition of the HET-COR spectrum indicates that DNP could be useful for selectively labelled samples. Interestingly the decreased molecular motion at low temperature brings up a new peak, centred on \(\delta = 32\) ppm (\(^{13}\)C), \(\delta = 121\) ppm (\(^{15}\)N), most likely from amino acid side chains in the protein which contain N–C pairs. The experiment was repeated at 170 K, where a full 2D spectrum could be obtained in about 4 h. The resolution was higher and the carbon peak around \(\delta = 32\) ppm was no longer visible indicating that there are significant dynamics in the system at this temperature, however the resolution was not high enough to be useful for subsequent NMR analysis.

The pharmaceutical furosemide has a long \(T_1\) (\(^1\)H) at room temperature (\(\approx 25\) s) making 2D experiments very time consuming or infeasible. The DNP build up curve for the furosemide with radical, shown in Fig. 4, fits well to a stretched exponential, 

\[
A = A_0 \left(1 - e^{-(t/T_0)^b}\right),
\]

where \(b\) is the stretching factor (0.70 in this case), indicative of a wide range of environments. The distribution in particle size results in a distribution of \(T_1\) values as the radical is further from some parts of the sample than others. As a consequence a similar S/N per unit time, also shown in Fig. 4, is obtained for build-up times between 5 s and 30 s. As suggested by Rossini et al. [25] the particle size distribution could be measured by comparing the signal build up with and without DNP. However this would be very time consuming in this case due to the long \(T_1\) of the bulk sample and the weak signal without DNP enhancement. Fig. 5 shows that the \(^{13}\)C enhancement obtained is approximately \(e = 5.5\) using a build-up time of 10 s. The effective improvement obtained by using DNP is given not just by the enhancement \(e\) but also by the Boltzmann factor and reduced noise in the coil, commonly taken as 3.6 [26], and the ability to pulse more rapidly.

Fig. 3. (a) \(^1\)H (\(^{13}\)C Detected) enhancement as a function of EIK output power for the bacteriorhodopsin sample using AMUPOL radical at 90 K. (b) \(^1\)H (\(^{13}\)C Detected) enhancement of the same sample as a function of temperature. Experiments were performed with a recycle delay of \(5 \times T_1\) and sufficient acquisitions (up to 128) to achieve a signal to noise ratio of greater than 20:1 in the unenhanced signal.

Fig. 4. \(^{13}\)C signal build-up of furosemide with 16 mM TEKPOL in dibromobutane, squares. The signal amplitude has been fitted to a stretched exponential with a \(T_1\) of 23 s and a stretching factor \(b = 0.70\). The circles show the signal per root second which is optimal for a build-up time of \(\approx 10\) s.

Fig. 5. \(^{13}\)C CP MAS Spectra of furosemide using 16 mM TEKPOL in dibromobutane as the polarising agent. 64 acquisitions with a 10 s build-up delay were acquired. The top spectrum is DNP enhanced. The enhancement achieved with this build-up time was approximately 5.5.
if the $T_1$ is reduced. However, as this sample had a large distribution of particle sizes and hence range of DNP build up times, although it is possible to pulse faster there is little extra gain in sensitivity and the total improvement is approximately 25, since the amount of sample and linewidths are almost unchanged. Comparison with the room temperature spectrum (see Fig. SI6), although in this case $\beta = 0.85$ is much closer to 1 (meaning a single exponential) than the furosemide sample ($\beta = 0.70$) indicating a smaller range of environments and/or distribution of radical distance from the surface. This is unsurprising given that the carbon being detected was exclusively at the surface, rather than throughout the bulk. However, it does indicate that the radical was not uniformly distributed with respect to the functionalised material. Optimum build-up times were around 4–10 s. Fig. 7 shows the $^{13}$C spectrum with and without microwave irradiation for a recycle time of 6 s, which gave $\epsilon = 6.7$. The $^{15}$N spectrum of this sample could be obtained in approximately 1 h of acquisition using DNP at 90 K (inset in Fig. 7) together with a room temperature spectrum which took 20 h of acquisition. The effective enhancement achieved was $\epsilon = 25$ as the amount of sample and linewidths are unchanged. As DNP offers over a factor 600 time saving for acquiring natural abundance $^{15}$N spectra of functionalised silica, DNP enables an otherwise prohibitive characterisation method to become routine.

6. Conclusion

A DNP NMR system based on an EIK microwave amplifier has been described that has significant demonstrated and potential advantages over gyrotron based systems. No additional superconducting magnet is required reducing space requirements, running and capital costs. The bandwidth of the amplifier allows easy change of frequency, sufficient to cover existing nitroxide based radicals without a change of magnetic field, and which is likely
to be of use when developing new polarisation radials. It also allows the possibility of frequency modulation [29] and pulsed DNP experiments to be undertaken, with such developments ongoing in our laboratory. The EIK is particularly easy to use, is very compact and can be readily incorporated into existing NMR systems. As previously demonstrated [8], the quasi-optic system, which projects a focussed beam of microwaves down the axis of the MAS rotor, together with the silver foil on the bottom rotor cap is very efficient and can be easily modified to increase functionality of the system. Due to the high efficiency of the quasi-optic system significantly lower microwave power (less than 3 W) is required than hitherto published for full DNP enhancement.

Significant DNP enhancements have been achieved in a wide range of samples with different radicals. For bacteriorhodopsin in the optic system significantly lower microwave power (less than the MAS rotor, together with the silver foil on the bottom rotor cap) was required than previously demonstrated [8]. The quasi-optic system, allowing the possibility of frequency modulation [29] and pulsed DNP experiments to be undertaken, with such developments ongoing in our laboratory. The EIK is particularly easy to use, is very efficient, water-soluble polarizing agents for dynamic nuclear polarization at high frequency, Angew. Chem. Int. Ed. 52 (2013) 10858–10861, http://dx.doi.org/10.1002/anie.201305647.

Acknowledgments

In conclusion, our work demonstrates that an existing conventional solid-state NMR system can be adapted to run DNP experiments via the use of an EIK microwave amplifier.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jmr.2016.01.021.

References


