

# One Small Acronym for Man, One Giant Leap for NMR



**Shortening run-times, lowering the limit of signal detection and producing a 10,000 fold increase in signal-to-noise ratio – Steven Reynolds and Damir Blazina at Oxford Instruments Molecular Biotools consider how liquid state DNP NMR is making pharma look twice at how it employs NMR analysis**

**Damir Blazina received a PhD in Chemistry from the University of York, UK, in 2003 and has five years' experience in the field of hyperpolarised NMR. He joined Oxford Instruments Molecular Biotools Ltd in September 2005, working as an Applications Scientist on the DNP project.**

**Steven Reynolds joined Oxford Instruments Molecular Biotools in February 2006 as an Application Chemist for the HyperSense platform, having previously worked in the field of hyperpolarised NMR with DNP. Since gaining his PhD, he has gained 10 years' experience in applying NMR, both in industry and academia, to fields as diverse as protein interaction and analytical radiochemistry.**

In today's target-rich but often lead-poor environment of drug design, there is an ever-growing need to isolate higher quality leads and focus resources on compounds with the greatest possible downstream potential. In the late 1980s and early 1990s, structure-based drug design seemed to hold the promise of delivering such results with the concept of rationally designing drug molecules to fit their target, virtually from the ground up. As time went on, however, many found that contemporary methods and technologies couldn't keep up with current needs, and so the industry moved towards faster, higher volume approaches such as combinatorial chemistry and high-throughput screening (HTS). But now HTS has revealed its own associated drawbacks, proving to be time consuming while yielding compounds with high attrition rates in the later stages of development. As a result, the industry is experiencing a resurgence in structure-based (or structure-guided) drug design and, more recently, fragment-based drug design, with the idea of combining information from these methods with HTS and combinatorial chemistry studies (1).

This new examination of structure and fragment-based design has been supported by recent advances in the technologies used to elucidate the structures of compounds and their key chemical fragments, namely X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy. Ideally, these two technologies are used as complements to one another, with X-ray analysis providing high resolution studies of the molecules being analysed and NMR producing highly detailed information on their molecular dynamics, 3D conformation and reaction kinetics, as well as structural information – all factors which have made NMR a favourite for those involved with fragment-based drug design.

Offering vast improvements in NMR sensitivity, a new combination technology known as liquid state dynamic nuclear

polarisation NMR (DNP NMR) is set to further enhance the applicability of NMR. The technique reduces the time it takes to analyse each compound, while requiring substantially smaller sample amounts (down to the  $\mu\text{g}$  level), enabling structure confirmation studies for chosen cases previously considered impractical. Combined with a significantly lower limit of signal detection, this not only expands the applicability of NMR in drug discovery and research, it also opens the door to using the same system for impurity detection and quality control in later development.

## THE BASICS OF NMR

Although an in-depth exploration of the physics and chemistry behind NMR is beyond our purposes here, a review of the

fundamentals provides context for the advantages brought by integrating it with DNP. NMR itself is a physical phenomenon based on the property of nuclei to produce magnetic fields associated with their spin angular momentum. Like electrons, nucleons (protons and neutrons) have an angular spin momentum. In atomic nuclei with an even number of nucleons, such as  $^{12}\text{C}$ , protons and neutrons with opposite spin pair with one another, and so generate a net spin of 0 for the nucleus, such that no NMR signal can be measured. In certain atoms, however, some of the nucleons will remain unpaired, each contributing  $+1/2$  or  $-1/2$  to the spin number of the nucleus and creating an associated magnetic moment,  $\mu$ . This condition can be thought of as a tiny magnet with a north and south pole. This is also the property that is manipulated during NMR experiments.

In a typical sample, nuclei can take on one of two possible orientations for  $\mu$  (known as  $\alpha$  and  $\beta$ ) and, in the absence of magnetic field, their rotational axes will be arranged randomly in space. When an external magnetic field is applied, the axes of the spins arrange themselves in either a spin aligned ( $\alpha$ ) or spin opposed ( $\beta$ ) direction, depending on whether the spins are parallel or anti-parallel to the direction of the magnetic field. In the energetically preferred situation, at thermal equilibrium, the spin-aligned state is slightly in excess. This difference in population is referred to as the polarisation of the sample, and is normally very small – typically in the order of less than 0.05 per cent. To detect these NMR signals, a pulse of the correct frequency is applied to simultaneously excite all the spins, making them oscillate together, which is then detected by an NMR spectrometer. Although NMR studies performed in this manner can provide comprehensive structural, chemical and electronic information on a wide range of molecules (including their inter- and intra-molecular interactions), the broader use of the technique has been hindered by the relative insensitivity of NMR compared to other spectroscopic and analytical methods. This inherent limitation arises from both the low magnetic energy of the bulk sample at room temperature (relative to their thermal energy) and the low abundance of some NMR active nuclei (such as  $^{13}\text{C}$  and  $^{15}\text{N}$ ). For example, in nature the abundance of  $^{13}\text{C}$  isotope of carbon is in only 1.1 per cent and  $^{15}\text{N}$  represents just 0.36 per cent of the natural population of nitrogen. Each NMR active nucleus can therefore be thought of as a tiny pinpoint of light in a vast dark space that NMR spectroscopists much search out.

For the purposes of drug discovery and research, NMR has often been thought of as time consuming, due to the high number of scans and long periods typically required to achieve sufficient sensitivity (that can be in the order of hours to days). Amplifying the NMR signal can normally only be achieved by using a much higher concentration of the sample (which isn't always available) so that there are more active nuclei to detect, or by increasing the strength of the applied external magnetic field, typically a costly endeavour. Although a number of sensitivity-enhancement methods have been developed to attempt to overcome this, they have each met with limited success. Some, for example, are able only able to work with

specific chemistries (such as para-hydrogen induced polarisation or PHIP), while others achieve only a marginal increase in signal strength, but add significantly to operational costs (such as cold probes).

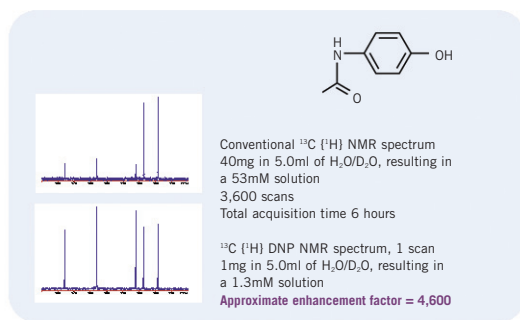
## FROM NMR PINLIGHT TO NMR SPOTLIGHT

Dynamic nuclear polarisation (DNP) is a nuclear spin enhancement technique in which the high spin polarisation levels of unpaired electrons (which are easily polarised due to their larger magnetic moment) are transferred to nearby nuclei. The process begins by cooling a sample/radical mixture down to temperatures approaching absolute zero, at which point electron spin polarisation approaches unity, while the nuclear spins remain less than one per cent polarised. With the application of microwave radiation, however, the two spin systems become able to intercommunicate and transfer of polarisation between the spin states occurs. The final result of this process is that the nuclear spin system becomes 'hyperpolarised', with polarisation levels increased by several orders of magnitude.

In terms of application, DNP has been principally used for polarisation enhancement in neutron scattering – a complementary technology to NMR and X-ray crystallography that produces lower resolution information on proteins and protein-DNA complexes. In NMR experiments, however, the use of DNP has been limited due to the fact that samples must be kept in a solid state in order to preserve hyperpolarisation. In solution (the preferred state for NMR experiments), polarisation levels rapidly return to normal and therefore the gains provided by DNP are just as quickly lost. Similarly, the spectral resolution of experiments performed with DNP-enhanced samples kept in the solid state is still less than that of standard solution state NMR. The challenge then, has been to integrate the two technologies in a manner that allows the introduction of solid-state, DNP-enhanced samples to solution-state NMR analysis.

Recently, a novel sample preparation instrument was developed to overcome this barrier, allowing researchers to obtain polarisations of 15 to 20 per cent for samples in solution (2). As with normal DNP processes, the new system starts by cooling sample mixtures down to about 1.4K in a pumped helium bath and exciting the electron spins with 94GHz microwave radiation. Hyperpolarisation builds up over the course of 15 minutes to four hours, depending on the nature and the amount of sample present. Once this is achieved, a unique injection system delivers a heated solvent charge into the cold space and rapidly dissolves the sample. An automated sample transfer mechanism then quickly delivers the sample to the NMR magnet for spectral acquisition in the solution state. This automated and seamless transfer of the sample from the DNP polariser to the NMR magnet forms the crucial bridge that links the two techniques.

Independent studies testing the threshold of detection with liquid state DNP NMR have shown that it is capable of generating greater than a 10,000 fold increase in baseline

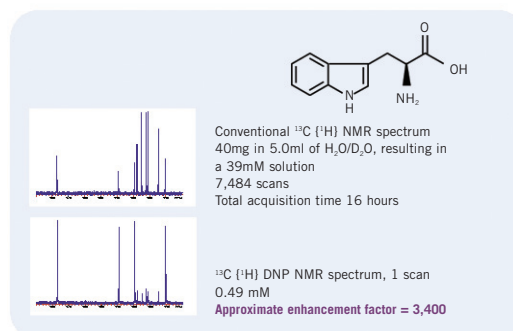
**Figure 1: Acetaminophen spectrum**

sensitivity compared to conventional NMR analysis (2). Just as important for the pharmaceutical community, experimental runtimes are substantially shortened, as only a single scan is required to capture a complete NMR spectrum. Figure 1 shows a comparative example using a sample of acetaminophen examined with both conventional NMR (1.a) and DNP NMR (1.b). In the first set up, the NMR spectrum was acquired from a 53mM sample after 3,600 scans performed over a six-hour period. In contrast, just a 1.3mM solution of the same sample was needed for examination by DNP NMR, which then produced a spectrum with substantially improved signal-to-noise ratio in only a single scan. A similar comparative test series performed with L-tryptophan is presented in Figure 2. Again, the S/N ratio was visibly improved with a single DNP NMR scan, compared to almost 7,500 scans taken with conventional NMR.

### CURRENT DNP NMR PROJECTS IN DRUG DISCOVERY AND RESEARCH

Beyond simply improving the signal-to-noise for conventional NMR applications, integrated DNP NMR systems are opening new doors for the use of NMR analysis in areas for which it was previously considered impractical. Potential applications for the technology include routine observation of low abundance nuclei (for instance  $^{15}\text{N}$ ), structural verification studies, ADME studies, impurity identification, ligand screening, real-time kinetic measurements and biomarker discovery. In each case, DNP NMR can offer researchers both richer and more accurate results than conventional NMR analysis, as well as a shorter time-to-results delivery.

To further create and expand the applications for DNP NMR in the pharmaceutical field, the Institute for Cancer Studies at the University of Birmingham, for example, will be using the technology in order to identify biological markers for a variety of cancer types and to develop new methods for determining the kinetics and dynamics of relevant protein-ligand interactions. At Queen Mary University of London, the technology is being applied to molecular structure determinations and to conduct research in several areas including fullerenes and carbon nanotubes, nitrogen containing drugs, and silicon in biofluids derived from implant materials. The improvement to NMR sensitivity enabled by DNP enhancement has also opened the

**Figure 2: L-tryptophan spectrum**

possibility of using NMR not just in research and discovery, but also in downstream pharmaceutical development processes. At Pfizer Global R&D, for instance, DNP NMR is being investigated for its applicability in the identification of trace chemical species in support of drug development, as well as a tool for the rapid characterisation of compound libraries.

The core application of DNP NMR in the pharmaceutical industry looks to structure and fragment-based drug design. The advantages brought by DNP NMR will allow researchers to potentially overcome the roadblocks and time constraints that have restricted the use of NMR in the past. The vastly enhanced signal intensity will also make possible the examination of molecules, which have historically been difficult to characterise with NMR. With DNP enhancement, far less sample material will be required to provide meaningful, high resolution results.

As the applications of liquid state DNP NMR continue to develop, it's important to remember that even experts in the field would not suggest that it becomes a replacement for X-ray crystallography. Rather, the two methods provide complementary information that helps researchers to produce better quality drug candidates with superior chemical and pharmacological properties, and lower rates of attrition in development. What DNP NMR does provide over the current generation of NMR technology is a dramatically lower limit of signal detection, reduced runtimes and up to a 10,000 times greater signal-to-noise ratio. By combining this with information from X-ray studies and combinatorial chemistry, it is possible to make significant gains in the efficiency and speed of drug discovery and, in the end, produce more effective therapies. ♦

*The authors can be contacted at  
molecularbiotools@oxinst.co.uk*

### References

1. Mitchell T and Cherry M, Fragment-based drug design, *Innovations in Pharmaceutical Technology* 16, pp34-36, 2005
2. Ardenkjær-Larsen JH *et al*, Increase in signal-to-noise ratio of >10,000 times in liquid state NMR, *PNAS* 100 (18): pp10,158-10,163, 2003