Cryogenically cooled probes—a leap in NMR technology

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Received 20 January 2005

Keywords: NMR (Nuclear Magnetic Resonance); Cryogenic probe technology; Sensitivity; Signal-to-noise ratio

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Abbreviations: 1D, 1-dimensional; 2D, 2-dimensional; 3D, 3-dimensional; ASTM, American Society for Testing and Materials; BPTI, bovine pancreatic trypsin inhibitor; CDCl$_3$, chloroform-$d$; CD$_3$CN, acetonitrile-$d_3$; CD$_3$OD, methanol-$d$; i.d., inner diameter; LC, liquid chromatography; MRI, magnetic resonance imaging; MS, mass spectroscopy; NMR, nuclear magnetic resonance; o.d., outer diameter; RDC, residual dipolar coupling; RF, radiofrequency; RP, reverse phase; SAR, structure-activity relationship; S/N, signal-to-noise ratio; SOD, superoxide dismutase; SPE, solid phase extraction; STD, saturation transfer difference; UV, ultra violet.

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doi:10.1016/j.pnmrs.2005.03.001
1. Introduction

During the 50 years of NMR, the technology has renewed itself again and again although the basic physical principles remain the same. As technical advances and novel materials boost the performance and stability of NMR spectrometers, the limits of the experiments, sample amounts and measuring time have been shifted beyond what was unthinkable just 10-years-ago. The technology advances in different ways. One is the continuous improvement of the performance of all parts of the NMR instrument. Another one is the occasional development of novel components that notably step up the performance of the NMR instrument and thus, benefit the user in many ways. The cryogenically cooled probes for high-resolution NMR spectroscopy are such an innovation. These enable typically a 3–4-fold enhancement of the detection sensitivity in high-resolution NMR compared to the corresponding conventional probes, enabling measurements of considerably smaller amounts of substances, or within an order of magnitude less time for a fixed sample concentration.

The sensitivity of the NMR-spectrometer is primarily limited by thermal noise in the signal detection pathway. A probe is a sensor positioned in the centre of the magnet containing the coil that is used both to send radiofrequency (RF) pulses to the sample and to detect the NMR-signals returning from the atomic nuclei. In addition to this coil a cryogenically cooled probe, usually, also houses the preamplifier to amplify the detected signal. The sensitivity or signal-to-noise enhancement in the cryogenic probe is accomplished by lowering the temperature of the coil and the preamplifier, thus essentially reducing the thermal noise in the receiver circuitry. The idea of lowering the coil temperature to improve the signal-to-noise ratio of a NMR instrument was initially proposed by Hoult and Richards in 1976 [1]. In 1984, Styles and co-workers [2] described a home-built probe where the receiver coil and the preamplifier were cooled with liquid helium. Cryogenic RF-probe techniques have also been applied to biomedical MRI [3,4].

In the 1990s, commercial products for high-resolution NMR based on the cryogenic probe technology [5] were developed and the first installations of cryogenic triple resonance probes at customer sites were done in 1999. Since then cryogenic probes for high-resolution NMR have been developed for spectrometers with operating frequencies ranging from 400 up to 900 MHz. Today these probes are available for operation with 5 and 3 mm diameter sample tubes and also for LC-NMR and flow injection modes.

At present the variety of available configurations comprises probes optimised for $^1$H- and $^{13}$C-detection, and probes for triple resonance involving either $^{15}$N or $^{31}$P as the third nucleus. The choice of different cryogenic probes is steadily expanding to meet the needs of different NMR applications.

Not only the NMR hardware, but also the NMR methodology has undergone a remarkable development towards increasingly complex and technologically demanding experimental schemes [6,7]. On the one hand, methodological advances pose technical challenges to the instrumentation, on the other hand technological innovation enables progress in the research methods. This reciprocity applies to all parts of a NMR spectrometer, including the magnet construction, probe construction, electronics and the software interface. As a result, the sensitivity, an important criterion of the NMR-instrument, has surged. The ever increasing sensitivity, illustrated in Fig. 1, also reflects the process of continuous innovation during the near four decades of commercial NMR technology. And, by all likelihood we are not near the end of this development.

The scope of the present review is to outline the basic theory and the current state of cryogenic probe technology in high-resolution NMR. In Section 2, we discuss the physical principles that govern the sensitivity of a NMR probe, and in particular the factors that influence the performance of a cryogenic probe. The construction of the necessary hardware, that is, the probe together with the cryogenic cooler, is presented schematically. In Section 3,
we describe a number of applications from different domains of research where the sensitivity gain achieved by using a cryogenically cooled probe has been particularly beneficial. The selection of examples presented here is by no means exhaustive. The applications continue to expand as cryogenic probes become more widely available. Currently, there are over 500 cryogenic probes installed world-wide and this number is rapidly increasing.

2. Sensitivity of the NMR experiment

The major limitation of NMR spectroscopy is the rather low detection sensitivity, rendering the experiments time-consuming compared to other methods used for molecular structure determination or verification such as X-ray crystallography or mass spectroscopy. This is because the sensitivity of the NMR signal depends on the small difference in the populations of the Zeeman energy levels. The separations between the nuclear spin states are small, corresponding to energies in the radiofrequency range. The population difference is given by the Boltzmann distribution. For $^1$H nuclei at room temperature and magnetic field of 10 T the difference in the population is in the order of 1 in $10^7$ which means that most of the nuclei do not contribute to the NMR signal. This is in contrast to optical spectroscopic methods such as, for instance, infrared (IR) spectroscopy where basically a single photon can be detected. For a high-resolution NMR investigation using a conventional probe operating at ambient temperature the required amount of substance is often milligrams. In many applications the available amount of sample is limited, or the inherent solubility of the substance of interest may be low, or a dilute solution is required because the sample may tend to aggregate at higher concentrations. In such cases, the cryogenic probe technology moves the lower limit of the feasible sample concentration to the microgram and micromolar range. For biological macromolecules, the change in the sample requirement from the millimolar to the micromolar sample concentration range greatly increases the number of compounds that can be studied by NMR.

The strength of NMR spectroscopy is given by its multifarious applications, which range from statistical analysis of mixtures to the determination of three-dimensional structures for molecules of biological interest. The information content of NMR at the atomic level is both comprehensive and diverse. Thus, to improve the sensitivity has always been an important development goal from the point of methodology and engineering. In an NMR experiment, the signal-to-noise ratio is usually augmented through computer averaging of accumulated transients. In the signal averaging, however, the signal-to-noise ratio is proportional to the square root of the number of transients. Consequently, a 3–4-fold sensitivity increase as provided by cryogenic probes, entails a 9–16-fold reduction in measurement time. This opens up completely new possibilities in high-resolution NMR research. In the following, an overview of the factors determining the sensitivity of the NMR experiment will be presented.

2.1. Milestones

For many NMR applications sensitivity is the most critical factor. During the past 40 years of commercial NMR technology, the sensitivity of the NMR instrument has increased dramatically, as illustrated in Fig. 1. In this figure, the specified signal-to-noise ratio for the methylene $^1$H signal of ethylbenzene—the standard $^1$H-sensitivity test—is plotted as a function of time, each point indicating the year when an increase in sensitivity was realised. The black dots denote the sensitivity of a conventional probe corresponding to the introduction of a magnet operating at a higher field, and the triangles mark the launches of novel cryogenic probes at different fields (all data from Bruker BioSpin). The magnetic field is indicated above the marker in terms of the $^1$H operating frequency. Fig. 1 illustrates both a constant drive to ever higher magnetic field strengths, $B_0$, to gain sensitivity and also spectral dispersion, and also dramatic improvements in probe construction. Theoretically, the sensitivity increase depends on $B_0^{7/4}$ whereas the second advantage, the improvement in spectral dispersion, is linearly proportional to $B_0$.

Noteworthy is not only the leap at the advent of the cryogenic probe technology, but also the on-going improvement of sensitivity of the conventional probes. This is highlighted by the specification of ethylbenzene sensitivity of the conventional inverse detection probe at 500 MHz currently, 900:1, marked with a dashed line in Fig. 1, to be compared to the value of 200:1 two decades earlier when this particular magnetic field strength was first introduced by commercial manufacturers. The sensitivity upsurge over the last decades has been driven by advances in both the probe and receiver technology. The most important milestones with respect to development of commercial NMR-spectrometers are:

- Introduction of superconducting magnets. The upper limit for the magnetic field strength that can be achieved by iron magnet technology is about 2.35 T. The discovery of superconducting materials which lose all their resistance when cooled below a certain temperature made it possible to manufacture higher field magnets resulting in increased dispersion and sensitivity.
- Introduction of Fourier transform (FT) NMR [8–11]. In the so-called continuous wave (CW) NMR spectroscopy, the absorption of radiofrequency was detected by sweeping the frequency of irradiation or the magnetic field over the spectral window of interest. This is similar to detecting the absorption in optical spectroscopy. The disadvantages of this approach are the long acquisition times and the inability to obtain distinct information through manipulation of the spin system, as is done, for
instance, in two- and three-dimensional NMR. FT-NMR uses RF-pulses to excite all transitions of interest simultaneously, followed by a Fourier transformation of the recorded signal. The time saving in the FT acquisition combined with signal averaging provide a much improved signal-to-noise ratio. Furthermore, the RF-pulses allowed selective editing of the spin systems and thus opened the way for devising a wide variety of different experiments.

- Introduction of pulsed field gradients. Long before their application in high-resolution NMR spectroscopy [12–18] pulsed field gradients were used in MRI (magnetic resonance imaging). In high-resolution NMR spectroscopy, the application of pulsed field gradients allow one either to select coherences of interest or to destroy unwanted coherences without the need of phase cycling. Thus the experiment time can be reduced dramatically. Further, signal detection with optimal dynamic range of the receiver system is possible because only the signal of interest reaches the receiver.

- Gradual improvement of hardware and higher overall stability of the NMR spectrometer. In addition to the availability of magnets operating at higher field strengths and advances in conventional probe technology, such as optimised geometry and materials of the receiver coil, sensitivity has been boosted by improved receiver technology, including low-noise preamplifiers, digital filters and oversampling [19]. The high instrumental stability has paved the way for the design and implementation of increasingly sophisticated pulse sequences.

- Development of cryogenically cooled signal detection circuitry. A significant increase in NMR-sensitivity is realised by this approach. The theoretical foundation will be described below.

Regarding the methodology, FT-NMR opened up the possibility of designing novel experiments, based on selective editing of spin systems by means of trains of RF-pulses. The most important discoveries with respect to higher sensitivity are:

- Polarisation transfer experiments based on the INEPT [20] and DEPT [21] pulse sequence. In these experiments, the population of a sensitive nucleus is transferred to an insensitive nucleus, thus enhancing the detection sensitivity.

- The transverse-relaxation-optimised spectroscopy (TROSY) principle [22–24]. NMR of large biomolecules is hampered by broad NMR signals due to fast relaxation. TROSY experiments select only the magnetisation components with the slowest relaxation and result in sharper resonance lines.

Apart from these further techniques exists a dramatic improvement of sensitivity of the NMR experiment.

The basic idea in these experiments is to enhance the difference in the population of the Zeeman levels through hyperpolarisation, that is, by transferring the angular momentum of laser photons to electronic and nuclear spin through so called optical pumping. Examples of these methods are the SPINOE-technique [25,26] for noble gases such as $^3$He and $^{129}$Xe, and dynamic nuclear polarisation (DNP) [27], a technique, which can increase the $^{13}$C sensitivity for certain samples by a factor of up to 10,000.

2.2. Theoretical considerations on the sensitivity of the NMR experiment

2.2.1. Signal-to-noise ratio and temperature

The sensitivity of the NMR experiment is given by the ratio between the signal and the noise of the entire receiver system [1,28]. The signal is the voltage induced in the receiver coil of the probe, while the noise consists of (i) thermal noise from the receiver coil in the probe, (ii) thermal noise from the preamplifier, (iii) thermal noise from the sample itself.

The most important contributions to the ratio of signal, $S$, to noise, $N$, in a NMR experiment, or, the ratio of the current of the induced signal, $U_s$, to the noise current, $U_N$, are [1,29]:

$$\frac{S}{N} = \frac{U_s}{U_N} \approx \frac{N \times \gamma_e \sqrt{T_c} \times B_0^4 \times (B_1/I_{coil})}{\sqrt{4k_B T_a \Delta f (T_c + T_e) - R_a(T_c + T_e)}}$$

Eq. (1) includes factors related to the sample and the particular experiment such as the number of nuclei in the sample $N$, the gyromagnetic ratio of the excited nucleus $\gamma_e$, and the gyromagnetic ratio of the detected nucleus $\gamma_d$. Significant for sensitivity are also the static magnetic field strength $B_0$ and the coil design, expressed through the ratio between the magnetic $B_1$-field in the RF-pulse $B_1$ and the current generated by the coil $I_{coil}$. The dependence of the sensitivity on the resistance and temperature of the detection coil, $R_c$ and $T_c$, and on the noise temperature of the preamplifier, $T_a$, are exploited in the cryogenically cooled probes and will be discussed in detail below. The resistance induced by the sample in the coil, $R_c$ and $T_c$, become an issue for solutions of high conductivity, these will be discussed in Section 2.3. In addition, Eq. (1) contains the receiver bandwidth in Hz, $\Delta f$, and the Boltzman constant $k_B$.

There are two ways of increasing the signal-to-noise ratio, either by increasing the factors influencing the signal voltage, $U_s$, or decreasing the factors influencing the noise voltage, $U_N$. The signal voltage can be raised by having a larger sample volume with more nuclei, by choosing a higher field strength $B_0$, or by selecting experiments with a favourable ratio of the gyromagnetic ratio of the excited and observed nucleus. The signal voltage can also be improved through optimising the coil design, here expressed through the ratio $B_1/I_{coil}$. This will be discussed in Section 2.2.2.

Further improvement of the signal-to-noise ratio can only be achieved by reducing the noise voltage. The term
the resistance of the entire receiver circuitry. Johnson noise, a synonym for thermal noise, results from the stochastic motion of electrons in any conducting medium. Since the electrical resistance of a conductor depends on the collisions of electrons, the resistance decreases at lower temperatures as less electron collisions occur, for instance, in this case within the wire of the coil. A quadratic approximation can be used to describe the temperature dependence of electrical resistance over a large temperature range. At low temperatures common metals have a residual and constant resistance that is temperature independent. This residual resistance is due to impurities in the conductor. Such factors will additionally influence the reduction of the thermal noise in the receiver coil.

In cryogenically cooled probes \( T_c \) and \( R_c \) are low, and thermal noise is reduced. For an optimum reduction of thermal noise in the detection pathway of a cryogenic probe, the preamplifier between the coil and receiver, and the connecting cables, should also operate at lowered temperature. If the output of a cold coil with low noise is directed to a preamplifier operating at ambient temperature, the latter will introduce additional noise. According to Eq. (1), the thermal noise from the preamplifier, \( T_n \), contributes to both the noise term related to the coil and that related to the sample.

It should be noted, however, that the sensitivity gain achieved by a cryogenically cooled probe is below the theoretical value. This is because the necessary thermal insulation between the sample and the receiver coil leads to a suboptimal filling factor \( \eta \) that is, the ratio of the sample volume to the volume of the receiver coil. In practical terms, a cold receiver coil reduces the thermal noise by a factor of about two. By using a cold preamplifier another factor of two may be gained. Thus, the overall reduction of thermal noise in a cryogenic probe is typically about a factor of four.

2.2.2. Other factors that influence sensitivity

In the theory of the conventional probe technology, the thermal noise contribution from the entire receiver circuitry is treated as constant. With this assumption, the sensitivity of a probe is commonly described by the quality factor, the \( Q \)-factor, of the probe. The \( Q \)-factor is determined by the resonance frequency \( \omega \), the inductance \( L \) and the resistance \( R \) of the entire resonant circuit [1]:

\[
Q = \omega L / R 
\]

The signal-to-noise ratio is proportional to the square root of the \( Q \)-factor notwithstanding power losses in the sample. For the best signal-to-noise ratio one obviously tries to raise the \( Q \)-factor. According to Eq. (2) this can be done by reducing the resistance \( R \). The inductance \( L \), however, is fixed for a given coil geometry. Hence, the \( Q \)-factor and the sensitivity of a probe can be approximated as a function of the resistance of the entire resonant circuit. According to Eq. (1), the resistance of the coil depends on the temperature of the coil. Thus, in the case of cryogenic probes, cooling the coil and reducing the resistance means that the \( Q \)-factor, and thereby the sensitivity of the probe, increases. The product of the \( Q \)-factor and the filling factor, \( \eta Q \), is a measure of how efficiently a coil can transfer RF-energy to the sample volume.

The impact of the geometry of the coil on the resistance can be explained in terms of the magnetic field generated by the electric current in the coil. As an example, sharp turns in a coil can increase the current at inside edges, thus reducing the amount of effective conductor and increasing the resistance. The effect of the coil geometry on the signal intensity is rather complex. The same coil is used for excitation (transmission) and detection (reception), hence, a good transmission efficiency means a good reception efficiency. In Eq. (1), this is expressed through the ratio of the magnetic \( B_1 \)-field of the RF-pulse and the current in the coil, \( B_1 / I_{coil} \). Good transmission efficiency requires good \( B_1 \) homogeneity of the coil, which can be expressed by the volume integral of the \( B_1 \) field. The theoretical basis is given by the reciprocity theorem [30,31]. The length of the 90° pulse at a given power can be taken as a contributing factor to the sensitivity. A coil with a short 90° pulse length will have higher sensitivity than a coil with a long 90° pulse.

The resistance of a probe operating at ambient temperature is defined by two components, these are the receiver coil and the capacitors of the resonance circuit. The resistance of the capacitors is related to the material used. The resistance of the coil is influenced by two factors, these are the so-called ‘skin effect’ and the geometry of the coil [1]. The skin effect states that electricity tends to flow in the outermost areas of a conductor. This can be understood through the principle that all electric currents generate magnetic fields that in turn can affect the current. With an alternating current the magnetic field tends to push the current towards the outside of the conductor. As the frequency increases, so does the effect until at very high frequencies the entire current flows in a very narrow skin on the conductor. The effect in a coil will be that the current is forced to flow in a smaller part of the coil, resulting in a smaller area for the current flow, thus increasing the resistance of the coil.

The so called quantisation noise is white noise introduced through the conversion of the analogue signal to a digital one as a part of the signal detection [19]. It is due to the inevitable rounding error in the process of digitalisation. The quantisation noise can be reduced in two ways, by using a digitizer with the highest possible digitizer resolution and using the technique of oversampling [19].

2.3. Sensitivity and conducting solvents

Eq. (1) contains additional terms that are related to the thermal noise contributed by the sample. These terms may become dominant for solutions of high conductivity.
Such circumstances are typical for samples in aqueous solution containing buffers or salt, whereas in most organic solvents the effect is small. If the terms related only to the thermal noise are extracted from Eq. (1) we obtain:

\[
\frac{S}{N} \propto \frac{1}{\sqrt{4k_B T \Delta f \left( R_c(T_c + T_a) - R_a(T_c + T_a) \right)}}
\]  

(3)

The term \( R_c(T_c + T_a) \) is the noise contribution related to the coil; the term \( R_a(T_c + T_a) \) depends on the sample. The resistance \( R_c \) is a resistance in the coil due to an inductive coupling between the sample and the coil [32]. \( R_s \) is directly proportional to the conductivity of the sample.

For conventional probes the terms \( R_c(T_c + T_a) \) and \( R_a(T_c + T_a) \) are of the same order of magnitude, as the coil and sample are typically at the same temperature. For such probes conducting solvents have a certain impact on the sensitivity, but a loss in sensitivity for highly conducting probes conducting solvents have a certain impact on the sensitivity, but a loss in sensitivity for highly conducting probes. Therefore, a reasonably linear relation is found between pulse length and sensitivity.

For a cryogenically cooled probe the noise contribution \( R_c(T_c + T_a) \) from the coil is low compared to the noise contribution \( R_a(T_c + T_a) \) from the sample. The resistance and temperature of the coil are low, while \( R_a \) of the sample, which is at room temperature, especially at elevated ionic strength and conductivity, is high. First, circular components of the Brownian motion of the electrical charges result in a randomly fluctuating magnetisation that is received by the coil, and second, the electric charge mobility in the sample dissipates RF power [4]. In order to gain maximum sensitivity at high ionic concentration, the noise contribution from the sample should be reduced. Several approaches have been proposed:

- Low conductivity buffers are used instead of salts with high conductivity [32]. The conductivity of a solution depends on both the ionic strength and the mobility of the ions. Therefore, buffers with large organic ions have a lower conductivity compared to buffers with small inorganic ions like sodium and chloride. The loss of the \(^1H\)-sensitivity of an inverse detection cryogenic probe at 500 MHz is only about 10% for samples buffered with 200 mM TRIS [32]. Because the sample resistance is proportional to the square of the frequency, the loss of sensitivity due to solution conductivity is less when nuclei of low resonance frequency such as \(^{13}C\) are detected [33], see also Fig. 9 in Section 3.2.
- By using a smaller solvent volume the noise contribution of the sample is reduced. In a 3 mm instead of a 5 mm tube, the solvent volume and consequently the noise contribution, are reduced by a factor of 2.7. With this approach one should also take into account the reduction of the sample amount, if the molar concentration for both sample diameters is identical.
- High conductivity solutions create a current in the sample. This current which flows in the \(XY\)-plane of a sample can be ‘broken’ by a compartmentalisation of the sample tube [34]. As this approach at the same time reduces the sample volume and is impractical for samples with high viscosity (like aqueous protein solutions) it seems to be of little practical use.

- Asymmetric geometry of the sample tubes and possibly also a coil tailored to yield an optimal filling factor improves theoretically the quality factor, \(Q\)-factor, of a coil and reduces the noise contribution from the sample [35].

To summarise, when working with high ionic concentrations, low conductivity buffers and/or sample tubes with smaller diameters should be favoured.

### 2.4. The cryogenic cooler and probe

The sensitivity gain obtainable by cryogenic cooling of the NMR probe was first demonstrated 20-years-ago [2] when a sensitivity improvement by a factor of eight for the \(^{13}C\)-signal at 45.9 MHz \(^{13}C\)-frequency was achieved. The probe was cooled with liquid helium and nitrogen by this approach. However, it took a long time for cryogenically cooled probes to become commercially available. The reason for this is the high engineering demands. Instead of liquid cryogens the modern cryogenic probes use cold helium gas, which requires an efficient thermal insulation. In addition to the probe itself, the cryogenic accessory consists of a cryogenic cooling unit and a helium compressor. The cooling unit supplies the entire infrastructure for the operation of the cryogenic probe and also continuously monitors the operation of the probe. These units form a closed-loop cooling system, cf. Fig. 2, where helium gas is compressed in one chamber and then chilled through expansion in a second chamber. This process of heat exchange follows the ideal gas law. This is rewritten in Eq. (4) to indicate the temperature change when pressure

![Fig. 2. Schematic presentation of the closed-loop cryogenic cooling system consisting of the cooling equipment, helium compressor and the cryogenic probe.](image-url)
and volume change:

\[ T = \frac{PV}{nR} \]  

Here \( P \) is the pressure, \( V \) is the volume, \( n \) is the number of moles of gas, \( R \) is the gas constant and \( T \) is the temperature. By this procedure a second helium Alux is cooled by means of a heat exchange. The cold helium is transferred to the probe assembly by means of a vacuum-insulated transfer line. The probe is a well-insulated Dewar system, housing both the receiver coil and preamplifier. These are fitted in one assembly in order to keep all the RF-connections between coil and preamplifier cold, so that additional thermal noise that might be introduced to the detector system is kept minimal. Typical temperatures for the receiver coil and the preamplifier in the cryogenically cooled probes are 20 K and 80 K, respectively. The spectrometer is operated in a routine manner while the cooling system works autonomously. The cool-down and warm-up procedures are initiated by push buttons or over controlling software, and they are completed in about 3 and 2 h, respectively. Fig. 2 schematically presents the different hardware units necessary for the operation of a cryogenic probe, and Fig. 3 shows a NMR instrument operating at 700 MHz equipped with a cryogenic probe.

3. Applications

3.1. Biomolecular NMR

Cryogenic probes substantially increase the signal-to-noise ratio in NMR spectra. In general, these probes offer an advantage when (i) the sample concentration is very low, (ii) NMR experiments with an intrinsic low sensitivity have to be performed or (iii) the experiment time needs to be reduced. In the research of biological macromolecules all three points are paramount. The sample concentration is often limited because of (i) low quantities available, for instance, as a consequence of low expression, (ii) intrinsic low solubility and aggregation, or (iii) oligomerisation at higher concentrations. Sometimes \(^{13}\)C- or \(^{15}\)N-labelling is not feasible, in such cases it might be possible to record heteronuclear three-dimensional experiments at natural abundance by using cryogenic probes. A large repertoire of NMR experiments for the resonance assignment and collection of conformational constraints of biomolecules exists, but the sensitivity of these varies highly and declines with increasing molecular weight of the sample.

3.1.1. High throughput structure determination

An approach for high throughput NMR-structure determination of larger proteins relying on cryogenic probe technology in combination with selective labelling techniques has been proposed by Fesik and co-workers [36]. The overall structural fold of the 180-residue antiapoptotic protein, Bcl-xL, was determined using data collected in only 4 days. Restraints solely from the hydrophobic core that are important for the overall fold of the protein were used. This was realised through a labelling strategy featuring protonated \(^{13}\)C-methyl groups of Val, Leu and Ile (delta1) residues and protonated aromatic rings of Phe and Tyr residues in a uniformly deuterated and \(^{15}\)N-labeled background. To improve the resolution of the calculated structure, restraints derived from a set of residual dipolar couplings (RDC) were subsequently included.
The additional RCD data were acquired in 3 days using a cryogenic probe at a 500 MHz field.

The efficiency of protein structure determination by NMR can be significantly augmented if rapid data collection is combined with automated spectral processing and analysis, as was shown by Montelione and co-workers [37]. By using cryogenic probes and integrated software the authors demonstrated that the backbone resonance assignment and the identification of the secondary structure elements could be done for small proteins optimally in less than 6 h. A set of six common triple-resonance experiments was recorded on 0.9 mM 59-residue BPTI at a 500 MHz 1H-field. The data were recorded with experimental settings optimised for short measurement times and 1 scan only. Based on a comparison of a cryogenic and a conventional probe the authors estimate that the timesaving in data collection is 10-fold for aqueous solutions at moderate ionic strength. For a visual comparison of the performance of the conventional and cryogenic probes the HN–C projections of HN(CO)CACB together with a representative trace through the spectrum are displayed in Fig. 4(A) and (B), respectively (courtesy of Monleón et al. [37]). In these spectra, the average peak enhancement by using cryogenic technology was found to be 3.3.

3.1.2. NMR in structural genomics

As abundant genomics sequence information becomes available structural characterisations of all the gene products are being undertaken in order to better understand the complexity and diversity of life at the molecular level [38–41]. To meet this goal proteins can be clustered according to their sequence homology, a typical threshold being 30% sequence identity [42]. The determination of one structure from each uncharacterised protein family gives about 16,000 non-membrane targets to the structural genomics initiative [43]. Based on the number of already deposited structures [42] the contribution by NMR can be expected to be 15–20%. Structural proteonomics aims to structurally characterise all proteins in a single organism in an effort to contribute to the understanding of protein functions [41]. The two techniques for secondary and tertiary structure elucidation, X-ray crystallography and NMR-spectroscopy, can be employed in a complementary fashion [44]. For two thirds of the NMR-structures deposited in Protein Data Bank a corresponding X-ray structure is not available [45]. Having a well-diffraction crystal and by using a synchrotron and MAD phasing techniques for the data collection, the determination of an X-ray crystal structure can be completed in one day [46,47]. By using NMR the crystallisation process is not needed, which can result in significant timesaving [45]. In addition, the foldedness of the target proteins can be rapidly determined by 1H–15N correlation NMR-spectra [44, 45,48]. This allows one to quickly identify samples suitable for full structural determination.

In response to the demands of structural genomics NMR spectroscopy is being optimised for high throughput operation [45,48]. In this respect the launch of the cryogenic
probe technology was a most significant and very timely hardware development. For NMR to contribute to high throughput structure determination, the collection, processing and analysis of data must be fast and automated [45]. Provided that the sensitivity is no longer the limiting factor for the data collection, it becomes topical to minimise the time needed for the data sampling process—NMR spectroscopy moves from sensitivity-limited to sampling-limited regime [49,50]. Several schemes for fast multidimensional NMR data sampling have been recently proposed (for an review, see [51]), and common to all these methods is the assumption that the instrumental noise can be neglected. Significant progress has also been made in the development of integrated software for semi-automated or automated analysis of NMR data and protein structure calculations [52–57]. Well resolved, high signal-to-noise data is a prerequisite for the successful application of these methods.

3.1.3. Biomolecular NMR with 13C and 15N at natural abundance

Isotope labelling of protein, DNA and RNA samples is usually necessary for successful heteronuclear NMR. This is either because low sample concentration results in low sensitivity or because the intrinsic insensitivity of some of the experiments which rely on magnetisation transfer over the 13C and 15N heteronuclei. The high sensitivity of cryogenically cooled probes, however, allows one to run experiments that formerly required full isotope labelling, on biomolecules containing one heteronucleus either at natural abundance or only partially labelled. This has several advantages: (i) working at natural abundance will reduce the overall cost as isotope enrichment may be expensive; (ii) initial investigations can be done with only uniformly 15N labelled protein, (iii) isotope labelling of DNA and RNA fragments can be avoided, this is often difficult as it is done through synthesis. Because of the higher natural abundance and intrinsic sensitivity of 13C, studies of natural abundance 13C are preferred over 15N.

The sensitivity of any pulse sequence is determined by the gyromagnetic ratios γe and γo of the excited and the observed nucleus, through γeγoω0, the abundance of the nuclei involved in the pulse sequence, and their relaxation rates. In inverse experiments the excited and observed nucleus is identical—the proton—while the gyromagnetic ratios of the heteronuclei do not influence the sensitivity. Thus, the sensitivity does not depend on the gyromagnetic ratio of 15N in the HSQC experiment or on the gyromagnetic ratios of 13C and 15N in a triple resonance experiment like HNCO. As a consequence, if relaxation effects are neglected, the 15N-HSQC and HNCO experiments have identical sensitivity for 100% 13C and 15N isotope labelled sample. Further, for a sample with 100% 15N isotope enrichment but 13C at natural abundance, the 13C-HSQC and HNCO experiments will, again, have identical sensitivity, notwithstanding relaxation effects. Thus certain backbone triple resonance experiments can be run with 13C at natural abundance. Here it is important to notice that relaxation effects can drastically reduce the sensitivity of triple resonance experiments, in this case, isotope enrichment of both 13C and 15N might still be required. Experiments that rely on magnetisation transfer between carbon nuclei, however, require uniform 13C labelling. Some examples of these are the protein backbone experiment HN(CO)CA and experiments for assignment of protein side chains such as HCCH-TOCSY and CBCA(CO)NH.

Already before the introduction of the cryogenically cooled probe technology, NMR experiments had been demonstrated on 15N-labelled protein samples with 13C natural abundance, such as 2D HNCO [58] and a dipolar coupling based strategy for the assignment and structure determination [59]. Further applications include protein ligand binding studies using 15N–HSQC experiments with 15N at natural abundance [60], and triple resonance experiments with 15N, 10–20% 13C labelled samples [61]. Recently, a simultaneous HNCA/HNCO experiment optimised for 15N labelled samples has been introduced [62]. This experiment works well even on reasonably dilute protein samples provided a cryogenically cooled probe is used.

The sensitivity of some inverse experiments recorded on non-labelled or singly labelled protein samples using cryogenically cooled probes is highlighted in Figs. 5–7. A 15N–HSQC spectrum of 2 mM lysozyme with 15N at natural abundance is shown in Fig. 5. This spectrum was recorded in 20 m at 600 MHz. Such a high sensitivity allows the initial screening of protein samples for their foldedness before producing isotope labelled samples, or the study of

![Fig. 5. 15N-HSQC spectrum recorded with 15N at natural abundance. The sample was a 2 mM solution of unlabeled lysozyme in 90% H2O/10% D2O, examined using a 600 MHz triple resonance CryoProbe. The data were collected with eight scans and 128 t1-increments yielding a total experiment time of 20 m.](image)
protein–ligand binding with non-labelled proteins. Triple resonance experiments that are not strongly prone to relaxation losses during the pulse sequence, such as HNCO and HNCA, can be recorded on $^{15}$N-labelled protein samples with $^{13}$C at natural abundance. Fig. 6 shows a HNCA spectrum of 2 mM $^{15}$N-labelled ubiquitin. The spectrum was recorded at 800 MHz using a cryogenic triple resonance probe in only 5 h. Even more impressive is the spectrum of Fig. 7 showing the $^{13}$C-plane of the HNCO experiment of 1 mM $^{15}$N-labelled ubiquitin recorded at 800 MHz using a cryogenic triple resonance probe. Within ten minutes of accumulation the spectrum already shows practically all the correlations.

3.1.4. Collection of conformational constraints

NMR structure determination of proteins and nucleic acids was originally based on the extraction of a sufficient number of interproton distances from the $^1$H–$^1$HN O E interactions and torsion angle constraints from the J-couplings [63]. More recently, several techniques have been introduced to obtain additional conformational restraints for biomolecular structure. These include the determination of the internuclear vector orientations with respect to a molecular frame from residual dipolar couplings in nematic media [64,65], pair-wise internuclear vector orientations from cross-correlated relaxation [66] and hydrogen-bond restraints from coupling over hydrogen bonds [67]. Common to these techniques is that the instrument sensitivity may become crucial for the determination of the constraints. Below two studies that were carried out by using cryogenic probes are described.

Residual dipolar couplings between nuclear spins in partially aligned media provide global conformational information, as they depend on the orientation of the internuclear vectors with respect to an order frame [68–70]. Use of the residual dipolar couplings (RDC) as conformational constraints allows for structure refinement with higher accuracy, for structural NMR studies of large systems, and for rapid determination of low-resolution folds. Residual dipolar couplings combined with the high sensitivity of the cryogenic probes can also rapidly yield low-resolution information about protein structure, provided that the polypeptide backbone assignments are available. Hence, their use in structural genomics for fast annotation of gene products through structural homology has been proposed [71–73]. Other applications of residual dipolar couplings include, for instance, docking of protein–protein or protein–ligand complexes [74,75].

Residual dipolar couplings are usually extracted from the frequency difference of the multiplet components, which are possibly recorded in two separate spectra to alleviate assignment issues [76]. As the residual dipolar couplings are typically small, their accurate determination may be jeopardised by broad line-widths particularly observed for large proteins and at higher fields. The interaction is inversely proportional to the cube of the internuclear distance, making residual dipolar couplings over two or more bonds considerably smaller than those over one bond [65,77,78,79]. The interaction is also directly proportional to the magnetogyratic ratios of the coupled spins, making

![Fig. 6. 3D HNCA spectrum recorded with $^{13}$C at natural abundance. The sample was 2 mM $^{15}$N-labelled ubiquitin in 50 mM phosphate buffer at pH 5.8. The data were collected on a 800 MHz triple resonance CryoProbe, total experiment time being 5 h.](image)

![Fig. 7. First $^1$H, $^{13}$C plane of a HNCO experiment recorded with $^{13}$C at natural abundance. The sample was 1 mM $^{15}$N-labelled ubiquitin in 50 mM phosphate buffer at pH 5.8. The spectrum was recorded on a 800 MHz triple resonance CryoProbe, the experiment time being 10 m.](image)
the determination of residual dipolar couplings for CC-pairs more challenging than that for HN-pairs [80]. Recently, Bax and co-workers [80] proposed an alternative method for the RDC determination based on quantitative J correlation [81]. This method relies on the determination of the amplitude ratio of the signals rather than on the quantification of their separation. Residual dipolar couplings between alpha-13C and carbonyl-13C were determined accurately and precisely for the 140-residue micelle-associated α-synuclein at 0.5 mM concentration, this is a protein characterised by a long rotational correlation time, $\tau_r = 15$ ns [80]. Triple resonance cryogenic probes operating at 500 and 600 MHz were used to record the necessary TROSY-HN-CO-CA-QJ and TROSY-HNCO-QJ spectra.

Cross-correlated relaxation is the interference of the relaxation mechanisms of two nuclear vectors leading to a non-exponential relaxation [82]. Cross-correlated relaxation rates depend on the relative orientation of the nuclear vectors involved and can be exploited as structural constraints for torsion angles [66,83]. Cross-correlated relaxation rates promise to be particularly useful in improving the accuracy of NMR-derived RNA structures [84]. Recently, a method was proposed for converting cross-correlated relaxation rates through a straightforward parameterisation into structural constraints for the glycosidic torsion angle in 13C, 15N-labelled RNA hairpin loops [85]. Cross-correlated relaxation can also be exploited to obtain the torsion angle over the hydroxyl bond in unlabeled carbohydrates [86,87]. These are cases where other structural constraints are scarce. A particular advantage of the cross-correlated relaxation is that the magnetisation transfer efficiency increases with molecular size because it depends mainly on zero-frequency spectral density terms [88]. Recently, a method was proposed for converting cross-correlated relaxation rates through a straightforward parameterisation into structural constraints for the glycosidic torsion angle in 13C, 15N-labelled RNA hairpin loops [85]. Cross-correlated relaxation can also be exploited to obtain the torsion angle over the hydroxyl bond in unlabeled carbohydrates [86,87]. These are cases where other structural constraints are scarce. A particular advantage of the cross-correlated relaxation is that the magnetisation transfer efficiency increases with molecular size because it depends mainly on zero-frequency spectral density terms [88]. Buck and co-workers [90] have used this approach to monitor the hydrogen bonding pattern in an anti-parallel β-sheet of a 1 mM triple-labelled plexin-B1, a 13.5 kDa protein. Fig. 8 shows two representative strips from an HNCO measurement for detection of H-bonds and a reference HNCO spectrum recorded by a cryogenic triple resonance probe operating at 600 MHz. The hydrogen bond data were used to confirm the proposed protein fold [91].

3.2. 13C-detection in biomolecular NMR

13C-detection for biomolecular applications is a topic that is gaining momentum because of the availability of cryogenic probes optimised for 13C-detection. The inherently lower sensitivity of 13C is due to the $\gamma^2/\gamma$ dependence of the signal intensity on the magnetogyric ratio of the detected nucleus. The magnetogyric ratio of 13C is one fourth that of 1H, which results in a factor of eight lower sensitivity for the 13C-detected experiments. In the past, low sensitivity and low natural abundance of 13C have limited 13C-observation mainly to applications in structure elucidation of low-molecular-weight substances available in large amounts. The 13C-detection in NMR-investigations of biomolecular structure has traditionally been confined to the study of paramagnetic proteins, where 1H-detection fails...
because of deleterious line-broadening near the paramagnetic centre. However, diamagnetic proteins and nucleic acids with high molecular weight also show signal loss due to rapid relaxation hampering NMR studies based on $^1$H-detection. Hence, $^{13}$C-detection has recently been proposed as an experimental approach for the investigations of large deuterated proteins, to circumvent low proton density due to partial or uniform replacement of non-exchangeable protons and fast transverse relaxation due to slow molecular tumbling [92]. Another potential application for $^{13}$C-detected methods is in the field of nucleic acid research where the signal dispersion is rather poor and becomes a major hindrance even for molecules of moderate size.

Provided that the lack of sensitivity can be overcome, $^{13}$C-detection has several advantages that render it a useful complement to $^1$H-detection. In addition to (i) favourable relaxation behaviour there is (ii) high dispersion of $^{13}$C-chemical shifts and (iii) detection of non-protonated carbons such as carbonyls, (iv) absence of large solvent or buffer peaks and (v) high salt tolerance. The introduction of cryogenic probes with high $^{13}$C-sensitivity is a key development for the practical realisation of $^{13}$C-detected experiments. In Fig. 9, the sensitivity specification for $^{13}$C-observe probes is depicted as a function of the year when a probe was first introduced at a particular magnetic field. The magnetic field, expressed as the $^1$H operating frequency, is indicated above the marker. Black dots denote the $^{13}$C-sensitivity of conventional probes that use the inner coil for $^{13}$C-detection. In Fig. 9, the specified $^{13}$C-sensitivity for two kinds of cryogenic probes is shown. Triangles indicate that of the so-called dual cryogenic probes that use the inner coil for $^{13}$C-detection and the outer coil for $^1$H-pulses. In addition, to meet the demands for more $^{13}$C-sensitivity without the need to change to a dedicated $^{13}$C-probe, a cryogenic triple resonance probe has been built. This probe has a proton inner coil and a heteronuclear outer coil, and cryogenically enhanced preamplifiers for both $^1$H- and $^{13}$C-detection. In Fig. 9 the $^{13}$C-sensitivity of these probes is denoted by diamonds.

Cryogenic probes optimised for $^{13}$C-detection were initially employed for biomolecular NMR by Dötsch and co-workers [93]. The authors presented a modified version of the 3D out-and-back HCACO experiment, eliminating the INEPT step that transfers magnetisation from the alpha-carbon back to the alpha-proton. As these two nuclei have short transverse relaxation times, to avoid their coherences in the transverse plane may partially compensate for the sensitivity losses due to direct $^{13}$C-detection. The authors applied post-acquisitional deconvolution methods to collapse splittings due to $^{13}$C-$^{13}$C couplings in the acquisition dimension [93]. For the side chain assignments a $^{13}$C-detected modification of 3D HCC-TOCSY was used [94]. In both experiments acceptable sensitivity was demonstrated for 17 and 14 kDa monomeric proteins at 0.6 and 0.8 mM concentrations, respectively. One of the advantages of $^{13}$C-detection is the relative insensitivity to high conductivity. Dötsch and co-workers [33] reported a comparison of the relative $^1$H- vs. $^{13}$C-sensitivity as a function of salt concentration, this is presented in Fig. 10. The measurements were done on a cryogenic probe dedicated for $^{13}$C-detection operating at 500 MHz $^1$H frequency. (Courtesy of Shimba et al. [33]. Reprinted with permission from Springer Science and Business Media, the Netherlands).

A novel strategy for the backbone assignment combining $^1$H-detected and $^{13}$C-detected experiments was proposed by Pervushin and Eletsky [92]. For such a strategy the cryogenic triple resonance probe with enhanced $^1$H- and $^{13}$C-sensitivity would be the probe of choice. These workers...
presented a new $^{13}$C-detected experiment, MQ-HACACO, which can be performed either as starting on $^1$H-magnetisation in the case of fractionally deuterated proteins or as starting on $^{13}$C-magnetisation in the case of perdeuterated proteins. This experiment connects the C-alpha and carbonyl-C resonance frequencies that are obtained from the two most sensitive triple resonance experiments, TROSY-HNCO and TROSY-HNCA. The applicability of this new assignment strategy was demonstrated for a 44 kDa trimeric $^{15}$N, $^{13}$C and 35% $^2$H-labelled 1 mM protein sample [92]. The same workers subsequently presented an extension of this strategy for fractionally deuterated protein samples again utilising both $^1$H- and $^{13}$C-detected experiments. In order to alleviate the possible remaining ambiguity of the C-alpha resonance assignment, two new $^1$H-detected experiments, 3D TROSY-HN(CA)HA and 4D TROSY-HACANH, were combined with the earlier mentioned $^{13}$C-detected HACACO experiment [95]. All the experiments were recorded with a cryogenic triple resonance probe optimised for $^1$H-detection operating at 600 MHz. The authors anticipate a potential for sequential resonance assignment for proteins up to 100 kDa by using this approach.

Subsequently, $^{13}$C-optimised cryogenic probes were used to obtain a nearly complete resonance assignment of the side-chain heteronuclei in the same deuterated 44 kDa trimeric protein [96]. A feature of these investigations is that deuteration equally affects the longitudinal and transverse relaxation times of $^{13}$C, the former being advantageous for the $^{13}$C-linewidth, but the latter making the $^{13}$C $T_1$-relaxation and thus the recycle delays between the scans unfavourably long. Pervushin and co-workers demonstrated that by adding a minor amount of a paramagnetic relaxation agent a compromise between these two effects can be found, which enables the optimal sensitivity per unit measuring time [96]. Another complicating feature of the $^{13}$C-observe spectra of labelled samples is that the numerous homonuclear $^{13}$C-$^{13}$C couplings are resolved in the acquisition dimension. To avoid the homonuclear splittings, the authors proposed a constant time version of the $^{13}$C-$^{13}$C-TOCSY experiment [96]. The constant time version is, however, about two times less sensitive than the real time version. The real time $^{13}$C-$^{13}$C-TOCSY is, in fact, the most sensitive $^{13}$C-$^{13}$C experiment up to molecular weights where the $^{13}$C-linewidth becomes comparable to the $^{13}$C-$^{13}$C coupling constant of about 35 Hz [97]. Both experiments are depicted in Fig. 11, the constant time experiment is displayed above the diagonal and the real time version below. For the collection of conformational constraints $^{15}$N-TROSY-NOESY of a 35% deuterated sample using a 600 MHz cryogenic probe and $^{13}$C-resolved NOESY at 900 MHz using a conventional probe, were recorded. The NOE spectra were analysed in a semi-automatic manner based on the over 90% complete set of heteronuclear assignments obtained from the $^{13}$C-experiments [96].

Fig. 11. 2D constant time $^{13}$C-$^{13}$C-TOCSY spectrum (above diagonal) and 2D real time $^{13}$C-$^{13}$C-TOCSY spectrum (below diagonal) of the uniformly $^2$H,$^{13}$C,$^{15}$N-labeled trimeric 44 kDa enzyme chorismate mutase from Bacillus subtilis. The measuring time for the constant time experiment was 96 h and that of the real time experiment was 36 h. (Courtesy of Eletsky et al. [96]. Reprinted with permission from Springer Science and Business Media, the Netherlands).

The numerous homonuclear $^{13}$C-$^{13}$C couplings tend to jeopardise the analysis of $^{13}$C-detected spectra of $^{13}$C-enriched samples. In an attempt to make use of these couplings Vögeli et al. [98] analysed a number of homonuclear $^{13}$C-$^{13}$C doublets and quadruplets in $^{13}$C-$^{13}$C-TOCSY spectra of uniformly deuterated ubiquitin. The authors were able to extract an extensive set of residual dipolar couplings to be used as structural constraints, excluding those from the highly mobile side chains. In this study, a cryogenic $^{13}$C-observe probe operating at 500 MHz was used for the measurements of aqueous and aligned samples of 1.4 mM ubiquitin. The authors showed that the set of RDCs correlates well with the known 3-dimensional structure, and also with independently determined backbone $^1$H–$^{15}$N RDCs. Thus, the alignment tensor can be determined using the $^{13}$C–$^{13}$C splittings [98].

3.2.2. Paramagnetic proteins

Direct detection of $^{13}$C enhanced by cryogenic technology becomes particularly interesting in NMR studies of paramagnetic metalloproteins where proton signals up to tens of Å from the paramagnetic centre can be broadened beyond detection. Bertini and co-workers [99,100] were able to monitor $^{13}$C-signals as close as 4 Å to a copper(II)ion by using the 2-dimensional $^{13}$C-experiments COCA-MQ, $^{13}$C-$^{13}$C-COSY and $^{13}$C-$^{13}$C-NOESY. The advantages of $^{13}$C-observe are pronounced; because the paramagnetic contribution to nuclear relaxation depends on the square of the gyromagnetic ratio of the observed nucleus, going from
$^1$H to $^{13}$C decreases the relaxation rate by a factor of 16. One of the experiments used was $^{13}$C–$^{13}$C NOESY [100]. Fig. 12 displays CO–$^{13}$C correlations in 2D $^{13}$C–$^{13}$C-NOESY experiments for the dimeric 32 kDa protein superoxide dismutase (SOD). The experiments were recorded with mixing times of (A) 300 ms and (B) 800 ms. In panel (C) the 800 ms mixing time spectrum is reprocessed through deconvolution of the homonuclear splittings. The NOE transfer between $^{13}$C-spins circumvents the problems of transverse relaxation during long scalar transfer times and, in fact, it becomes more efficient with increasing molecular size and magnetic field. A comparison of the magnetisation

Fig. 12. The CO–$^{13}$C region from 2D $^{13}$C–$^{13}$C-NOESY spectra recorded on a dimeric superoxide dismutase of 32 kDa using a $^1$H and $^{13}$C-optimised cryogenic triple resonance probe operating at 500 MHz $^1$H-frequency. The three panels show spectra recorded with (A) 300 ms mixing time, (B) 800 ms mixing time and (C) 800 ms mixing time and maximum entropy reconstruction to remove the CO–$^{13}$C splittings. (Courtesy of Bertini et al. [100]. Reprinted with permission from American Chemical Society).

Fig. 13. $^{13}$C–$^{13}$C-COSY spectrum recorded at 500 MHz using a CryoProbe dedicated for $^{13}$C-detection. The sample was 4 mg (~2 mM) $^{15}$N–$^{13}$C-labelled RNA 14-mer, 5’ GGCAUUCCGUGCC 3’ (produced by Silantes), in H$_2$O/D$_2$O. The signal regions of the different ring carbons are indicated.
transfer efficiency in the $^{13}$C–$^{13}$C-NOESY and $^{13}$C–$^{13}$C-TOCSY experiments by Fischer et al. [97] shows that the transfer in the two experiments can be expected to become about equally strong for diamagnetic proteins with molecular weights of 20 kDa examined at 600 MHz.

3.2.3. Oligonucleotides

The $^{13}$C-detected NMR-methods boosted by the cryogenic probe technology are potentially a useful complement to the traditional $^1$H-detected approach for the heteronuclear resonance assignment of oligonucleotides. The low proton density in the nucleotide bases makes the $^1$H-detected NMR experiments of limited utility for the assignment of the base nuclei. Additionally, the low chemical shift dispersion of sugar protons severely complicates the assignment of the ribose protons. Direct $^{13}$C–$^{13}$C and $^{13}$C–$^{15}$N correlated experiments can facilitate these tasks. To demonstrate this, the heteronuclei in a $^{15}$N, $^{13}$C-labelled 14-mer RNA hairpin were assigned by relying solely on 2-dimensional $^{13}$C-detected correlation experiments, using cryogenic probes at magnetic fields of 11.7 and 14.1 T (corresponding to 500 and 600 MHz proton frequency) [101]. The $^{13}$C–$^{13}$C-TOCSY experiment [96] was used to assign the $^{13}$C-signals from the fourteen ribose rings, cf. Fig. 13. At 11.7 T this experiment was recorded with band-selective homonuclear $^{13}$C-decoupling during acquisition in order to enhance the resolution through spectral simplification [102]. The heteronuclear resonance assignment within the individual nucleotide bases was obtained from $^{13}$C–$^{13}$C-COSY combined with $^{13}$C–$^{15}$N-HSQC spectra, cf. Fig. 14. The latter experiment also yielded correlations between the base nitrogens and sugar carbons of each nucleotide. In addition, sequential connectivities between the nucleotides were observed by means of $^{13}$C–$^{31}$P-HSQC experiments, cf. Fig. 15, recorded by using a cryogenic triple resonance probe equipped with $^{31}$P nucleus. Thus, this set of $^{13}$C-detected experiments allowed a direct and unambiguous assignment of the majority of the heteroatoms and the identification of the individual bases, sugars and their sequential order in the 14-mer RNA hairpin [101].

3.3. NMR screening and ligand binding

One of the very first applications where the cryogenic probe technology had a significant impact was high-throughput NMR-based screening in the pharmaceutical industry. In the initial stage of drug discovery protein targets are screened against large libraries of low-molecular-weight compounds in order to identify potential ligands. High affinity binders are selected as lead compounds in order to be optimised further through medicinal chemistry or structure-based drug design. Lower affinity ligands ($k_d$ ranging 10–1000 μM) with favourable physical properties may also be of interest, and these can be readily detected by NMR-screening [103]. A number of protocols for NMR-based screening exist [103–106].

3.3.1. Heteronuclear screening for drug design

The cryogenic probe technology was adopted early on by Fesik and co-workers at Abbott Laboratory [107] to accelerate the throughput in the screening approach called SAR by NMR, structure-activity relationships by nuclear magnetic resonance. In this method molecular fragments that interact at different sites on the protein are found and subsequently linked to form a single molecule with heightened specificity and affinity. The practical part of this strategy involves the acquisition of 2-dimensional $^1$H–$^{15}$N correlation spectra of a $^{15}$N-labeled protein target to monitor shifting of the NH signals corresponding to the protein backbone amides that accompanies protein-ligand interactions. As molecular interaction takes place on the protein surface, the subset of amide NH signals close to

Fig. 14. $^{15}$N–$^{13}$C-HSQC spectrum recorded at 600 MHz using a triple resonance with enhanced $^{13}$C-detection. The sample was 4 mg (~2 mM) $^{15}$N–$^{13}$C-labelled RNA 14-mer, 5′ GGCACUUCGGUGCC 3′ (produced by Silantes), in H$_2$O/D$_2$O. The regions containing the resonances of the different nucleotide bases are indicated.
the binding site shift due to alterations in their chemical environments whereas the amide NHs further away remain unaffected. The method provides information on both the affinity and the location of the binding site.

The advantages of heteronuclear screening by NMR are that it allows a straightforward determination of site-specific binding information and detection of low-affinity ligands. Its usefulness is, however, limited by the necessary relatively large substance concentrations and long measuring times. Cryogenic probes make it possible to record the $^{1}\text{H}--^{15}\text{N}$ correlated spectra in a few minutes on a $^{15}\text{N}$-enriched sample at the concentration of 50 $\mu$M and at 500 MHz $^{1}\text{H}$ frequency. Fesik and co-workers demonstrated that up to 100 low-molecular-weight test compounds from a compound library may be added to the protein solution and examined at one time as a mixture with a total concentration of 5 mM [107], while retaining the specificity and reliability of the screening method. Using this approach combined with cryogenic probes the throughput of the assay could be enhanced 10-fold, such that compound libraries of up to 200,000 compounds can be screened in one month.

The sidechain methyl groups rather than backbone amides can also be used in NMR-based screening. This was shown by Hajduk and co-workers [108] who combined cryogenic probe technology with selective $^{13}\text{C}$-labelling of the methyl groups of the same protein [108] and screening by recording $^{1}\text{H}--^{13}\text{C}$-correlated spectra. The advantages of monitoring the methyl groups are the three-fold stronger signal because of the three methyl-protons, favourable relaxation and often increased chemical shifts because of the methyl group proximity to the ligand. In another study, NMR-screening was employed to identify weakly binding ligands for protein targets with unknown biological function [109]. The screening library consisted of a diverse set of substrates and cofactors that are known to bind to different proteins and enzymes. A total of 160 compounds were tested against the target protein HI-0033 from Haemophilus influenzae by monitoring perturbations in $^{1}\text{H}--^{15}\text{N}$-HSQC spectra, using cryogenic probes at 500 MHz. This investigation showed that NMR-based screening can potentially assist in the discovery of biological function of gene products in functional genomics.

### 3.3.2. Ligand binding detection through saturation transfer difference

Whereas heteronuclear screening is based on monitoring changes in the chemical shifts of the target protein, ligand-based binding studies focus on the changes in the NMR-observable properties of the ligand upon interaction. These may include changes in NOE interactions, chemical shifts, relaxation or diffusion properties [104]. In the saturation transfer experiment (STD) of Mayer and Meyer [110], a protein signal is first selectively irradiated, whereafter this saturation rapidly spreads through spin diffusion to all...
protein hydrogens. It is also transferred to a tightly bound ligand. The saturation signal is detected on the free ligand as this returns back to solution through exchange. The broad signals from the protein are eliminated by subtracting a reference spectrum without saturation or through relaxation. Only the sharp signals of the ligand due to saturation transfer from protein are detected. The STD method can be incorporated into any experiment such as TOCSY or HSQC \[104,110\]. Nevertheless, often a 1-dimensional spectrum suffices to identify the ligand protons that have been in contact with the protein.

Three main factors influence the saturation transfer efficiency from protein to ligand, namely a long irradiation time, an appropriate dissociation constant and large excess of ligand \[104\]. Since the decay time of the ligand saturation can be assumed to be similar to the ligand proton relaxation time, an irradiation time of about 2 s is commonly used. Reasonably high ligand dissociation constants (100 nM < $K_d$ < 10 mM) enable the saturation of many ligands at a single binding site. High excess of ligand (ligand:protein molar ratio of roughly 100:1) is favourable as it makes it unlikely that a saturated ligand re-enters the binding site. The STD experiment is extensively used to identify functional groups and epitopes responsible for ligand binding. Other applications of STD are in competitive binding studies \[111\] for compound library screening, in determination of dissociation constants ($K_d$) and ligand based screening of carbohydrate binding to proteins \[104\].

The STD experiment is a useful tool for investigating carbohydrate recognition by lectins, an important step in the immunocascade. An example of such a study is the characterisation of the polysaccharide motif present in glycoproteins on tumour cells that is recognised by the lectin wheat germ agglutinin, as reported by Lycknert and co-workers \[112\]. For high NMR sensitivity the STD experiments were recorded with a cryogenic probe operating at 500 MHz $^1$H frequency. The concentrations of the disaccharide ligand and the trimeric 36 kDa lectin (consisting of three slightly dissimilar sub-units) were 4.7 mM and 54 mM, respectively, giving a ligand:protein molar ratio of $\approx$ 86:1 \[112\]. The ligand excess with respect to the primary binding site was $\approx$ 22:1. A 1D STD spectrum with a saturation time of 5 s and T$_{1p}$-filter of 30 ms to remove the protein background, was recorded with 8192 transients, the total experiment time being 24 h, cf. Fig. 16. The identification and relative intensity of the STD signals in this spectrum allowed the mapping of the binding epitope on the disaccharide.

### 3.4. Metabonomics

NMR is used in multiple ways in metabonomics investigations. Liquid chromatography combined with NMR (LC-NMR) is a standard tool used to identify drug metabolites in biofluids \[113–121\]. For these applications sensitivity is critical. A second important area is the screening of biofluids. This is done in order to monitor toxicological effects of drug candidates, drug efficacy, disease recognition or for food screening \[122–137\]. In screening, speed is vital to allow hundreds of samples to be measured in a day. While 1D-spectra from screening are usually analysed by statistical methods to identify metabolites or metabolic changes, 2D-spectroscopy is employed for yet another metabonomics application, the structural identification of new biomarkers. Here heteronuclear inverse experiments become mandatory. They are needed to resolve the overlap of proton resonances, as biofluids like urine or plasma may contain more than 1000 NMR visible compounds. Since the concentration of indicative biomarkers can be very low (in the nanomolar range, that is), NMR sensitivity is critical also for this application. In addition, the screening based on examination of 1D spectra presently relies only on proton observation, and this suffers from substantial overlap problems. Carbon detection would be an attractive alternative because of the larger chemical shift range and the higher signal dispersion. The sensitivity of carbon at natural abundance is, however, two orders of magnitude lower than proton sensitivity. By using a cryogenic probe optimised for carbon detection, measurement times can be reduced substantially and medium speed screening based on 1D $^{13}$C-spectra of samples with a maximum of 500 μl of urine or plasma becomes possible \[138\].
3.4.1. Development of cryogenic flow probes for LC-NMR

While in direct biofluid screening the sample amount is typically not limited and 5 mm probes can be used successfully, the LC-NMR coupling method involves smaller detection volumes. The best compromise to fulfill both the requirements of on-flow, direct stop-flow and loop collection is a flowcell with an active volume of 60 μl. Such a flowcell can also be connected to 2 mm LC-columns. In the conventional probes, the detection coils are directly wound around the flowcell leading to an optimal filling factor. Using such a flowcell in a 5 mm cryogenic probe results in a much lower filling factor. Therefore, the need for a small inner diameter (i.d.) cryogenic probe with flow capabilities became acute.

The initial approach was to convert an existing 3 mm cryogenic probe into a dedicated cryogenic flow probe. With this probe (with an active volume of 40 μl), it was possible to increase S/N compared to a conventional 4 mm flow probe (120 μl active volume) by a factor of ~8, while having the same amount of sample in the active volume. This cryogenic probe connected to a on-flow LC-NMR/MS (mass spectroscopy) system was used to investigate a sample of human urine. The sample was obtained 4 h after dosage of 500 mg of paracetamol [139], one of the most thoroughly investigated drugs. Besides the already known metabolites, it was possible to identify a new metabolite, a methoxylated glucuronide of paracetamol. In previous tests with the same sample the new metabolite could be identified only after long stop-flow experiments, here it was possible to detect it by using only 16 scans per time slice in the on-flow mode. The exact position of the methoxy group was subsequently determined by a 2D-NOESY experiment.

3.4.2. LC-NMR combined with solid phase extraction

While the dedicated cryogenic flow probe was being tested, a new accessory to LC-NMR was introduced; post-column solid phase extraction (SPE) [140–142]. The flowpath of this set-up is shown in Fig. 17. The peaks eluting from the LC-column are first transferred to small solid phase extraction cartridges. During this transfer water is added to allow trapping of peaks even at high organic solvent ratio in reversed phase separations. After the peaks are trapped on the cartridges (2 mm i.d., 1 cm length), the solvent is removed by drying with nitrogen gas. After drying the peaks are removed from the SPE cartridge using completely deuterated organic solvent like CD$_3$CN or CD$_3$OD. This means that the chromatography can be done in completely non-deuterated solvents.

The elution volume from such a 2 mm cartridge is about 25 μl. The sample is thus considerably more concentrated than the eluting volume of the peak coming directly from the LC-column, which is typically between 100 and 300 μl depending on the LC-column diameter used. The higher concentration leads to higher NMR sensitivity. A single trapping step can lead to a S/N gain up to a factor of 4. Another advantage of the SPE approach is the possibility to perform multiple trappings, where the same sample is injected several times and the peaks of interest are collected on the same trapping cartridge. This results in a linear increase of the NMR S/N ratio with the number of trappings until the holding capacity of the cartridge is reached. Hence, if the single trapping gives a factor of 4 increase in NMR S/N, a triple trapping results in a 12-fold increase.

With an eluting volume of 25 μl it became necessary to have a flow cell with a correspondingly small active volume. Therefore, a 30 μl cryogenic flow probe was constructed. Typically an 8-fold S/N increase is observed in comparison to a 60 μl conventional flow probe, when transferring a peak from a SPE cartridge into either probe. For the LC-NMR reference sample of para-hydroxybenzoicacidpropylester the following results were obtained after injection of 5 mg of the ester on to a 2 mm reversed phase LC-column. The S/N ratio for the aromatic signals using different NMR probes at 600 MHz is reported.

(1) S/N after loop collection, conventional 3 mm flow probe (60 μl) 9:1.

![Fig. 17. The flowpath of the post-column solid phase extraction (SPE) that is used in LC-NMR.](image-url)
(2) S/N after single trapping on 2 mm cartridge, conventional 3 mm flow probe (60 μl) 23.5:1.
(3) S/N after single trapping on 2 mm cartridge, cryogenic flow probe (30 μl) 170:1.
(4) S/N after 4-fold trapping on 2 mm cartridge, cryogenic flow probe (30 μl) 660:1.

The power of the LC-SPE-NMR combination with the cryogenic flow probe is demonstrated on the example of dichlofenac metabolites in human urine. A sample was collected 4 h after a dosage of 50 mg dichlofenac to a woman. 100 μl of the urine were injected on to a 4.6 mm reversed phase column in a solvent gradient of acetonitrile/water. The peaks for SPE trapping were identified with on-line mass spectrometry and trapped on 2 mm i.d. SPE cartridges. The spectrum in Fig. 18 shows the dichlofenac O-glucuronide signals obtained after a single trapping and transfer from the cartridge. The spectrum was recorded with 128 scans and a line broadening of 1 Hz was applied. The spectrum in Fig. 18 indicates that the sensitivity is sufficient to run all 2D experiments needed for a structure elucidation. Using conventional LC-NMR and room temperature probes it is hardly possible to collect useful 1D-NMR data with such a low dose.

The flow probe with 30 μl active volume optimised for SPE is appropriate also for on-flow, direct stop-flow or loop collection with a 1 mm LC-column. Nevertheless, miniaturisation is progressing also in liquid chromatography, and the current range of cryogenic flow probes still covers only a small fraction of LC-applications. A further development of the SPE process has allowed the use of cartridges with a 1 mm i.d. and an eluting volume of only 10 μl. It can be seen that from the LC-NMR standpoint alone cryogenic flow probes with three different optimised volumes are needed. These are as follows.

(1) Conventional LC-NMR with on-flow, direct stop-flow and loop collection on 2 mm or larger i.d. LC-columns with 60 μl active volume.

3.4.3. Convertible cryogenic flow probes

Keeping in mind that there are also high throughput flow injection applications, the need for a larger i.d. transfer capillary emerges. For LC-NMR applications, the LC-peak shape has to be maintained during the transfer into the flow cell. This requires small i.d. capillaries (0.25 mm or smaller). For flow injection NMR, however, the emphasis is on high throughput. This can only be achieved through rapid transfer with flow rates up to 15 ml/min. Using a small i.d. capillary as is appropriate for LC-NMR, may create high back pressure and damage the system. Therefore, in flow injection NMR applications, the transfer capillary i.d. of 0.5 mm is customary.

It is obvious that a dedicated cryogenic flow probe only fulfils one of the above needs at a time. As the cost of a cryogenic probe and its infrastructure is higher than that of a conventional probe, a solution that allows changing the flowpart of the cryogenic probe was required. Even when using different dedicated cryogenic flow probes, the cooling and warming procedures between a probe change wastes expensive instrument time. As a solution to this problem, an insert was constructed that can be easily exchanged without having to warm up the probe. Thus, it became possible to change the mode of operation within less than 10 min, for example, from LC-SPE-NMR to flow injection NMR. The flow insert also allows the use of discrete NMR tubes.

One of the major challenges in the development of the convertible flow insert was to retain the sensitivity of the dedicated cryogenic flow probe. Fig. 19 shows a spectrum of...
2 mM sucrose in D$_2$O, a sample that is used to specify S/N on flow probes. The measurement was done at 600 MHz using a selective inverse $^1$H/$^{13}$C cryogenic probe and a flow insert with a 30 $\mu$l active volume. A S/N ratio of 218:1 was achieved on the signal from the anomeric proton with 1 scan and a line broadening of 1 Hz. Using the dedicated cryogenic flow probe with the same active volume a S/N of 216:1 was obtained.

Since both models of cryogenic probes used for flow experiments have two completely cold channels the performance on carbon should remain the same on going from a dedicated flow probe to a probe with exchangeable flow insert. At 600 MHz the ASTM $^{13}$C-sensitivity test yielded a S/N on the benzene signal of 370:1 with the 30 $\mu$l flow insert, while the dedicated flow probe gave a S/N of 320:1. With this carbon sensitivity it is possible to obtain carbon NMR data from LC-NMR runs, especially if SPE is used in addition.

The sensitivity of the cryogenic probe is highlighted by two results obtained in the flow injection mode of operation (using the 30 $\mu$l flow insert). It is noteworthy that the high throughput screening used in most metabonomics applications is also based on this mode of operation. A gradient COSY spectrum was acquired with a single scan on a sample of 1 $\mu$g of tryptophane in 30 $\mu$l D$_2$O. This corresponds to a 0.189 mM solution. Further, a $^1$H-decoupled carbon spectrum was acquired with the same flow insert on a sample of 20 $\mu$g of cinnamicacid-ethylester in 30 $\mu$l CDCl$_3$. This corresponds to a 3.79 mM solution. In 70 min a S/N ratio of $\sim$8:1 was obtained. This demonstrates the capabilities of the cryogenic flow probes to acquire...
spectra on the smallest amounts of sample in high throughput mode.

3.5. Structure verification of low-molecular-weight compounds

3.5.1. Structure determination of natural products

For the complete structure elucidation of small molecules it is necessary to obtain high-resolution $^1$H and $^{13}$C 1D-spectra as well as 2D homonuclear and heteronuclear chemical shift correlation spectra. For small amounts of natural products such experiments may take several days of measurement time, particularly if $^{13}$C-detection is mandatory. A cryogenic probe with cold preamplifiers on both $^1$H and $^{13}$C channels offers not only optimum sensitivity for proton detection and inverse experiments, but also enhanced sensitivity for the carbon-detected experiments. Figs. 20 and 21 illustrate that by using such a cryogenic probe directly observed $^{13}$C-spectra can be successfully recorded on sample concentrations in the mg regime. The sample used was 8.1 mg quinine (mw. 324 g/mol). The 1D $^{13}$C-direct observe and DEPT-135 spectra obtained are shown in Fig. 20 and the 2D $^{13}$C-$^{13}$C correlation INADEQUATE spectrum is shown in Fig. 21.

In an industrial central analytical laboratory (Discovery Technologies, Novartis Institutes for Biomedical Research, in Basel, Switzerland) the laboratory manager, Lukas Oberer, has reported a five-fold increase in the number of their structure elucidations of natural products per year since the installation of the cryogenic probe at 500 MHz. This is because smaller sample amounts suffice. For a set of 2D experiments consisting of COSY, HSQC, HMBC and ROESY, which are typically run for 4–8 h in total, 1 mg of substance is sufficient if the cryogenic probe is used. The available substance amount may be as low as 0.2 mg. In many cases $^{13}$C-detected experiments are mandatory. With cryogenic probe technology even the spectra from the notoriously insensitive $^{13}$C-$^{13}$C correlation experiment INADEQUATE can be recorded with a substance amount in the low mg range. Fig. 22 shows a 1D $^{13}$C-spectrum recorded on 0.3 mM of a 921-mw compound extracted from blue algae in DMSO-d$_6$, using a cryogenic probe dedicated for $^{13}$C-detection at 400 MHz. Experiment time was 13 h. (Courtesy of Oberer and von Elert [143]).

The chromatogram shown in Fig. 23 was obtained using UV-detection. Two peaks are highlighted. These were further investigated through SPE transfer to the NMR-spectrometer equipped with a cryogenic flow probe. The main peak was identified as the monoterpene carvacrol based on the NMR and MS information. Other identified peaks were several flavonoids, phenolic acid and rosmarinic acid. The 1D-proton spectrum of carvacrol obtained after triple trapping and transfer is shown in Fig. 24 (A). Here 20 μl of the extract at a time were injected to a 150 × 6 mm reversed phase column. The excellent S/N of the proton spectrum already indicates that direct carbon observation would also be feasible. The $^{13}$C-spectrum obtained in 50 min at 600 MHz, cf. Fig. 24(B), shows clearly all carbon signals in addition to the solvent signals of acetonitrile.

3.5.2. Investigations using cryogenic flow probes

Natural product extracts are often investigated by NMR in the search for possible new drugs. This is another area where LC-NMR plays an important role. As an example, an acetone extract of oregano was investigated by LC-SPE-NMR/MS.

The chromatogram shown in Fig. 23 was obtained using UV-detection. Two peaks are highlighted. These were further investigated through SPE transfer to the NMR-spectrometer equipped with a cryogenic flow probe. The main peak was identified as the monoterpene carvacrol based on the NMR and MS information. Other identified peaks were several flavonoids, phenolic acid and rosmarinic acid. The 1D-proton spectrum of carvacrol obtained after triple trapping and transfer is shown in Fig. 24(A). Here 20 μl of the extract at a time were injected to a 150 × 6 mm reversed phase column. The excellent S/N of the proton spectrum already indicates that direct carbon observation would also be feasible. The $^{13}$C-spectrum obtained in 50 min at 600 MHz, cf. Fig. 24(B), shows clearly all carbon signals in addition to the solvent signals of acetonitrile.
Inverse 2D proton–carbon direct and long range experiments were used to confirm the assignment.

Based on the NMR results, the small peak marked on the chromatographic trace, cf. Fig. 23, could be shown to be a coelution of two flavanoids, namely naringenin and apigenin. Fig. 25(A) and (B) display the 2D inverse detected direct and long range proton–carbon correlations which allowed the assignment of the two flavanoids. The data were recorded in an overnight measurement period. Without the cryogenic probe, it would have been extremely difficult to obtain data comparable to the above. Further trapping would have been necessary. This, however, might not have been possible due to limited loading capacity of the trapping cartridges.

4. Conclusions

This article discusses the primary factors governing and limiting the sensitivity in high-resolution NMR spectroscopy in general, and their implications for the cryogenic probe technology in particular. Further, the importance of the cryogenic probes for NMR-research is illustrated in terms of examples from different fields of NMR applications, some of these being at their early stage but already strongly benefiting from the boosted sensitivity of the cryogenic probes.

In an NMR spectrometer every component in the receiver path is a source of noise. The thermal noise from the entire receiver circuitry has a strong influence on the final spectrum. The cryogenic probes operate at lowered...
temperatures where the thermal noise of the coil and the noise figure of the preamplifier are reduced. The quality factor of the probe, the Q-factor, being mainly dependent on the resistance of the coil, also improves. The filling factor, volume ratio of sample and coil, cannot be fully optimised as extensive thermal isolation between the sample and the coil is necessary. As the thermal noise is dramatically reduced, the noise contribution from the sample, particularly at high ionic concentrations, becomes pronounced. This can be avoided to some extent by the use of low conductivity buffers and NMR-tubes with smaller diameters.

The cryogenic probe technology can be regarded as an example of the interdependence of technological innovation and market demand. In several emerging fields of research the contribution of NMR highly depends on the sensitivity of the technique. The launch of this innovation practically coincided with the boom in genomics, which brought about the need for high throughput investigations of the gene products, this being the objective of structural genomics and proteonomics. There is also a general trend towards miniaturisation in chemical synthesis and screening, which places increasingly strong demands on the sensitivity of analytical techniques. Further, the cryogenic equipment has been rapidly adopted for the NMR-investigations of body fluids where minute amounts of metabolites are detected. The impact of the cryogenic probe technology is equally important for traditional NMR applications, such as studies of low-molecular-weight compounds where sample quantities often are in the submicrogram range, and in other diverse applications of biomolecular NMR. Since the first installations in 1999 the number of cryogenically cooled probes in use worldwide has increased to several hundreds today (2005). These probes are already considered a standard accessory in both academic and industrial NMR-laboratories. They are used routinely for a variety of high-field solution NMR applications, and in the future, as the cryogenically cooled probe technology matures, the applications for high-resolution NMR-spectroscopy are likely to expand.

Acknowledgements

The authors would like to express their appreciation to Mr Oskar Schett, Mr Urs Seehofer, Dr Daniel Marek and Dr Michael Fey at Bruker BioSpin Switzerland, for enlightening discussions and to Dr Kim Colson at Bruker BioSpin U.S., for a careful reading of the manuscript. The authors also thank Drs. Daniel Monleón, Matthias Buck, Volker Dötsch, Konstantin Pervushin, Ivano Bertini, Göran Widmalm and Mr. Lukas Oberer together with their co-workers for kindly making Figs. 4, 8, 10–12, 16 and 22, respectively, available.

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