6 Sensitivity Enhancement by Inverse Detection in Solids

Kay Saalwachter and Ayyalusamy Ramamoorthy

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6.1 INTRODUCTION

Solid-state nuclear magnetic resonance (NMR) spectroscopy has proven to be a powerful method to obtain atomistic-level information from a variety of crystalline, noncrystalline, and amorphous samples.\(^1\) Recent technique and instrumental advancements further strengthened the scope of the technique. For example, the advent of higher magnetic fields, the art of sample preparation and fast MAS capabilities dramatically increased the sensitivity and resolution of spectra even from biological complexes such as membranes and amorphous materials.\(^2\,^4\) Although spectral resolution rendered by solid-state NMR experiments is on par with that of high-resolution solution NMR experiments, the sensitivity is still a major concern. The techniques demand a rather large (milligrams) quantity of biological solids in spite of their capability to provide impressive “solution-like” spectra from most solids. However, the mandatory requirement of long experiments to enhance the sensitivity suffers because of instabilities of the spectrometer and the sample. For example, typically, signal acquisition for more than a day is necessary to obtain a two-dimensional PISEMA\(^3\,^5\,^11\) spectrum of a mechanically aligned lipid bilayer.
sample containing a few milligrams of $^{15}$N-labeled membrane-associated protein. Thus, the development of techniques that further enhances the sensitivity of solid-state NMR experiments is of great importance.

Highly abundant nuclei with large gyromagnetic ratios (such as $^1$H and $^{19}$F) are very NMR sensitive and therefore should be the first choice for detection in NMR experiments. Although this has become true for solution NMR experiments in which $^1$H-detected (or inversely detected) multidimensional techniques are routinely applied, it is still under development for solid-state studies. The key factors that have been preventing the application of $^1$H-detected solid-state NMR are the large $^1$H-$^1$H dipolar interaction, short relaxation ($T_2$ and $T_1\rho$) times, and need for high rf power. Some of these difficulties have been overcome by the recent developments in the field. As a result, several reports have demonstrated the feasibility of $^1$H-detected solid-state NMR experiments as well as significant gain in sensitivity. Because this remarkable methodology, based on coherence transfer (CT) between coupled nuclear spins, can in principle be applied to any heteronuclear spin system, in this chapter we refer to it as “inverse detection” instead of “$^1$H-detection.”

Although most of these inverse-detected techniques are efficient under fast MAS conditions, some of the techniques for static or slow spinning speeds are also reported. Most of these techniques employ a reverse polarization transfer step based on ramp or Lee-Goldburg (LG) CP as well as other coherence transfers, possibly involving some type of recoupling.

This happy circumstance is naturally a major advancement in the field and certainly will have a major effect on NMR studies of biological solids. In addition, the development of a plethora of new multidimensional solid-state NMR techniques based on inverse detection is also in progress in several laboratories. In this chapter, $^1$H-detected methods reported in the literature are reviewed with a special emphasis on the basic concepts that led to the development of $^1$H-detected solid-state NMR experiments under static and MAS conditions. Advantages, disadvantages, and hardware requirements for each method are briefly discussed with some examples from the literature. In addition, possible applications of these remarkable techniques to investigate small molecules and large proteins are highlighted.

### 6.2 BASIC CONCEPTS AND TECHNIQUES

The sensitivity of an NMR experiment depends on the gyromagnetic ratio ($\gamma$) of the nuclei that are being prepared and the $\gamma$ of the detected nuclei. This sensitivity is further increased if the nuclei under preparation and detection are highly abundant. The potential to significantly enhance the sensitivity of heteronuclear correlation experiments via inverse detection, particularly of low-$\gamma$ nuclei such as $^{15}$N and $^{13}$C, was recognized at the early stages of the development of two-dimensional NMR.

The basic idea is to make use of the large $\gamma$, which, first of all, provides a large magnetic moment that leads to a large induction voltage in the receiver coil. Second, most solution-state inverse-detected experiments take advantage of the high equilibrium magnetization of protons by coherent transfer of polarization. Because the transverse relaxation has negligible effects on the timescale needed for CT via isotropic scalar couplings, transfer efficiencies close to 100% are possible in solution.
samples. Therefore, the magnitude of the acquired signal in an optimized inverse detected experiment will not depend on the low gyromagnetic ratio ($\gamma_S$) of the heteronucleus, and thus inverse detection may lead to a gain proportional to $(\gamma_H/\gamma_S)^{3/2}$.

A serious limitation of this approach in $^1$H-detected solution NMR spectroscopy arises when the heteronucleus is not highly abundant. This results in receiver saturation resulting from unwanted (uncoupled and also solvent) proton signal. However, this problem can be overcome by employing a suitable phase cycle to suppress the solvent signal. As the wanted signal is relatively weak and appears as a difference of large numbers, significant amounts of noise are introduced into the spectrum, thus often spoiling the sensitivity gain because of inverse detection. This difficulty was solved by the use of pulsed field gradients for coherence selection as well as by dephasing unwanted coherences, which allow the receiver to be optimized for the wanted signal.

In solid-state NMR spectroscopy, however, inverse detection was not regarded as a useful procedure for a long time. First, the high filling factor of solid rather than solution samples, along with polarization enhancement by CP, line narrowing by moderate MAS, high-power dipolar decoupling, and possibly isotopic labeling, makes low-abundance heteronucleus spectroscopy quite feasible in many relevant cases. Second, the signal-to-noise ratio ($S/N$) gained by an inverse detection depends on the quality factor of the detection circuits, $Q$; the effective line width, $W$, of the two heteronuclei and the efficiency of the additional CT step, $f_{XH}$, as given by the following equation

$$
\xi = \frac{(S/N)_{dd}}{(S/N)_{dd}} \propto f_{XH} \left( \frac{\gamma_H}{\gamma_X} \right)^{3/2} \left( \frac{W_X}{W_H} \right)^{1/2} \left( \frac{Q_H}{Q_X} \right)^{1/2}
$$

Although $Q_H/Q_X$ is usually greater than unity even in most commonly used commercial double-resonance probes (and can certainly be further improved), the proton line width was traditionally considered prohibitively large (i.e., on the order of 40 kHz even at moderate MAS). In addition, CT via scalar couplings in solids is not as efficient as in solution. Although full CT via $J$ couplings is theoretically possible in solids and has experimentally been demonstrated, it is subject to large losses resulting from strong $T_2$ relaxation. All other alternatives rely on the use of orientation-dependent dipolar couplings. Apart from experiments in ordered samples or single (liquid) crystals, the inevitable random orientation of polycrystallites will always limit the efficiency of CT to values on the order of 50%. As an alternative, adiabatic transfer schemes may be considered, but these often suffer from the need to have short and selective single-bond transfers.

Even though first reports of $^1$H-detected double-resonance experiments date back much further than the above-mentioned milestones in solution-state NMR, few applications were reported, mainly in the field of CP, involving quadrupolar nuclei. A first notable gain in sensitivity was described for the indirect detection of rare-spin resonances such as $^{113}$Cd, $^{77}$Se, and $^{29}$Si via their $J$ coupling to $^{31}$P in MAS NMR of inorganic solids, using a method that is essentially a variant of the original
P represents a favorable case of a comparatively sensitive nucleus with rather weak $T_2^*$ relaxation times, and thus narrow lines in MAS spectra.

Renewed interest in sensitivity enhancement of solid-state experiments has mainly been spawned by the growing interest in studying biological solids, where even when the heteronuclei of interest are isotopically labeled and very low $S/N$ are common because of the high dilution of specific nuclei in a given macromolecule. Also, in the case of membrane-associated proteins, labeled proteins are often need to be reconstituted in artificial membranes, possibly stacked between thin glass plates, which further lowers the effective filling factor. Significant technological advances, particularly the possibility to do fast MAS (spinning speed > 20 kHz) and the development of other efficient proton line-narrowing techniques have finally opened avenues to successful inverse detection via protons in solids.

A schematic representation of various types of inverse-detection experiment is depicted in Figure 6.1. During the first stage, initial $^1$H polarization is transferred to the heteronucleus ($S$) either using CP or some other CT scheme. In the second step, spectral information on $S$, most commonly its chemical shift but also dipolar couplings or quadrupole coupling, is encoded in an indirect dimension ($t_1$). In addition, when significant polarization is on the $S$ spin (and possibly stored along the $z$-axis to avoid loss resulting from $T_2$), specific measures can be taken to remove any unwanted proton magnetization. This is particularly important when isotopically dilute systems are investigated, in which leakage of such signals into the detection stage is often deleterious. Finally, the $S$ spin polarization needs to be transferred to protons for detection on the proton channel, possibly under heteronuclear decoupling.

Most approaches discussed in the next three subsections follow this scheme. Sections 6.2.1 and 6.2.2 comprise techniques designed to work under fast MAS, where the CP-based techniques (given in Section 6.2.1) are mostly used to establish chemical-shift correlations and semiquantitative distance constraints. Although the REDOR-based heteronuclear CT (Section 6.2.2) give high-precision information on heteronuclear dipolar couplings, it is also useful for the investigation of local structure and dynamics. More specialized approaches applicable in static samples are summarized in Section 6.2.3.

### 6.2.1 Techniques Based on CPMAS

A sensitivity gain in a solid-state inverse $^{15}$N-$^1$H shift correlation experiment was successfully demonstrated under fast MAS conditions. For both heteronuclear polarization transfer steps in the pulse sequence (Figure 6.1), a standard CP sequence was used. An optimized polarization transfer can be achieved by the use of adiabatic spin-lock pulses during CP on one of the rf channels. This is particularly important at fast MAS frequencies of around 30 kHz, which are crucial, as the observed gain is only possible with rather narrow proton lines [Equation 6.1]. Although an adiabatic transfer is certainly recommendable for the initial enhancement of $^{15}$N polarization, actual applications like the resonance assignment in proteins might require a more specific transfer at the second stage; in such applications, polarization transfer among directly dipolar coupled spin pairs is desired, as relayed polarization transfer or spin diffusion defeat the purpose of heteronuclear correlation experiments. In many cases,
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FIGURE 6.1 Basic steps of a typical inverse-detected 2D experiment and a survey of techniques discussed in this chapter. Spectral information included in $t_1$ can be chemical shifts or dipolar or quadrupolar coupling. The three different schemes are discussed in Sections 6.2.1–6.2.3. In some cases, two or more stages are combined into one, and in three cases, the location of the clean-up stage differs, as indicated by the arrows. The abbreviations are CP, cross polarization; ZF, z-filter; RRR, rotary resonance recoupling; REDOR, rotational-echo double-resonance; HSQC, heteronuclear single-quantum coherence; DQ, double-quantum; PFG, pulsed field gradient; SEMA, spin exchange at the magic angle; MP-dec, multiple-pulse decoupled; HMQC, heteronuclear multiple-quantum coherence; PSLD, pulsed spin-lock detection.
a short, ramped CP\textsuperscript{32} has proven to be suitable at fast MAS. However, an off-resonance spin-lock\textsuperscript{33,34} can be used for effective polarization transfer among directly dipolar coupled spin pairs under static or slow spinning conditions.

When this technique is to be applied at low isotopic abundance, surplus proton signals from uncoupled protons that are bound or close to \textsuperscript{14}N or \textsuperscript{12}C must be removed. A simple and also rather robust approach was presented later for the case of \textsuperscript{13}C-\textsuperscript{1}H correlation at natural abundance,\textsuperscript{22} where two half-millisecond pulses at the rotary resonance recoupling condition (\(\nu_1 = n\nu_R\)) were applied during a \textsuperscript{13}C z-filter delay to convert the dipolar-coupled \textsuperscript{1}H spin bath into nonobservable higher-quantum coherences. A sample spectrum acquired with this technique is given in Figure 6.2, in comparison with a conventional CP-based HETCOR. Another interesting option was also demonstrated: When only one-dimensional spectra of the heteronucleus are sought, it is possible to employ traditional stroboscopic observation while the transverse \textsuperscript{1}H magnetization is locked with short pulses (i.e., pulsed spin-lock detection, PSLD). In this procedure, even though the \textsuperscript{1}H chemical shift information is lost, the length of the FID is increased and the effective line width is consequently small. The main disadvantage of this method is that a two-dimensional acquisition is mandatory to construct the S nucleus spectrum. Nevertheless, a twofold signal gain per unit time could be obtained.\textsuperscript{32}

Rotary resonance recoupling pulses to remove unbound protons are only efficient in rather immobile solids. In samples with significant molecular motions such as semisolids, such as hydrated lipid bilayers, pulsed field gradients applied during a z-filter are one method of choice, although it requires special hardware. This method was demonstrated on a uniformly labeled microcrystalline SH3 protein sample at only 10 kHz MAS.\textsuperscript{30} This condition offers additional narrowing of \textsuperscript{1}H spectral lines. Another important strategy is to use isotopic dilution of protons that can be achieved by perdeuteration of a protein, followed by the back-exchange of N-Ds to N-Hs.

![FIGURE 6.2 Directly detected (a) and inverse-detected (b) \textsuperscript{1}H-\textsuperscript{13}C correlation spectra of poly(methyl methacrylate) with \textsuperscript{13}C in natural abundance, measured with a double CP technique at 31 kHz MAS, demonstrating sensitivity gains for the different signals of about 2–3. Reproduced from reference 16, with permission from the Journal of the American Chemical Society.](image-url)
A recent study demonstrated that pulsed field gradients are not even necessary to remove excess water signal. Introduction of a constant-time interval with a prolonged heteronuclear decoupling in the pulse sequence (Figure 6.1) effectively dephases the water signal. The constant-time interval comprises the $t_1$ evolution time and the $z$-filter, where simply the filter store pulse is moved from $t_1 = 0$ to $t_{1,\text{max}}$. As all pulses on the proton channel stay exactly the same during this procedure, any residual water signal is suppressed by a simple two-step phase cycle on the $S$-channel pulse. In fact, this study showed that it is possible to obtain a two-dimensional $^{15}$N-$^1$H chemical shift correlation spectrum of a uniformly $^{15}$N labeled protein in about 10 min with no additional phase cycling. At 20 kHz MAS, proton line widths as narrow as 0.2 ppm were reported.

### 6.2.2 REDOR-BASED TECHNIQUES

A second family of inverse-detection schemes was devised using a quantifiable dipolar transfer step at the very fast spinning speeds necessary to achieve high proton resolution. Earlier work has shown that REDOR, originally designed to recouple dipolar interactions and transfer polarization between isolated pairs of heteronuclei, is surprisingly efficient even for $^1$H-$S$ systems. REDOR-type CT among $^1$H and $S$ nuclei was made possible mainly because fast MAS on the order of 30 kHz effectively suppresses $^1$H-$^1$H dipolar couplings. In this limit, the $^1$H-$S$ spin system can, to a very good approximation, be analyzed, using simple expressions derived using product operator formalism in conjunction with the simple REDOR average Hamiltonian and a complete neglect of $^1$H homonuclear couplings.

Apart from the replacement of free scalar-coupling evolution periods by REDOR $\pi$ pulses spaced by half the rotor period, this whole family of techniques closely resembles the structure of solution-state inverse-detected experiments. In liquid-state, CT via the scalar coupling is usually restricted to protons directly bound to the $S$ nucleus, such that $H_n \rightarrow S$ and $S \rightarrow H_n$ transfer periods are considered equivalent and are optimized for a specific $J$ value and the multiplet type (or a compromise when different kinds of multiplets are measured at the same time). However, more subtleties arise in the solid-state: although the evolution of $S$-spin transverse magnetization is influenced by the joint dipolar field of differently positioned $^1$H nuclei that are close to the $S$ nucleus, an evolving $^1$H transverse coherence typically feels the dipolar field of only one $S$ nucleus. The former situation is commonly referred to as separated local field (SLF), and the latter is termed proton-detected local field (PDLF) and has the advantage that the theoretical description embodies a simple summation over spin pairs. For this reason, the details of the CT process in a solid-state REDOR experiment depend sensitively on the nucleus that represents the transverse part of the coherence evolving under REDOR recoupling. The modulation of the final signal intensities observed as a function of the recoupling time or $t_1$ rotor encoding (see following) can be very sensitive to the coupling constants as well as the local coupling topology, and the choice of transfer pathway should not just be made on the basis of sensitivity considerations. In fact, when directly detected and inverse-detected experiments are combined, valuable information on the local coupling topology (“spin triangulation”) becomes accessible.
There are essentially four possible permutations of transfer pathways, which are shown in Figure 6.3 for the specific case of HSQC experiments, in which evolution of a heteronuclear antiphase coherence is probed in $t_1$. An HSQC experiment derived from the traditional REDOR scheme is shown in Figure 6.3a. The intensities of the cross signals in such an experiment can be analyzed in terms of the strongest couplings of $S$-spin to its surrounding protons, and as the $S$-spin coherence is always transverse during this experiment, it has the lowest $T_2^*$ losses during recoupling. When the channels are switched, the initial CP can be omitted, however, at the expense of larger losses by $T_2^*$ of protons during recoupling (Figure 6.3b). This experiment is conceptually identical to one of the first inverse HSQC experiments in solution, and its use in solid-state shift correlation has been demonstrated. It has been proven to provide 5–10-fold sensitivity enhancements over a directly detected version (Figure 6.3c), which also does not require an initial CP. It was further demonstrated that one could omit $t_1$ and use the sequence only as a heteronuclear editing filter in front of a $^1$H homonuclear DQ shift correlation experiment.
The experiment in Figure 6.3c is very similar to the TEDOR experiment, the only difference being that HSQC coherence is monitored in the middle of the transfer process as opposed to observing single $^1$H-spin coherence before excitation and reconversion. It combines PDLF and SLF coupling topologies, and calculations show that this type of CT is only efficient for single $S$-$H$ moieties at the shortest possible recoupling times (one or two rotor periods at 30 kHz MAS). Under these conditions, its total performance is still somewhat inferior to a well-optimized CP, but this disadvantage is compensated for by the possibility to determine the actual heteronuclear coupling with high precision.

Finally, the experiment in Figure 6.3d is the only one that was successfully used to obtain $^{15}$N-$^1$H correlation spectra and determine their distances in small molecules with $^{15}$N natural abundance. A more detailed scheme can be inspected in Figure 6.4. The initial CP is essential in that it creates $^{15}$N polarization, which can be stored along the $z$-axis during the time needed for the removal of the overhead $^1$H magnetization. There are two important differences: first, the chemical shift modulation occurs before the REDOR excitation period, and second, the coherence present during a second indirect dimension $t_1$ is heteronuclear dipolar order, which is rotor-encoded when $t_1$ is incremented in fractions of the rotor period. Moving the chemical shift dimension up front has the advantage that the $t_1$ increment can be chosen freely to suit the required spectral width. This is not possible in the HSQC variants, in which increments are restricted to integer rotor periods. This is because noninteger rotor period increments between REDOR excitation and reconversion lead to the appearance of a special kind of side-band spectra with spinning side-bands separated by $2\nu_R$. These increments depend sensitively on the heteronuclear dipolar coupling constant. Their large frequency separation is not compatible with the simultaneous encoding of chemical shifts, which would require an extremely high number of slices in the indirect dimension when considering the concept in the case of HSQC. This problem is overcome by encoding heteronuclear dipolar order (which does not evolve) during $t_1$ simultaneously with the initial $t_1$, but with different increments. In this way, the side-band pattern is folded into the chemical shift range, and its apparent frequency spread is scaled by the ratio of $\Delta t_1/\Delta t_1$.

Results obtained using this experiment are shown in Figure 6.5. Note that heteronuclear dipolar order rotor encoding (HDOR) can also be performed without the additional chemical shift dimension (Figure 6.5d). Efficient processing strategies help to further minimize the acquisition time and yield precise coupling information within reasonable experimental times. Because the combined shift–side band spectrum of Figure 5b takes about a day of acquisition time on a 700-MHz spectrometer, this technique, when applied at natural abundance, is clearly limited to the study of small molecules. Experiments on labeled proteins are certainly feasible and promise rich structural insights via the exact measurement of amide-NH distances, which provides information on hydrogen bonds.

Finally, we note that probably the most promising inverse experiment of Figure 6.3b has not been applied at high isotopic dilution. It does not require an initial CP and features an efficient symmetric REDOR transfer pathway, which is expected to more than compensate its increased losses resulting from proton $T_2^*$. The HSQC dimension is easily split into a separate HSQC $t_1$ and an HDOR $t_1$. The only major
An inverse-detected $S$-$H$ correlation experiment to study samples without isotopic labeling. Unwanted magnetization is purged either by a gradient or by a pair of RRR pulses. There are two separate $t_1$ evolution periods, which can be incremented simultaneously (with different step sizes). The first leads to $S$ chemical shift encoding, and the second splits the signal into a spinning side-band manifold that depends on the $S$-$H$ dipolar coupling.
FIGURE 6.5 Inverse-detected $^1\text{H}-^{15}\text{N}$ correlation spectra of L-histidine-HCl·H$_2$O at 700 MHz. (a) Shift correlation with incremented $t_1$, (b) additional rotor encoding by also incrementing $t_1$, (c) side-band spectra extracted from (b), and (d) side-band spectra obtained by pure rotor ending in $t_1$. The extracted distance compare favorably with results from other methods. Reproduced from reference 22, with permission from the Journal of the American Chemical Society.
requirement would be the application of pulsed field gradients for coherence pathways selection, as $^{15}$N is never present in a pure magnetization state, and a simple $z$-period for clean up cannot be implemented.

### 6.2.3 Inverse Detection in Static Samples

The foregoing sections have presented widely useful experiments that yield high-resolution shift and dipolar coupling spectra under fast MAS. In this section, we review the inverse-detection techniques that are specifically designed for studies under static or slow spinning conditions. In one of the techniques, an off-resonance spin-lock\(^3\) (i.e., using LG\(^5\)\(^1\),\(^2\) or flip-flop Lee-Goldburg [FFLG]\(^5\)\(^3\),\(^4\) pulse sequence) is used to transfer the amide-$^{15}$N transverse magnetization to its dipolar coupled $^1$H spin. This polarization transfer step via SEMA (spin exchange at magic angle) avoids the relay of polarization transfer as well as suppresses spin diffusion via $^1$H-$^1$H dipolar couplings.\(^3\),\(^4\) Employing this step in an inverse-detection experiment (Figure 6.1), the selective acquisition of amide $^1$H chemical shifts under multipulse decoupling (or CRAMPS-type detection\(^1\)) has been demonstrated.\(^2\) A spin-lock pulse in the $S$-spin channel after the $t_1$ period is used to suppress the $^1$H channel before the second polarization transfer step. This sequence can also be used to obtain $S$ nuclei chemical shift and heteronuclear dipolar coupling in the $t_1$ dimension. Because this method involves CRAMPS-type acquisition of the $^1$H signal,\(^3\) it is applicable to solids under slow spinning condition and to study static aligned samples such as mechanically/magnetically aligned bilayers and liquid crystalline materials.\(^7\)

A recent study has demonstrated that the PISEMA sequence can be modified using the inverse-detection procedure.\(^7\) Because transverse magnetization is exchanged between $I$ and $S$ nuclei during the $t_1$ period (or SEMA sequence) of the PISEMA sequence, a stroboscopic observation of the $^1$H signal can be used to observe the heteronuclear dipolar coupling. This method has been successfully demonstrated on site-specifically $^{15}$N-labeled single-crystalline and polycrystalline peptide samples. The main disadvantage of this sequence is the requirement of the sampling window during a multiple-pulse sequence like FFLG. Because FFLG is a windowless sequence, the insertion of rf-free delays reduces the efficiency of the sequence. In addition, the bulk magnetization from residual solvent (mainly water) or from protons that are not involved in the polarization transfer process result in a zero-frequency peak in the $IS$ dipolar coupling spectrum. To avoid this difficulty, it is possible to use a two-dimensional version of this technique that will suppress the nonparticipating $^1$H magnetization as well as use the spin-lock detection in the acquisition dimension. Such sequences will be useful in structural studies of membrane proteins.

A proton inverse-detected deuteron (PRIDE) NMR experiment is demonstrated for the measurement of $^2$H wide line spectra from a small amount of sample (on the milligram scale) within 2 h.\(^2\) This technique is expected to be useful in studying molecular motions in complex organic solids. Evolution under heteronuclear dipolar coupling although suppressing the homonuclear dipolar couplings, is used to create and reconvert HMQC coherences, which are modulated by the $^2$H quadrupolar coupling in an indirect dimension. Proton magnetization is detected using PSLD,
which is essential for the observed enhancements on the order of $10^{-20}$. An important feature is that the shape of the $^2\text{H}$ spectra is free of artifacts and independent of the HMQC excitation/reconversion times. This would be expected for a power-average dependent transfer via single $^1\text{H}-^2\text{H}$ dipolar couplings but is not observed because the $^2\text{H}$ obtains its polarization from many surrounding protons. No specific clean-up stage was introduced, yet good suppression of large amounts of mobile signal components was also demonstrated. Its suitability to do $^2\text{H}$ spectroscopy in natural abundance will need to be examined.

Two-dimensional correlation of $^1\text{H}-^{15}\text{N}$ dipolar coupling with $^{15}\text{N}$ chemical shift using inverse detection was demonstrated for a static sample. It employs a simple double CP, between which a conventional SLF-type $^{15}\text{N}-^1\text{H}$ dipolar coupling dimension featuring a partially multipulse-decoupled Hahn echo is combined with a second, isotropic $^{15}\text{N}$ chemical shift dimension. PSLD is the preferred choice for $^1\text{H}$ detection, resulting in a three-dimensional measurement protocol, in which, of course, only two dimensions are of interest. Proton inverse-detected nitrogen static (PRINS) NMR has been demonstrated on a labeled peptide and a membrane channel domain protein, with about twofold sensitivity enhancements. Successful applications in the field of aligned biological samples are anticipated.

Another recent study has revived the early experiments of Grannell et al., in which the whole process of cross-polarization to an $S$ nucleus, its chemical shift evolution, and the back transfer to protons is combined within a single extended proton spin lock period, during which an $S$ spin lock is interleaved with multiple $\tau_1$ evolution periods. A phase cycling on the $S$ spin lock pulses is apparently sufficient to remove large overheads of uncoupled proton magnetization, such that it provides static $^1\text{H}$ wide line spectra in natural abundance (0.015%). This is remarkable considering that the broad $^1\text{H}$ signal was detected directly, without taking advantage of the large potential gain of PSLD used in other cases when $^1\text{H}$ chemical shift information is of minor importance. Line shape distortions are apparent in the resultant spectra, but it is proposed that use of modern hardware would eliminate such artifacts. The main drawback of this sequence is the use of long spin-lock periods, which restricts the applications to samples with a long $T_{1p}$.

6.2.4 Which Experiment to Use?

The above sections provided an overview over published approaches to inverse detection in different samples, in which different types of polarization transfer steps have been employed. Apart from the examples concerning static samples, all other techniques benefit from fast MAS conditions in excess of 20 kHz, for which special equipment is mandatory. The choice between CP- and REDOR-based techniques is simple: Given a reasonable spectrometer stability, CP approaches appear to be the most efficient ones and are the methods of choice when qualitative distance constraints are sought. LG-CP certainly represents a very promising alternative for a quantifiable transfer, but a slight modification to meet the Hartmann-Hahn condition under MAS will be useful for studies under fast spinning frequencies (Figure 6.6). Because FFLG is efficient in suppressing $^1\text{H}-^1\text{H}$ dipolar couplings, SEMA-type sequence is preferred to selectively transfer coherence from an $S$ nucleus.
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FIGURE 6.6 Off-resonance \(^1\)H spin-lock pulse sequences that have been used for coherence transfer among heteronuclei via dipolar coupling in inverse-detected experiments. (a) Spin exchange at magic angle (SEMA) for static condition,\(^{28}\) (b) SEMA under MAS with a spinning speed \(v_R\),\(^{3,51}\) (c) time-averaged nutation spin exchange at magic angle (TANSEMA) for static conditions, and (d) TANSEMA under MAS. In (b), the \(S\)-spin-lock power \((B_{eff,S})\) is set to equal to \(B_{eff,I} \pm v_R\). In TANSEMA under static condition, \(B_{eff,S} = (\tau_1 \tau_2)B_{eff,I}/(\tau_1 + \tau_2)\), whereas under MAS, \(B_{eff,S} = (\tau_1 \tau_2)B_{eff,I}/(\tau_1 + \tau_2) \pm v_R\). Half the duration of the sequences in (a) and (b) can also be used for coherence transfer.\(^{28,50}\) If needed, supercycles and ramp spin-lock pulses can be used to overcome effects caused by offset and mismatch in rf power.\(^{48}\)

to its dipolar coupled \(^1\)H spins.\(^{34}\) The TANSEMA sequence (Figure 6.6) has been shown to dramatically reduce the rf power needed in the polarization transfer process.\(^{55}\) Although CRAMPS-type homonuclear dipolar decoupling sequences under slower spinning conditions might also provide sufficient proton line narrowing to make inverse detection feasible, it seems that the simplicity of fast MAS will favor this approach. This method will particularly be useful to study wet biological solids that cannot be spun faster. However, the use of small rotors for fast MAS is often not a serious restriction, as most biological macromolecules are often not available in large amounts.
Pulsed field gradients have been used in some cases to remove mobile water signals and surplus uncoupled $^1$H magnetization in samples with a low abundant $^S$ nucleus. However, both problems can be solved with conventional probes and clean-up based on rf irradiation (i.e., prolonged $^1$H decoupling during a z-filter in a constant time protocol and RRR pulses, respectively). Gradients might, however, prove indispensable when recoupling pulse sequences, such as the symmetric REDOR-HSQC, do not allow for the introduction of $^S$ nucleus z-filter delays, during which clean-up rf schemes can be applied.

The REDOR-based schemes are at present the best choice when precise dipolar couplings between the heteronuclei are to be determined. Also, in this case, we stress the fact that such studies do not depend on a scaling factor, as REDOR is rather robust and forgiving of experimental errors resulting from nonideality of $\pi$ pulses. This holds in particular when the rotor-encoding spinning side-bands, instead of spectral intensities, are used for the determinations. In the following section, we present a model study in which not only precise distances but also a full coupling topology was elucidated by these methods.

6.3 APPLICATIONS

In general, most of the techniques discussed herein have just recently been developed, and not many actual applications have emerged until now. The sensitivity gain is the key factor that will enable many studies to be completed within a fraction of the time needed to do the experiments in the conventional way. We here append a few further examples that highlight the great potential of the new methods, which we expect to have a significant effect on the routine toolbox of the solid-state NMR spectroscopists.

6.3.1 APPLICATIONS TO STUDY SMALL MOLECULES AND MATERIALS

In Section 6.2.2, it was indicated that the REDOR-based approaches not only hold promise for remarkable sensitivity enhancements by inverse detection but also serve to elucidate local coupling geometries by combining inverse- and directly detected experiments and making use of the different influence of SLF- and PDLF-type coupling topologies on the measured signals. We highlight this aspect here by reviewing a study concerned with the structure of N-H...N hydrogen bonds in the enol form of N-butylaminocarbonyl-6-tridecyl-isocytosine. A dimer structure (Figure 6.7a) is stabilized by four hydrogen bonds, and we focus on the two equivalent central ones between a urea-N and a pyrimidine-N. Figure 6.7b shows a directly detected side-band spectrum using the pulse scheme of Figure 6.3a, in which HDOR rather than HSQC was selected and rotor-encoded during $t_1$ and was then Fourier-transformed. Each of the urea-N has a relatively tightly bound $^1$H with secondary couplings being comparably weak; thus, the bond length is easily determined from the side-band intensities using a simple spin-pair solution for the fit. When analogous side-band spectra are taken with the inverse-detected version of the same experiment (Figures 6.7c and 6.7d), the local coupling topology around the evolving and detected $^1$H coherence is strongly influenced by both nitrogens in the hydrogen bond. The
FIGURE 6.7 (a) Hydrogen bonds in N-butylaminocarbonyl-6-tridecyl-isocytosine. (b–d) Side-band patterns obtained from HDOR experiments measured in a $^{15}$N-enriched sample at 30 kHz MAS following the experimental schemes in Figure 6.3. The REDOR excitation and reconversion times are (b) $\tau_{\text{exc}} = \tau_{\text{rec}} = 8\tau_R$, (c) $\tau_{\text{exc}} = 3\tau_R$ and $\tau_{\text{rec}} = 6\tau_R$, (d) $\tau_{\text{exc}} = 4\tau_R$ and $\tau_{\text{rec}} = 8\tau_R$. Reproduced from reference 41, with permission from Solid State Nuclear Magnetic Resonance.
side-band modulation then depends not only on the two distances but also on the angle between the two coupling tensors. This unique correlation between distances and angles can be further amplified by using different REDOR recoupling times for excitation and reconversion. This leads to some intensity loss (as this is not the condition for optimum transfer), but still the obtained side-band spectra are considerably less noisy than the one in Figure 6.7b as a result of inverse detection.

The missing longer distance and the hydrogen bond angle can be deduced by comparison with simulated side-band spectra plotted in Figure 6.8. The accuracy of the determination can be increased by analyzing further side-band spectra taken under different recoupling conditions and fitting them to the same set of angle and distance. Obviously, this approach bears a large potential for applications in the biological and material sciences, in which hydrogen bonds are probably the most important supramolecular structure-directing interaction. NMR thus provides access to structure–function relationships in materials that do not need to be crystalline.

### 6.3.2 Biomolecular Applications

Most of the presently used solid-state NMR experiments require a large quantity of sample or a long acquisition time to obtain a reasonable S/N multidimensional spectrum from biological solids. Because most of the interesting biological molecules, such as membrane-associated proteins and RNA, are not available in large quantities, applications of existing NMR techniques to such systems are highly restricted. However, in many cases, even when obtaining large quantities of the system of interest is feasible, the use of a large quantity of such a system does not provide biologically relevant information. For example, in the structural studies of membrane-permeating peptides (such as antimicrobial peptides, toxins, fusion peptides, and channel forming peptides), there is considerable interest in obtaining data from biologically relevant concentrations of the peptide in lipid bilayers, as the increase in the peptide concentration can lead to the disruption of the lipid bilayer structure. Ligand-binding studies are another example in which the concentration of the ligand cannot be increased to acquire signal from the bound ligand. In addition, the mandatory sample size restriction is a heavy price to pay for static as well as MAS experiments with a higher magnetic field. Long signal acquisitions used to avoid these concerns suffer from spectrometer instability and sample denaturing resulting from rf heat dissipation. Therefore, the remarkable sensitivity gained through an inverse-detected experiment could be the long-awaited “magic wand” for NMR applications on biological solids.

Inverse-detected NMR experiments for studies on biosolids fall under two categories: aligned or unaligned crystalline samples. Experiments on aligned samples are usually performed under static condition, whereas unaligned samples are studied under MAS. Experiments need to be chosen on the basis of the nature of the sample under study. The advantages of using fast sample spinning and REDOR-based coherence transfer cannot be used for experiments on aligned samples or on samples that cannot be spun faster. However, the rate of CT among heteronuclear spins and the extent of line broadening caused by homo- and heteronuclear dipolar couplings depend on the rigidity of the sample. For example, because of molecular motions
FIGURE 6.8 Sample side-band patterns calculated for different coupling topologies of an N-H-N hydrogen bond, based on the experiment in Figure 6.3b with $\tau_{1}=4\tau_{R}$ and $\tau_{2}=8\tau_{R}$. The short NH distance was taken to be 115 ppm. Reproduced from reference 41, with permission from *Solid State Nuclear Magnetic Resonance*.
in wet samples, dipolar couplings are partially averaged out, and therefore the contact
time for optimum cross polarization needs to be carefully chosen. In addition, the
use of constant time period is not possible if $T_2$ is not favorable.

Among the methods proposed for inverse detection under MAS, the most
recently demonstrated $^1$H-detected 2D $^1$H/$^{15}$N HSQC MAS experiment is straight-
forward to implement in any solid-state NMR spectrometer. In this experiment, a
two-step phase cycle is used to suppress water. This experiment is expected to be
applicable to any crystalline proteins. It provides a sevenfold increase in sensitivity.
2D spectra that correlate the chemical shifts of amide-$^1$H and amide-$^{15}$N in ubiquitin
nanocrystals are given in Figure 6.9. Experiments on a perdeuterated protein under
fast MAS with $^{15}$N decoupling using WALTZ-16 provided a $^1$H line width of about
0.2 ppm. This experimental plan can be used to further design a family of multidimen-
sional MAS experiments to determine the three-dimensional structure of crys-
talline proteins.

Inverse-detection experiments designed based on the SEMA concept are well-
suited to studies on aligned samples. In these experiments, in the absence of MAS,
homonuclear dipolar couplings are suppressed using multiple pulse sequences. For
this purpose, FFLG sequence is chosen, as it can be used to accomplish coherence
transfer among dipolar coupled heteronuclear spins selectively. This technique was
used to obtain an amide-$^1$H spectrum of a single-site $^{15}$N-labeled magainin2 peptide.
Therefore, this experiment will also be useful in determining chemical shift tensors
of amide-$^{15}$N, $^{13}$C$_\alpha$, and amide-$^1$H that can be used in the dynamic studies.

A recent study demonstrated the application of the PRIDE technique to deter-
mine the slant angle of helices in membrane proteins. This is so far mainly done
using the (directly detected) PISEMA experiment, which is used to measure $^1$H-$^{15}$N
dipolar frequencies in oriented samples. Because the coupling constant is known,
one can directly convert the dipolar frequency into the tilt angle of the NH axis with
respect to the membrane normal and derive the helix orientation from this. By
exchanging the amide protons by deuterium (which is done by just adding heavy
water), one can take advantage of the large quadrupolar interaction, which has exactly
the same symmetry axis as the heteronuclear dipolar tensor. The spectra in Figures
6.10a and 6.10b show that directly detected $^2$H spectroscopy is not at all suited to
measuring the quadrupolar splitting associated with the amide groups of the peptide,
as the signal is dominated by residual D$_2$O. PRIDE efficiently removes this signal
and separates out the amide-$^2$H signals. The spectrum is consistent with the in-plane
orientation of the helix reported earlier. Notably, the central peak is not an artifact
but is associated with N-D axes that are oriented close to the magic angle with
respect to the membrane normal. This orientation represents a “blind spot” for
PISEMA, in which the polarization is transferred via the N-H coupling and is
therefore zero. PRIDE transfers magnetization of surrounding protons to the deu-
teron in question, therefore circumventing this type of problem.
FIGURE 6.9 $^{15}$N-detected (a) and $^1$H-detected (b) 2D $^1$H-$^{15}$N chemical shift correlation spectra of ubiquitin nanocrystals prepared using perdeuterated 2-methyl-2,4-pentanediol (MPD) under 25 kHz MAS. The directly detected spectrum in (a) was acquired with eight times as many scans. Reproduced from reference 25, with permission from the *Journal of the American Chemical Society*. 
FIGURE 6.10 Static 1H spectra of 2.5 mg back-exchanged ovispirin, constituted in membrane lipids (mole ratio 1:27). (a) Directly detected spectrum, (b) the same, magnified 1000 times vertically, and (c) proton inverse-detected spectrum, experimental time: 6 h. Reproduced from reference 23, with permission from the Journal of Magnetic Resonance.
REFERENCES
