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Multiple acquisition of magic angle spinning solid-state NMR experiments using one receiver: Application to microcrystalline and membrane protein preparations

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ABSTRACT

Solid-state NMR spectroscopy of proteins is a notoriously low-throughput technique. Relatively low-sensitivity and poor resolution of protein samples require long acquisition times for multidimensional NMR experiments. To speed up data acquisition, we developed a family of experiments called Polarization Optimized Experiments (POE), in which we utilized the orphan spin operators that are discarded in classical multidimensional NMR experiments, recovering them to allow simultaneous acquisition of multiple 2D and 3D experiments, all while using conventional probes with spectrometers equipped with one receiver. POE allow the concatenation of multiple 2D or 3D pulse sequences into a single experiment, thus potentially combining all of the aforementioned advances, boosting the capability of ssNMR spectrometers at least two-fold without the addition of any hardware. In this perspective, we describe the first generation of POE, such as dual acquisition MAS (or DUMAS) methods, and then illustrate the evolution of these experiments into MEIOSIS, a method that enables the simultaneous acquisition of multiple 2D and 3D spectra. Using these new pulse schemes for the solid-state NMR investigation of biopolymers makes it possible to obtain sequential resonance assignments, as well as distance restraints, in about half the experimental time. While designed for acquisition of heteronuclei, these new experiments can be easily implemented for proton detection and coupled with other recent advancements, such as dynamic nuclear polarization (DNP), to improve signal to noise. Finally, we illustrate the application of these methods to microcrystalline protein preparations as well as single and multi-span membrane proteins reconstituted in lipid membranes.

1. Introduction

Magic angle spinning solid-state NMR (MAS ssNMR) spectroscopy is emerging as a unique method for the atomic resolution structure determination of biomacromolecules, such as membrane proteins and fibrils that are recalcitrant to crystallization. Recent reviews have illustrated the most important achievements in this field [1–3]. The advancements of this spectroscopy are due to the concomitant developments in high-field magnet technology, advances in probe design, isotopic-labeling schemes, and sample preparations, as well as pulse sequence design. However, sensitivity and resolution of the ssNMR spectra still limit the routine application of these techniques, particularly for membrane proteins, which necessitate lipids to maintain their native fold.

There is no doubt that ssNMR structure determination of biomacromolecules is time consuming. Many hours or several days of signal averaging are required for a series of multi-dimensional experiments. In the classical experiments used for membrane proteins, low gyromagnetic ratio nuclei ^{13}C and ^{15}N are acquired, which are intrinsically insensitive. In the past few years, several approaches have been introduced to speed-up ssMAS NMR experiments, including dynamic nuclear polarization (DNP) at ultra-low temperatures [4–6], paramagnetic relaxation enhancement (PRE) [7–9], and ^{1}H detection using ultra-fast MAS probes with or without perdeuteration [10,11]. However, all these methods rely on sample modifications such as doping the samples with chemical compounds with unpaired electrons, metals, or use of low temperatures. While these methods have been successfully used for crystalline proteins, their application to membrane proteins has

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been limited. To maintain their tertiary fold and function, membrane proteins need to be reconstituted in lipid membranes [12,13]. Lipid types as well as lipid to protein ratio are crucial parameters to maintain their functional integrity [12,13]. Also, high spinning speeds can in several cases limit the application of MAS ssNMR techniques [14]. For instance, enzyme function can be significantly reduced in the case of high spinning rates, although moderate spinning speeds can still be used to analyze the structural features of these enzymes.

In recent years, our group has been revisiting the classical MAS ssNMR experiments for resonance assignment and distance determination in biological solids. Inspired by the previous work of Pines et al. [15], we asked ourselves: how much of the polarization generated by the experiments is actually converted into observable signals? Can we optimize the pulse sequences by recovering the *orphan spin operators* that are neglected or purged by the current pulse schemes?

Toward this goal, we developed a class of new experiments called Polarization Optimized Experiments (POE) that utilize orphan spin operators to generate multiple NMR spectra from one pulse sequence [16–19]. These experiments are carried out using commercial solid-state NMR probes for bio-solids and require only one receiver. To accomplish this task, we worked on the three main building blocks of the pulse sequences, including creation of the polarization, evolution, and detection. To generate extra spin operators, we introduced the simultaneous cross polarization (SIM-CP) [18], generating multiple spin operators to be utilized in the generation of additional experiments. We introduced the concept of bidirectional specific cross polarization [17], and the use of long-living ^{15}N z-polarization [20] to generate parallel pathways of spin operators to detect several (up to four) multidimensional spectra [17], with at least twofold time saving.

In this perspective, we describe the most common experiments, such as dual acquisition MAS (DUMAS) [18,19] and MEIOSIS (Multiple ExperIments via Orphan SpIn operatorS) [17], that enable the recording of three to four multi-dimensional experiments, explaining the principles and the technical details for concatenating multiple experiments together. We also project the use of these experiments in conjunction with other techniques, including ^{1}H detection experiments. In the following synopsis, we will illustrate the optimization of the various steps and the implementations that make possible the simultaneous acquisition of multiple 2D, 3D, and 4D spectra.

2. Simultaneous cross-polarization

A central element in an MAS ssNMR experiment is the Hartmann-Hahn (HH) cross-polarization (CP) [21], which enables the polarization transfer from high (^{1}H) to low gyromagnetic ratio nuclei (^{13}C or ^{15}N) (Fig. 1A). The optimization of this first step is crucial for enhancing the sensitivity of the ssNMR experiments. For the POE such as DUMAS and MEIOSIS, we create the initial polarization using a SIM-CP on three different channels. This scheme makes it possible to create both ^{13}C and ^{15}N polarization by satisfying the Hahn-Hartmann conditions for ^{1}H and ^{13}C as well as ^{1}H and ^{15}N (Fig. 1B). The rotating frame radio frequency (RF) amplitudes for SIM-CP transfer are given by $\omega_1 \pm n \cdot \omega_r$, ω_1, and ω_1 for ^{1}H, ^{13}C, and ^{15}N, respectively, where ω_1 is ^{13}C and ^{15}N RF amplitudes, ω_r is MAS spinning frequency, and n is set to ±1 or ±2 to satisfy first or second sideband matching conditions, respectively. To test the efficiency of polarization transfer during SIM-CP, we performed a series of experiments on microcrystalline protein preparations or membrane proteins, varying spinning speeds and RF amplitudes. The ^{13}C and ^{15}N spectra are acquired separately using a single receiver. Importantly, we found that the ^{13}C sensitivity is almost identical for CP and SIM-CP, whereas for ^{15}N we observed ~5–10% loss. Moreover, the HH matching conditions for SIM-CP are identical to those of conventional CP. Therefore, the experimental setup for the SIM-CP is relatively straightforward. As an example, we report a comparison of CP and SIM-CP optimization profiles carried out with U-^{13}C, ^{15}N ubiquitin (Fig. 1C and D). The data points indicate the normalized integrated intensities of ^{13}C and ^{15}N at a spinning rate of 12 kHz. Notably, the build-up rates of the polarization transfer for both CP and SIM-CP are very similar, with SIM-CP showing only marginal loss (less than 10%) of sensitivity for ^{15}N spectra with respect to conventional CP.

3. Bidirectional specific-CP and residual polarization

In the conventional ssNMR pulse sequences for resonance assignments, ^{1}H–^{13}C or ^{1}H–^{15}N double resonance CP creates the initial ^{13}C or ^{15}N polarization. Thereafter, a specific-CP scheme [22] transfers the polarization between ^{15}N and ^{13}C from N to CA or CA to N in a unidirectional manner. We discovered that, using SIM-CP followed by specific-CP, it is possible to obtain a bidirectional polarization transfer between ^{13}C (either CA or CO) and ^{15}N. This additional polarization can be detected using the sequence shown in Fig. 2A, in which, after SIM-CP, the polarization is transferred from N to CA and, simultaneously, from CA to N. The CA polarization is detected in the first acquisition (blue FID); while the ^{15}N polarization is stored along *z*-direction and then transferred to ^{13}CO for a second acquisition (red FID). Fig. 2B shows the two spectra obtained for U-^{13}C, ^{15}N ubiquitin using the dual acquisition pulse sequence reported in Fig. 2A, which utilizes SIM-CP and the bidirectional specific-CP for NCA and CAN transfers.

Similarly, it is possible to achieve bidirectional NCO and CON transfers using NCO specific-CP prior to first acquisition. In fact, the ^{15}N polarization from a CON transfer can be stored during first acquisition and subsequently transferred to CA for a second acquisition time. For the optimization of the N–C specific-CP experiments, we utilize the 1D pulse sequences reported in Fig. 2A. These sequences are used as building blocks for the 3D DUMAS experiments [19] that we will describe below. To cancel the residual polarization of ^{13}C and ^{15}N, we implemented a specific-CP sequence with a four-step phase cycle on ^{13}C and ^{15}N spin-lock pulse phases (see φ_2 and φ_3 in Fig. 2A). Later on, we found that the residual polarization of ^{13}C and ^{15}N along with bidirectional polarization transfer creates *four* different polarization transfer pathways. These new coherences can also be detected and utilized to generate additional 2D and 3D experiments. This represented the genesis of MEIOSIS, a pulse scheme that enables one to detect four FIDs simultaneously. The one-dimensional experiment is shown in Fig. 3A. Although conceptually similar to the experiment depicted in Fig. 2A, the MEIOSIS pulse sequence has phase φ_2 of the specific-CP set to *x* and φ_3 switched between *x* and –*x*. This phase scheme enables one to decode the polarization into four data sets that are then stored into separate files representing four different polarization pathways: NCA, NN, CAN, and CC, where NCA and CAN refer to polarization transfer from N to Cα and vice versa, and NN and CC refer to the residual magnetization on ^{15}N and ^{13}C that is not transferred to ^{13}C and ^{15}N during specific-CP. The first acquisition gives the ^{13}C spectrum resulting from the NCA and CC pathways (Fig. 3A). The ^{15}N polarization resulting from the other two pathways (i.e., CAN and NN) is stored along the *z* direction, and at the end of the first acquisition, is transferred to ^{13}CO. At this point, a second acquisition records the coherences resulting from the CAN and NN pathways. In order to decode the two pathways in each acquisition, we switch the specific-CP ^{15}N spin-lock phase between +*x* and –*x* in alternate scans. The resultant