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Compensated second-order recoupling: application to third spin assisted recoupling†

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Abstract

We consider the effect of phase shifts in the context of second-order recoupling techniques in solid-state NMR. Notably we highlight conditions leading to significant improvements for the Third Spin Assisted Recoupling (TSAR) mechanism and demonstrate the benefits of resulting techniques for detecting long-distance transfer in biomolecular systems. The modified pulse sequences of PAR and PAIN-CP, Phase-Shifted Proton Assisted Recoupling (AH-PS-PAR) and Phase-Shifted Proton-Assisted Insensitive Nuclei Cross Polarization (ABH-PS-PAIN-CP), still rely on cross terms between heteronuclear dipolar couplings involving assisting protons that mediate zero-quantum polarization transfer between low- γ nuclei (^{13}C – ^{13}C , ^{15}N – ^{15}N , ^{15}N – ^{13}C polarization transfer). Using Average Hamiltonian Theory we show that phase inversion compensates off-resonance contributions and yields improved polarization transfer as well as substantial broadening of the matching conditions. PS-TSAR greatly improves on the standard TSAR based methods because it alleviates their sensitivity to precise RF settings which significantly enhances robustness of the experiments. We demonstrate these new methods on a 19.6 kDa protein (U–[^{15}N , ^{13}C]-YajG) at high magnetic fields (up to 900 MHz ^1H frequency) and fast sample spinning (up to 65 kHz MAS frequency).

A. Introduction

Over the last decade solid-state nuclear magnetic resonance (SSNMR) rapidly evolved (thanks to the development of advanced pulse sequences, optimized sample-preparation protocols, access to high magnetic fields, *etc.*) and ascertained its role as a powerful approach to tackle three-dimensional structure determination of systems not accessible *via* solution-state NMR or X-ray crystallography, such as membrane proteins, amyloid-forming

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peptides and microcrystallized/precipitated proteins, through the estimation or the measurement of internuclear distances and torsion angles.¹ The high resolution necessary for the study of such large systems is obtained *via* magic angle spinning (MAS), which averages out anisotropic interactions such as chemical shifts. However, MAS also attenuates the dipolar interactions thus significantly reducing the available structural information. Consequently, a prerequisite for structure determination under MAS is the development of techniques that reintroduce dipolar couplings. To this aim, numerous homonuclear or heteronuclear “recoupling techniques” have been introduced in recent years. However, measurements of long distances in uniformly and densely isotopically labelled samples are complicated by a phenomenon of dipolar truncation: the presence of large dipolar interactions characteristic of short distances strongly attenuates the magnetization transfer due to weak dipolar couplings characteristic of long distances. Among the homonuclear recoupling sequences that have been proposed, only a few of them permitted the 3D structure determination of proteins. Proton-driven spin diffusion (PDS),² which relies on a $^1\text{H}-^{13}\text{C} \times ^{13}\text{C}-^{13}\text{C}$ cross term, has the advantage of attenuating the dipolar truncation phenomenon.³ This technique was used for the first 3D structure determination by solid-state NMR of a selectively labelled protein (SH3, 62 amino acids)⁴ and more recently for the structural study of uniformly labelled ubiquitin.⁵ Dipolar Assisted Rotational Resonance (DARR)^{6,7}/RF Assisted Diffusion (RAD)⁸ have been used for the structure determination of small proteins and fibrils (ubiquitin (76 amino acids),⁹ GB1 (56 amino acids),¹⁰ HET-s amyloid fibrils (79 amino acids),¹¹ Crh (2 × 85 amino acids)¹²). The structure calculation of the first protein bigger than 100 amino acids (MMP12 (159 amino acids)¹³) was made possible using PDS and DARR among other recoupling techniques. However, these two techniques have the disadvantage that their efficiency rapidly degrades with increasing MAS frequencies and magnetic field. In the early 2000s Baldus *et al.* developed a new recoupling sequence that combines the observation of proton–proton contacts and indirect detection.^{14–17} This method alleviated the problem of the limited spectral dispersion and the poor spectral resolution in the proton dimension whilst allowing the measurement of valuable structural details. This technique led to the structure determination of the small protein kalitoxin¹⁸ and of the dimeric Crh.¹² Techniques based on rotational resonance (R^2) and variants have the advantage that they attenuate the dipolar truncation phenomenon through selectivity and that they allow measurements of accurate distances.^{19–24} However, this selectivity restricted these techniques to small peptides until Peng *et al.* demonstrated that rotational resonance combined with PDS could be used to determine the structure of GB1.²⁵

Various heteronuclear dipolar recoupling sequences have also been developed. Methods based on the REDOR experiments (J-decoupled REDOR,²⁶ FS-REDOR,²⁷ TEDOR,²⁸ REPT,²⁹ ZF-TEDOR,³⁰ BASE-TEDOR³⁰) enable the measurement of accurate long distance interactions but their application has so far been limited to rather small uniformly labelled systems^{31,32} or selectively labelled larger systems.³³ The accurate measurement of internuclear distances in uniformly labelled large biomolecules remains very challenging. Methods based on double cross-polarization^{34–37} are limited to the measurement of short distances (one-bond transfer).

Third Spin Assisted Recoupling (TSAR) is a second-order mechanism where polarization is transferred between two spins using the cross term involving dipolar couplings to an assisting spin (typically protons).^{38,39} In the context of biological NMR, this mechanism can be used to design pulse sequences (see Fig. 1) that transfer polarization between homonuclear spins, *e.g.* ^{13}C – ^{13}C PAR (Proton Assisted Recoupling) or ^{15}N – ^{15}N PAR, and heteronuclear spins, *e.g.* ^{15}N – ^{13}C PAIN-CP (Proton-Assisted Insensitive Nuclei Cross-Polarization).^{13,38–42}

Previously, we discussed the theory of the TSAR mechanism for both the homonuclear and heteronuclear cases.^{13,38} We also showed that TSAR-based methods can provide numerous medium- to long-range ^{13}C – ^{13}C and ^{15}N – ^{13}C contacts, which are essential for biomolecular structure determination, under a wide range of spinning frequencies (up to 65 kHz)⁴¹ and magnetic fields (up to 900 MHz). These methods have also been applied to obtain ^{15}N – ^{15}N PAR 2D correlation spectra both under MAS⁴² and static conditions.⁴³ Finally, the TSAR-based techniques were recently used in several structural studies.^{13,44–47}

Here we discuss radio-frequency phase inversion as means of significantly improving the performance and robustness of second-order TSAR-based techniques. The RF-phase inversions can change the sign of some unwanted cross terms, whilst preserving a very efficient TSAR mechanism. With these new techniques the conditions yielding optimum TSAR polarization transfer are broadened which considerably simplifies optimizations and improves overall stability—the sequences are more robust with respect to RF amplifier fluctuations, probe detuning, *etc.*

Note that the PAR experiment was recently modified by Scholz *et al.* yielding the RESORT pulse sequence (“resonant second order transfer”).⁴⁸ The RESORT sequence consists in replacing the CW irradiation on the ^1H channel in the PAR experiment by a rotor synchronized phase alternation (also called XiX scheme). The phase-modulation frequency is typically matched to a small integer multiple n (typically $n = 1, 2$) of the MAS frequency to produce a resonant second-order homonuclear dipolar coupling Hamiltonian. At this point it is important to emphasize the differences with the approach presented in this work. The π phase-shift considered here is applied in the middle of the TSAR mixing period (which is typically much larger than the rotor period). It is either applied on the channels corresponding to the “recoupled” spins, or on the ^1H channel (assisting spins), or on all channels. As demonstrated in the rest of the paper, this does not induce the reintroduction of resonant conditions, but is designed to compensate for autocross terms contributions which are present both in PAR and in RESORT pulse sequences (with different weighting factors).

B. Theory

1. TSAR principles

The TSAR experiment can be analyzed using Average Hamiltonian Theory (AHT)⁴⁹ and the induced spin dynamics can be visualized in the corresponding TSAR subspace, which greatly facilitates the understanding of the mechanism. We consider a three-spin system, consisting of two spins A and B between which we want to transfer polarization and an assisting ^1H spin, all three subjected to a continuous-wave (CW) RF field of strength

$\omega_{1A}/2\pi$, $\omega_{1B}/2\pi$ and $\omega_{1H}/2\pi$ for the A, B and ^1H channels, respectively. The internal Hamiltonian can be written as:

$$H = \overbrace{\omega_{AB} 2A_z B_z}^1 + \overbrace{\omega_{AH} 2A_z H_z}^2 + \overbrace{\omega_{HB} 2H_z B_z}^3 + \overbrace{\omega_A A_z}^4 + \overbrace{\omega_B B_z}^5 + \overbrace{\omega_H H_z}^6 + \omega_{1A} A_x + \omega_{1B} B_x + \omega_{1H} H_x \quad (1)$$

where ω_A , ω_B and ω_H denote shift tensors and resonance offsets of the A, B and ^1H nuclei, respectively (terms 4 to 6), and ω_{AB} , ω_{AH} , and ω_{HB} are heteronuclear dipolar couplings (terms 1 to 3). Note that rotation at the magic angle induces a time dependence of the spatial anisotropy of the interactions.

In the following we focus on the zero-quantum (ZQ) δp_0 TSAR conditions (corresponding to the PAR experiment and the δp_0 PAIN-CP experiment reported previously), for which the RF-field amplitudes on spins A and B are kept equal, $\omega_{1A} = \omega_{1B}$. Following ref.¹³ and ³⁸ we apply AHT to the second order in the interaction frame (IF) defined by the three RF irradiations, paying attention to avoid first-order recoupling conditions such as Hartmann–Hahn matching, R^3 , *etc.* The TSAR recoupling term arises from cross terms involving dipolar couplings A– ^1H and ^1H –B (terms 2 and 3 in eqn (1)), and can be written in the tilted frame (defined by $I_{\tilde{x}}$, $I_{\tilde{y}}$, $I_{\tilde{z}}$)^{38,39}

$$\begin{aligned} \overline{\tilde{H}}_{ZQ, \delta p=0}^{(2), \text{TSAR}} &= 2\omega_{ZQ, \delta p=0}^{\text{TSAR}} (T_{11}^A T_{1-1}^B) T_{10}^H + 2\omega_{ZQ, \delta p=0}^{\text{TSAR}} * (T_{1-1}^A T_{11}^B) T_{10}^H \\ &= \text{Re}(\omega_{ZQ, \delta p=0}^{\text{TSAR}}) 2I_{\tilde{x}}^{\text{AB}, (23)} H_{\tilde{z}} \\ &\quad + \text{Im}(\omega_{ZQ, \delta p=0}^{\text{TSAR}}) 2I_{\tilde{y}}^{\text{AB}, (23)} H_{\tilde{z}} \end{aligned} \quad (2)$$

The expression of the effective TSAR coupling element $\omega_{ZQ, \delta p=0}^{\text{TSAR}}$ can be found in ESI[†], Section 1. In order to complete the description of the TSAR mechanism given by eqn (2), we need to calculate additional contributions arising from an important class of cross terms, referred to as auto-cross terms. These are cross terms of interactions with themselves. The different auto-cross terms resulting from terms 2 to 6 in eqn (1) all yield longitudinal T_{10} contributions (along the $\tilde{z}$ axis in the TSAR subspace, see Fig. 2a) that induce a tilt of the effective recoupling axis. The relevant Hamiltonian can be written as follows:

$$\overline{\tilde{H}} = \overline{\tilde{H}}_{ZQ, \delta p=0}^{(2), \text{TSAR}} + \overline{\tilde{H}}_{ZQ, \text{AUTO}}^{(2)} \quad (3)$$

with the first term given by eqn (2) and the second by:

$$\overline{\tilde{H}}_{ZQ, \text{AUTO}}^{(2)} = \omega_{ZQ}^{\text{AUTO}} I_{\tilde{z}}^{\text{AB}, (23)} \quad (4)$$

Analytical expressions of $\omega_{ZQ}^{\text{AUTO}}$ can be found in ref. 38.

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2. Effect of RF-phase shift on the TSAR mechanism

RF-phase shifts have been successfully used in the context of decoupling and first-order recoupling experiments to compensate for unwanted cross terms.^{50–52} In this work we extend the use of such phase-shifts to second-order recoupling mechanisms. In particular for the first time we show that the use of non-rotor synchronized π -phase shifts applied in the middle of the TSAR mixing period can significantly improve the performance of the polarization transfer mechanism.

In the previous section we recalled the second-order average Hamiltonian that governs the TSAR spin dynamics during the application of CW irradiations (which corresponds to the pulse sequence S_0 in Fig. 2a). Based on this derivation, we can calculate the effect of π -phase shifts applied in the middle of the mixing time (*e.g.* Fig. 2(e) and (f)). As illustrated in Fig. 2(b)–(d), the effect of the π -phase shift on the effective Hamiltonian can be considered when applied on all the channels (corresponding to the pulse sequence block noted S_π^{ABH} in Fig. 2b), on the A and B channels only (noted S_π^{AB} in Fig. 2c), or on the H channel only (noted S_π^H in Fig. 2d).

The effect of the π -phase shifts can be understood by evaluating the propagator over the two blocks S_0 and S_π (phase shifted by π) of duration τ each:

$$U(S_0 S_\pi) = U_{\text{RF}}^{S_\pi}(\tau, 2\tau) \exp(-i\tau \widetilde{H}_{S_\pi}) U_{\text{RF}}^{S_0}(0, \tau) \exp(-i\tau \widetilde{H}_{S_0}) \quad (5)$$

where U_{RF}^S is defined as follows:

$$\frac{d}{dt} U_{\text{RF}}^{S_x}(t_0, t) = -i H_{\text{RF}}^{S_x}(t) U_{\text{RF}}^{S_x}(t_0, t) \quad (6)$$

and $\widetilde{H}_{S_x}$ represents the effective Hamiltonian in the interaction frame defined by the RF pulses S of phase x . We choose the τ period equal to a multiple n of the sample-rotation period T_r such as:³⁸

$$U_{\text{RF}}^{S_0}(0, \tau) = U_{\text{RF}}^{S_\pi}(\tau, 2\tau) = 1 \quad (7)$$

We can thus write:

$$U(S_0 S_\pi) = \exp(-i\tau \widetilde{H}_{S_\pi}) \exp(-i\tau \widetilde{H}_{S_0}) \quad (8)$$

The effective Hamiltonian in the IF, $\widetilde{H}_{S_\pi}$, can be estimated using a Magnus expansion to the second order since we assume that the first order is zero:

$$\widetilde{H}_{S_x} = \underbrace{\widetilde{H}_{S_x}^{(1)}}_{=0} + \widetilde{H}_{S_x}^{(2)} + \underbrace{\cdots}_{\text{neglected}} + \quad (9)$$

Let us calculate $\widetilde{H}_{S_\pi}^{(2)}$. By definition we have:

$$\tilde{H}_{S_\pi}(t) = U_{\text{RF}}^{S_\pi}(0, t) H(t) (U_{\text{RF}}^{S_\pi}(0, t))^{-1} \quad (10)$$

The π -phase shift induces the following relationship between $U_{\text{RF}}^{S_0}(t)$ and $U_{\text{RF}}^{S_\pi}(t)$:

$$U_{\text{RF}}^{S_\pi}(0, t) = \exp(-i\pi I_z^{A/B/H}) U_{\text{RF}}^{S_0}(0, t) \exp(i\pi I_z^{A/B/H}) \quad (11)$$

$I_z^{A/B/H}$ represents I_z^A , I_z^B , I_z^H , $I_z^A + I_z^B$, or $I_z^A + I_z^B + I_z^H$, depending on which channels the π -phase shift is applied: on channel A only, B only, H only, A and B, or all channels, respectively. We can then replace eqn (11) in eqn (10):

$$\begin{aligned} \tilde{H}_{S_\pi}(t) &= \exp(-i\pi I_z^{A/B/H}) U_{\text{RF}}^{S_0}(0, t) \\ &\quad \times \underbrace{\exp(i\pi I_z^{A/B/H}) H(t) \exp(-i\pi I_z^{A/B/H})}_{=H(t)} \\ &\quad \times (U_{\text{RF}}^{S_0}(0, t))^{-1} \exp(i\pi I_z^{A/B/H}) \\ &= \exp(-i\pi I_z^{A/B/H}) \tilde{H}_{S_0}(t) \exp(i\pi I_z^{A/B/H}) \end{aligned} \quad (12)$$

From the above relation we can determine the contribution to the second order of cross terms involving two interactions λ and κ :

$$\begin{aligned} \overline{\tilde{H}}_{S_\pi}^{(2), \lambda\kappa} &= \exp(-i\pi I_z^{A/B/H}) \overline{\tilde{H}}_{S_0}^{(2), \lambda\kappa} \exp(i\pi I_z^{A/B/H}) \\ &= \exp(i\pi I_z^{A/B/H}) \overline{\tilde{H}}_{S_0}^{(2), \lambda\kappa} \exp(-i\pi I_z^{A/B/H}) \end{aligned} \quad (13)$$

Owing to eqn (13), we can write the effect of a π -phase shift on the various terms present in the TSAR Hamiltonian, including the TSAR effective terms (eqn (2)) and the auto-cross terms (eqn (3)). The results are summarized in Table 1.

We now use the BCH expansion (Baker–Campbell–Hausdorff) of eqn (8) to evaluate the effective Hamiltonian over the two blocks of duration τ ($=nT_r$) (denoted S_0 and S_π):

$$H_{\text{eff}} = \frac{1}{2} (\overline{\tilde{H}}_{S_0}^{(2)} + \overline{\tilde{H}}_{S_\pi}^{(2)}) - \frac{\tau}{4} [\overline{\tilde{H}}_{S_0}^{(2)}, \overline{\tilde{H}}_{S_\pi}^{(2)}] + \dots \quad (14)$$

We consider the following cases:

- i. no phase shifts (Fig. 2a):

$$\begin{aligned} H_{\text{eff}} &= \text{Re}(\omega_{ZQ, \delta p=0}^{\text{TSAR}}) 2I_{\tilde{X}}^{\text{AB}, (23)} H_{\tilde{Z}} + \text{Im}(\omega_{ZQ, \delta p=0}^{\text{TSAR}}) 2I_{\tilde{Y}}^{\text{AB}, (23)} H_{\tilde{Z}} \\ &\quad + \omega_{ZQ}^{\text{TSAR}} I_{\tilde{Z}}^{\text{AB}, (23)} \end{aligned} \quad (15)$$

This corresponds to the regular TSAR Hamiltonian. The TSAR mechanism is active when the magnitude of the TSAR recoupling terms is larger than the longitudinal auto-cross terms. If we plot the magnetization transfer for a given mixing time as a function of the RF-field strengths, we can typically find two areas where the transfer is efficient:^{38,39}

1. Just below the diagonal where the magnitude of the autocross terms is minimized.³⁸ Note that in this area the TSAR recoupling term is dominated by:

$$\text{Im}(\omega_{ZQ,\delta p=0}^{\text{TSAR}})2I_{\tilde{Y}}^{\text{AB,(23)}}H_{\tilde{Z}}$$

2. At small RF-field strengths on the A and B channels combined with $p_H > 2.5$, where p_H denotes the ^1H RF-field strength in units of the spinning frequency. Note that in this area the TSAR term is dominated by:

$$\text{Re}(\omega_{ZQ,\delta p=0}^{\text{TSAR}})2I_{\tilde{X}}^{\text{AB,(23)}}H_{\tilde{Z}}$$

- ii. π -phase shift on all channels (ABH-PS-TSAR) (Fig. 2e):

$$H_{\text{eff}} = \underbrace{\text{Im}(\omega_{ZQ,\delta p=0}^{\text{TSAR}})2I_{\tilde{Y}}^{\text{AB,(23)}}H_{\tilde{Z}} + \frac{\tau}{2}\text{Re}(\omega_{ZQ,\delta p=0}^{\text{TSAR}})\left\{-\text{Im}(\omega_{ZQ,\delta p=0}^{\text{TSAR}})I_{\tilde{Z}}^{\text{AB,(23)}} + \omega_{ZQ}^{\text{TSAR}}2I_{\tilde{Y}}^{\text{AB,(23)}}H_{\tilde{Z}}\right\}}_{\text{Correction terms}} \quad (16)$$

To first-order in the BCH expansion, the π -phase shift on all channels averages out the longitudinal auto-cross terms and projects the TSAR component onto the Y -axis in the TSAR subspace. For most of the applications reported here the first term in eqn (16) is larger than the correction terms. Note that the effect of these correction terms can be minimized if necessary for RF settings leading to $\text{Re}(\omega_{ZQ,\delta p=0}^{\text{TSAR}}) \approx 0$.

- iii. π -phase shift on A and B channels (AB-PS-TSAR) (Fig. 2f):

$$H_{\text{eff}} = \underbrace{\text{Re}(\omega_{ZQ,\delta p=0}^{\text{TSAR}})2I_{\tilde{X}}^{\text{AB,(23)}}H_{\tilde{Z}} + \frac{\tau}{2}\text{Im}(\omega_{ZQ,\delta p=0}^{\text{TSAR}})\left\{\text{Re}(\omega_{ZQ,\delta p=0}^{\text{TSAR}})I_{\tilde{Z}}^{\text{AB,(23)}} - \omega_{ZQ}^{\text{TSAR}}2I_{\tilde{Y}}^{\text{AB,(23)}}H_{\tilde{Z}}\right\}}_{\text{Correction terms}} \quad (17)$$

To first-order in the BCS expansion, the π -phase shift on both A and B channels averages out the longitudinal auto-cross terms and projects the TSAR component onto the X -axis in the TSAR subspace. For most of the applications reported here the first term in eqn (17) is larger than the correction terms. Note that the effect of these correction terms can be minimized if necessary for RF settings leading to $\text{Im}(\omega_{ZQ,\delta p=0}^{\text{TSAR}}) \approx 0$.

- iv. π -phase shift on the proton channel only (H-PS-TSAR):

$$H_{\text{eff}} = \omega_{ZQ}^{\text{AUTO}}I_{\tilde{Z}}^{\text{AB,(23)}} - \frac{\tau}{2}\omega_{ZQ}^{\text{AUTO}}\left(\text{Im}(\omega_{ZQ,\delta p=0}^{\text{TSAR}})2I_{\tilde{X}}^{\text{AB,(23)}}H_{\tilde{Z}} - \text{Re}(\omega_{ZQ,\delta p=0}^{\text{TSAR}})2I_{\tilde{Y}}^{\text{AB,(23)}}H_{\tilde{Z}}\right) \quad (18)$$

To first-order in the BCH expansion, the π -phase shift on the proton channel averages out the $H_{\tilde{Z}}$ terms but also the effective recoupling TSAR component.

Terms that can drive polarization transfer appear in higher-order terms in the BCH expansion.

In summary, the theory predicts that a π -phase shift applied in the middle of the TSAR irradiation on both A and B channels, either with or without concurrent π -phase shift on the proton channel (further referred to as, respectively, ABH-Phase-Shifted TSAR (ABH-PS-TSAR) and AB-Phase-Shifted TSAR (AB-PS-TSAR)), will project the TSAR effective component onto the *Y*- or *X*-axis, respectively, whilst averaging out the longitudinal auto-cross terms' contributions. This results in a significant broadening of the RF settings that can be chosen to record TSAR-based polarization-transfer experiments. So far these settings were restricted to narrow conditions where auto-cross terms were smaller than the TSAR component. The PS-TSAR approach thus alleviates the main limitation of the TSAR-based techniques.

The theory detailed here assumed that the Magnus expansion converges and that higher-order terms in the BCH expansion can be neglected. In the following section, we perform simulations that clearly demonstrate the validity of this approach.

C. Numerical simulations and experimental applications to biomolecular NMR

The first set of simulations were performed on spin systems composed of only one assisting proton. This allows investigating the influence of higher-order terms in the effective Hamiltonian (*i.e.* >2) and to appreciate the dipolar truncation strength for long distance transfers. Such simulations were performed in both the homonuclear and the heteronuclear case. The corresponding spin systems are reported in the figures.

The second set of simulations discuss multiple assisting spins effect (here ^1H s), influence of ^1H – ^1H dipolar couplings, and more generally assess the applicability of the theory depicted above.

Note that all simulations include standard anisotropic chemical shift interactions (~ 20 ppm and ~ 7 ppm for carbons and protons respectively).

Overall, the agreement between theory and simulations is excellent. In all cases the use of a π -phase shift in the middle of the TSAR mixing period significantly broadens the range of RF settings, yielding maximum polarization transfer.

1. Homonuclear PS-TSAR: three spin simulation

The first set of simulations were conducted on the three spin system shown in Fig. 3(a) in order to measure the effect of a π -phase shift on the TSAR mechanism. The magnetization starts on the C_α carbon (with 10 ppm offset frequency) and is detected on either the directly bonded C_β carbon (see Fig. 4 and Fig. ESI[†], S1) or on the remote carbon C_{remote} (see Fig. 3 and Fig. ESI[†], S2) which both have the same offset frequency (-10 ppm).

These simulations were performed for two sets of ^1H Larmor frequency ($\omega_{\text{H}}/2\pi$) and sample spinning frequency ($\omega_r/2\pi$) which also correspond to the experimental settings used

in the work: $\omega_{\text{H}}/2\pi = 400$ MHz, $\omega_{\text{T}}/2\pi = 20$ kHz (see Fig. 3 and ESI[†], S1) and $\omega_{\text{H}}/2\pi = 900$ MHz, $\omega_{\text{T}}/2\pi = 60$ kHz (see Fig. 4 and ESI[†], S2).

Panel (c) of Fig. 3 corresponds to the regular PAR experiment described in detail recently.³⁸ The polarization transfer between carbons is efficient for conditions where the effective PAR recoupling terms (eqn (2)) dominate over the longitudinal auto-cross terms (eqn (4)). As can be seen in the simulated contour plot of Fig. 3(c), this is the case for two different sets of RF-field strengths: area 1, just below the diagonal (carbon RF-field strength, p_{C} , slightly larger than proton RF-field strength, p_{H}), and area 2, for small p_{C} values ($p_{\text{C}} < 1$ but different from 0.5) with $p_{\text{H}} > 2.5$. As explained in detail in previous work,³⁸ the first area corresponds to an effective PAR Hamiltonian lying almost along the Y -axis of the TSAR subspace, whereas it is mostly along the X -axis for the second area.

The theory outlined above predicts that the effective Hamiltonian can be either projected onto the X -axis with a π -phase shift on channels A and B (which are the same channel in the homonuclear case) or onto the Y -axis with a π -phase shift on all channels. This is in good agreement with the contour plots of Fig. 3(d) and (e), since the TSAR transfer is mainly occurring in the area 2 and area 1, respectively. Thus, depending on the chosen pulse sequence, we are able to enhance polarization transfer for low p_{C} values (narrowband settings) by applying a π -phase shift on the ^{13}C channel (C-PS-TSAR), or to enhance the polarization transfer just below the diagonal (area 1) by applying a π -phase shift on both the proton and carbon channels (CH-PS-TSAR). The latter case is illustrated in Fig. 3(b) which displays slices through panels (c) and (e) along the arrows. The introduction of the π -phase shift significantly improves the polarization transfer efficiency (by a factor of ~ 2) and broadens the “matching” conditions (also by a factor of ~ 2). Both these effects are crucial for a successful application of the TSAR methodology to probe long distance contacts (in this case 4.5 Å) in biomolecular systems.

Fig. 3(f), which corresponds to the application of a π -phase shift on the proton channel only, is more difficult to interpret. The plot seems closer to panel (c) at first sight. Indeed we do observe polarization transfer for both area 1 and area 2. However, the polarization transfer mechanism is much more complicated (see eqn (18)) and its interpretation is beyond the scope of this article. All the above comments concerning Fig. 3(c)–(f) are also relevant for the simulations of Fig. S1 (ESI[†]) which shows the one-bond polarization transfer from the C_{α} to the C_{β} spin under the same experimental conditions.

Fig. 4 and ESI[†], S2 show analogous simulations but for very high spinning frequencies ($\omega_{\text{T}}/2\pi = 60$ kHz) and high magnetic field (^1H Larmor frequency $\omega_{\text{H}}/2\pi = 900$ MHz). The observations and conclusions are similar to the slower spinning case detailed above (Fig. 3). Essentially, the introduction of a π -phase shift significantly broadens the TSAR RF-field strength settings that can be used.

2. Heteronuclear PS-TSAR: three spin simulation

Fig. 5 illustrates the effect of the π -phase shift in the heteronuclear case for a three spin system. The magnetization starts on the nitrogen and is detected on the carbon spin. Setting up a PAIN-CP experiment typically requires one to adjust the nitrogen and carbon power

levels in order to equalize the nutation frequencies (within less than 1 kHz precision) and then to optimize the ^1H power level such that the corresponding nutation frequency is slightly below both the carbon and the nitrogen ones. As already seen in the homonuclear case, the π -phase shift leads to an improvement of the maximum efficiency and a broadening of the transfer conditions (approximately a factor of 2). More specifically, the π -phase shift not only broadens the ^1H RF settings that can be chosen (Fig. 5d) but also the allowed mismatch between the nitrogen and carbon nutation frequencies (Fig. 5e). This is really important for the application of such techniques to sophisticated systems since it should significantly aid in compensating for RF inhomogeneity and/or amplifier instabilities.

3. PS-TSAR: multiple spin effect

(i) Multiple spin simulation: effect of ^1H – ^1H dipolar couplings—Fig. 6 represents polarization transfer curves as a function of the mixing time for PAR and CH-PS-PAR. The RF settings are indicated with crosses in panels of Fig. 3c and e respectively (*i.e.* for $p_{\text{C}} = 2.5$ and $p_{\text{H}} = 2.3$). The red and green lines correspond to simulations performed on the same spin system SS1 (see Fig. 6a) as the ones reported in Fig. 3 and 4, and represent polarization transfer efficiency to the directly bonded carbon C_{β} and to the remote carbon C_{remote} respectively. The brown and blue simulations correspond to equivalent simulations when three additional protons are included. The corresponding spin system SS2 is represented in Fig. 6b and thus includes ^1H – ^1H couplings. Finally, solid lines correspond to PAR polarization transfer efficiencies (Fig. 6c), whereas dotted lines correspond to CH-PS-PAR (Fig. 6d).

As expected from the theoretical derivations reported above, polarization transfer profiles are different with and without π -phase shift. Overall the maximum transfer is higher with the introduction of the π -phase shift on both the proton and the carbon channels. The maximum buildup time is slightly longer for ABH-PS-TSAR and rather flat around this point which is an interesting feature from an experimental point of view since this alleviates the careful optimization of the optimum mixing time.

Such observations are true for simulations performed on SS1 and SS2 (which includes strong ^1H – ^1H dipolar couplings). Furthermore comparison between buildup curves on SS1 and SS2 not only shows that ^1H – ^1H couplings do not destroy the TSAR mechanism or the beneficial effect of the ABH π -phase shift but also shows that higher polarization transfer to the remote carbon can be reached on densely protonated systems compared to the three spin case.

At this point, it is important to stress that simulations performed on SS1 (four spin system that only include one directly bonded proton) do not represent an ideal and easier case scenario for the TSAR transfer compared to SS2 (for instance). As explained in the theoretical section, heteronuclear dipolar interactions also induce auto-cross terms in the second-order Hamiltonian. Such terms lead to longitudinal contributions of the forms C_z on each of the carbon spin. Since the TSAR mechanism discussed here is a ZQ process, only the difference between these longitudinal contributions at two given carbon sites matters in the polarization transfer. Obviously this difference is minimized if each carbon has a strongly coupled proton attached.

Concerning the ^1H – ^1H dipolar couplings they can manifest themselves in the TSAR polarisation transfer spin dynamics through auto-cross terms or cross terms with the ^1H – ^{13}C dipolar interaction. The auto-cross terms lead to longitudinal contribution of the form H_z , which commute with the TSAR terms and thus do not destroy the ^{13}C – ^{13}C polarization transfer. Cross terms between $^1\text{H}_i$ – $^1\text{H}_j$ and $^1\text{H}_i$ – ^{13}C dipolar interactions lead to terms of the forms $H_z^j C_z^i H_z^i$ when the condition $|p_C \pm p_H| \in \{0, 1, 2, 3, 4\}$ is matched. These terms can thus potentially drive some magnetization transfer from carbons to protons if the TSAR conditions chosen is too close to $|p_C \pm p_H| \in \{0, 1, 2, 3, 4\}$. Such leakage terms are reduced by the application of an ABH π phase shift.

(ii) Robustness with respect to carrier frequency—As mentioned above, the polarization transfer is enhanced by the introduction of an ABH π -phase shift (for both SS1 and SS2). More importantly, ABH π -phase shift also significantly broadens the bandwidth of the polarization transfer for both the directly bonded C_β and the remote carbon, as shown in Fig. 6e. Note that these simulations include chemical shift anisotropy for each of the carbons, that the C_α carbon has a 10 ppm offset frequency and the C_β and C_{remote} spins a -10 ppm offset frequency respectively. Overall these numerical results are in perfect agreement with the theory that predicts a compensation of off-resonant auto-cross terms.

4. Application to a biomolecular system

The results of the numerical simulations are confirmed by experiments. Fig. 7 presents experimental PAR polarization-transfer profiles as a function of RF settings with and without π -phase shift on alanine. The polarization transfer from the C' to the C_α carbon is consistently improved with the introduction of the π -phase shift. Details can be found in the figure caption and in the materials and methods section.

Fig. 8 shows a 2D ^{13}C – ^{13}C correlation spectrum obtained on the ~ 20 kDa YajG protein using the CH-PS-PAR method. The experiment was recorded using about 5 mg of protein in 2 days on a 900 MHz spectrometer. Experimental details can be found in the materials and methods section.

As another example of the introduced here approach Fig. 9 shows a ^{15}N – ^{13}C heteronuclear TSAR experiment (NCH-PS-PAIN-CP) using a π -phase shift on the YajG protein (20 kDa). Experimental details can be found in the figure caption.

D. Materials and methods

Sample preparation

Natural abundance L-alanine was purchased from Merck. Uniformly ^{13}C , ^{15}N -labelled L-alanine was purchased from Cambridge Isotope Laboratories, diluted to 10% in natural abundance material, re-crystallized in Milli-Q water and center-packed in a 2.5 mm rotor (9 mg). Uniformly labelled ^{13}C – ^{15}N YajG protein was slowly precipitated in water and directly centrifuged into the NMR rotor.⁵³

Solid-state NMR

All experiments were carried out using two high-field Bruker instruments, operating at 9.4 T ($\omega_{\text{H}}/2\pi = 400.13$ MHz) and at 21.1 T ($\omega_{\text{H}}/2\pi = 900.18$ MHz). For experiments at 9 kHz MAS rate, a Bruker 4 mm MAS triple-resonance probe was used. The bearing gas temperature was set to 260 K, corresponding to a sample temperature of 275 K. For the experiments at 20 kHz MAS rate, a Bruker double-resonance 2.5 mm MAS probe was used. The sample temperature was stabilized at 268 K (bearing gas at 260 K). The experiments at 60 kHz MAS rate were performed on a Bruker 1.3 mm MAS triple-resonance probe. The bearing gas temperature was 235 K leading to a sample temperature of approximately 270 K. For all studies, the temperature was regulated using a Bruker BCU-X unit.

The ^{13}C magnetization profiles presented in Fig. 7 were obtained using a pulse sequence starting with a ramped cross-polarization (CP) step^{54,55} of 1750 μs , performed at an average RF field strength of 80 kHz on the ^1H channel and 60 kHz on the ^{13}C channel. The CP step was followed by a flip back Q5⁵⁶ selective pulse of 1260 μs and a flip down E-burp²⁵⁷ selective pulse of 2240 μs prior to the PAR mixing time and detection under heteronuclear decoupling. The length and frequency offset of the selective pulses were adjusted to selectively excite the CO resonance of the alanine, whereby the RF carrier frequency was set in the middle of the ^{13}C spectrum. Between the two selective pulses a 3 ms delay was inserted in order to dephase all magnetization except that of CO. The proton RF field strength was set to 140 kHz for hard pulses, and to 120 kHz for SPINAL heteronuclear decoupling⁵¹ during both acquisition and selective pulses. The acquisition time was 30 ms. For each 1D spectrum, 400 scans preceded by 16 dummy scans were acquired. The recycle delay was set to 3 s.

The 2D ^{13}C – ^{13}C CH-PS-PAR correlation spectrum of [U- ^{13}C , ^{15}N]-YajG protein (Fig. 7) was recorded at $\omega_{\text{H}}/2\pi = 900$ MHz and $\omega_{\text{T}}/2\pi = 60$ kHz with a PS-PAR mixing time of $\tau_{\text{mix}} = 7$ ms performed at the RF-field strengths of $\omega_{\text{C}}/2\pi = 85.5$ kHz ($p_{\text{C}} = 1.425$) and $\omega_{\text{H}}/2\pi = 66$ kHz ($p_{\text{H}} = 1.1$). Initial ^{13}C magnetization was obtained by an adiabatic cross-polarization step of 800 μs ,⁵⁴ performed at averaged RF-field strengths of 15 kHz on the ^1H channel and 75 kHz on the ^{13}C channel. The proton RF-field strength was set to 100 kHz for hard pulses and to 15 kHz for low-power TPPM heteronuclear decoupling^{58,59} during both evolution and detection periods. Acquisition times were 20 ms in the direct dimension, and 10 ms in the indirect dimension, with spectral widths of 75 kHz and 20 kHz, respectively. The ^{13}C RF carrier was set at 37 ppm for the entire experiment. 192 scans were added for each t_1 increment. Quadrature detection in the indirect dimension was achieved using States-TPPI. The recycle delay was set to 2.1 s. The total experimental time was 2 days.

The 2D ^{15}N – ^{13}C NCH-PS-PAIN-CP correlation spectrum of [U- ^{13}C , ^{15}N]-YajG protein was recorded at $\omega_{\text{H}}/2\pi = 400$ MHz and $\omega_{\text{T}}/2\pi = 9$ kHz with a PS-PAIN mixing time of $\tau_{\text{mix}} = 8$ ms performed at the RF-field strengths of $\omega_{\text{C}}/2\pi = \omega_{\text{N}}/2\pi = 40$ kHz ($p_{\text{C}} = p_{\text{N}} = 4.4$) and $\omega_{\text{H}}/2\pi = 37.35$ kHz ($p_{\text{H}} = 4.15$). Initial ^{15}N magnetization was obtained by an adiabatic cross-polarization step of 1500 μs ,⁵⁴ performed at an average RF-field strength of 59 kHz on the ^1H channel and 50 kHz on the ^{15}N channel. The proton RF-field strength was set to 90 kHz for both hard pulses and SPINAL heteronuclear decoupling used during evolution and

acquisition. Acquisition times were 15 ms in the direct dimension, and 13 ms in the indirect dimension, with spectral widths of 30 kHz and 2.6 kHz, respectively. RF-carriers were set to 44 ppm on the ^{13}C channel and 122 ppm on the ^{15}N channel. 696 scans were accumulated for each t_1 increment. Quadrature detection in the indirect dimension was achieved using States-TPPI. The recycle delay was set to 3 s. The total experimental time was 1.5 days.

Simulations and data analysis

All numerical simulations were performed with SPINEVOLUTION.⁶⁰ Spin systems used in the simulations are shown in each figure. Simulations used typical anisotropic chemical shift tensor values encountered in protein systems.

E. Conclusions

In this contribution we have introduced the use of π -phase shifts in the middle of the TSAR recoupling period. The resulting effect has been described theoretically and the benefits were demonstrated both experimentally and using numerical simulations. Notably the ABH-PS-TSAR and AB-PS-TSAR mechanism (π -phase shift in the middle of the mixing period applied on all channels or on the heteronuclei only, respectively) improves both the efficiency and the robustness of the polarization transfer mechanism. This is especially true for long-distance transfers.

PS-TSAR greatly improves on standard TSAR based methods because it alleviates their sensitivity to the precise RF settings which significantly enhances the robustness of the approach. This new approach is demonstrated on a 19.6 kDa protein (U- ^{15}N , ^{13}C]-YajG). The original TSAR methodology, in combination with other experiments, already enabled the structure determination of a 17.6 kDa protein.¹³ The improved robustness of PS-TSAR should thus enable tackling even more challenging systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

(a) AH-PS-PAR and (b) ABH-PS-PAIN-CP pulse sequences that use a π -phase shift in the middle of the mixing period on all channels at the same time. Phase cycling for both sequences was performed as follows: $\phi_1 = x, -x$; $\phi_2 = y$; $\phi_3 = x$; $\phi_4 = x, x, -x, -x$; $\phi_5 = -x, -x, x, x$; $\phi_{\text{rec}} = x, -x, x, -x$.

Fig. 2.

Visualization of the TSAR spin dynamics subspace. The space can be seen as a coupled basis between a ZQ fictitious spin I^{AB} (involving a spin A and a spin B) and a proton spin H (assisting spin). The total effective field can be decomposed into two components: a red arrow which indicates the transverse TSAR recoupling axis and a blue arrow which represents the longitudinal off-resonance contribution (arising from auto-cross terms). The effect on the TSAR effective field of an inversion by 180° of the phase of irradiation on the different channels is summarized in Table 1 and can be visualized in panels (b)–(d), whereby the result obtained with the reference phase is given in (a). The dotted line in (b)–(d) is given to guide the eye and represents the original position of the TSAR recoupling field. Notably the TSAR recoupling field in panels (b) and (c) is symmetric with respect to the y- and x-axis, respectively, compared to the one in panel (a), whereas the TSAR recoupling field is shifted by π around the z-axis in panel (d), compared to (a). Regarding the auto-cross terms, they are inverted in cases (b) and (c) with respect to their original position given in (a). Panels (e) and (f) show the result of ABH-PS-TSAR and AB-PS-TSAR, respectively. Expressions for the ZQ fictitious spin operator can be found in the ESI[†] Section 1b.

Fig. 3.

Numerical simulations of the TSAR-based polarization-transfer efficiency with and without phase shift at $\omega_H/2\pi = 400$ MHz and $\omega_r/2\pi = 20$ kHz. The magnetization starts on the C_α spin and is detected on the C_{remote} spin after 5 ms of mixing time, according to the geometry of the spin system considered given in (a). (b) Polarization transfer efficiency for PAR (black) and CH-PS-PAR (red) as a function of the proton RF-field strength p_H , with p_C fixed at 2.5 (expressed in units of the spinning frequency), corresponding to slices through the simulated contour plots (c) and (e) at the position of the black and red arrows. (c)–(f) 2D maps of the polarization-transfer efficiency as a function of the carbon (p_C) and proton (p_H) RF-field strength for (c) PAR, (d) C-PS-PAR, (e) CH-PS-PAR, and (f) H-PS-PAR. The corresponding polarization transfer to the directly bonded C_β spin is presented in Fig. S1 (ESI[†]).

Fig. 4.

Numerical simulations of the TSAR-based polarization-transfer efficiency with and without phase shift at $\omega_{0H}/2\pi = 900$ MHz and $\omega_r/2\pi = 60$ kHz. The magnetization starts on the C_α spin and is detected on the C_β spin after 5 ms of mixing time according to the geometry of the spin system considered given in (a). (b) Polarization-transfer efficiency for PAR (black) and CH-PS-PAR (red) as a function of the proton RF-field strength p_H , with p_C fixed at 1.425 (expressed in units of the spinning frequency), corresponding to slices through the simulated contour plots (c) and (e) at the position of the black and red arrows. (c)–(f) 2D maps of the polarization transfer efficiency as a function of the carbon (p_C) and proton (p_H) RF-field strength for (c) PAR, (d) C-PS-PAR, (e) CH-PS-PAR, and (f) H-PS-PAR. The corresponding polarization transfer to the remote spin C_{remote} is presented in Fig. S2 (ESI[†]).

Fig. 5.

(a) Spin system used for the numerical simulations given in (b)–(e) which are all performed at $\omega_{0H}/2\pi = 400$ MHz, $\omega_r/2\pi = 9$ kHz. The magnetization starts on the nitrogen spin and is transferred to the C_α spin in the presence of a proton spin. (b) and (c) 2D contour plots of the polarization-transfer efficiency at a mixing time of 5 ms as a function of the different RF-field strengths p_H , p_C , and p_N (with $p_C = p_N$) for (b) PAIN-CP and (c) NCH-PS-PAIN-CP. (d) Polarization-transfer efficiency at a mixing time of 3 ms for PAIN-CP (black) and NCH-PS-PAIN-CP (red) as a function of the ^1H RF field strength with p_C and p_N both fixed at 2.5. (e) Polarization-transfer efficiency at a mixing time of 3 ms for PAIN-CP (black) and NCH-PS-PAIN-CP (red) as a function of the ^{13}C RF-field strength with fixed $p_N = 2.5$ and $p_H = 2.2$ or 2.4, respectively.

Fig. 6.

Effects of ^1H - ^1H couplings and offsets on different variants of PAR. Spin system used in the simulations: (a) four spins system composed of C_{α} , C_{β} , C_{remote} and H_{α} , (b) same as (a) with three additional protons. Magnetization starts on the C_{α} spin. Polarization transfer efficiency as a function of the mixing time for (c) PAR and (d) CH-PS-PAR at the resonance conditions $p_C = 2.5$ and $p_H = 2.25$ (expressed in units of the spinning frequency) at $\omega_H/2\pi = 400$ MHz and $\omega_r/2\pi = 20$ kHz. (e) Profile of the polarization transfer efficiency with respect to the ^{13}C carrier frequency for PAR and CH-PS-PAR.

Fig. 7.

Experimental polarization-transfer efficiency recorded on $[\text{U-}^{13}\text{C}, ^{15}\text{N}]$ -alanine at $\omega_0\text{H}/2\pi = 400$ MHz and $\omega_1/2\pi = 20$ kHz. The C_α peak integral is plotted as a function of the CW RF-field strength applied on the proton channel during the mixing time of a CH-PS-PAR (red line) and a regular PAR experiment (black line). The mixing time τ_{mix} was set to 1.5 ms and $\omega_1\text{C}/2\pi = 50$ kHz.

Fig. 8.
2D ^{13}C - ^{13}C CH-PS-PAR correlation spectrum acquired on the protein $[\text{U-}^{13}\text{C}, ^{15}\text{N}]\text{-YajG}$ at $\omega_{\text{H}}/2\pi = 900$ MHz and $\omega_{\text{T}}/2\pi = 60$ kHz with $t_{\text{mix}} = 7$ ms, $\omega_{\text{C}}/2\pi = 85.5$ kHz and $\omega_{\text{H}}/2\pi = 66$ kHz.

Fig. 9. 2D ^{15}N - ^{13}C NCH-PS-PAIN-CP correlation spectrum of $[\text{U-}^{13}\text{C}, ^{15}\text{N}]$ -YajG protein at $\omega_{\text{H}}/2\pi = 400$ MHz and $\omega_{\text{r}}/2\pi = 9$ kHz with $t_{\text{mix}} = 8$ ms, $\omega_{\text{C}}/2\pi = \omega_{\text{N}}/2\pi = 40$ kHz and $\omega_{\text{1H}}/2\pi = 37.35$ kHz.

Table 1

Effect of a π -phase shift on the different elements of the TSAR effective Hamiltonian (see also Fig. 2(b)–(d)) given in the ZQ subspace spanned by the fictitious spin I^{AB} (involving a spin A and a spin B) and a proton spin H (assisting spin)

Hamiltonian terms	π -Phase shift on all RF channels	π -Phase shift on A and B channels	π -Phase shift on ^1H channel only
$2I_{\tilde{X}}^{\text{AB},(23)}H_{\tilde{Z}}$	$-2I_{\tilde{X}}^{\text{AB},(23)}H_{\tilde{Z}}$	$2I_{\tilde{X}}^{\text{AB},(23)}H_{\tilde{Z}}$	$-2I_{\tilde{X}}^{\text{AB},(23)}H_{\tilde{Z}}$
$2I_{\tilde{Y}}^{\text{AB},(23)}H_{\tilde{Z}}$	$2I_{\tilde{Y}}^{\text{AB},(23)}H_{\tilde{Z}}$	$-2I_{\tilde{Y}}^{\text{AB},(23)}H_{\tilde{Z}}$	$-2I_{\tilde{Y}}^{\text{AB},(23)}H_{\tilde{Z}}$
$I_{\tilde{Z}}^{\text{AB},(23)}$	$-I_{\tilde{Z}}^{\text{AB},(23)}$	$-I_{\tilde{Z}}^{\text{AB},(23)}$	$I_{\tilde{Z}}^{\text{AB},(23)}$
$H_{\tilde{Z}}$	$-H_{\tilde{Z}}$	$H_{\tilde{Z}}$	$-H_{\tilde{Z}}$