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Communication

Repetitive cross-polarization contacts via equilibration-re-equilibration of the proton bath: Sensitivity enhancement for NMR of membrane proteins reconstituted in magnetically aligned bicelles

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ABSTRACT

Thermodynamic limit of magnetization corresponding to the intact proton bath usually cannot be transferred in a single cross-polarization contact. This is mainly due to the finite ratio between the number densities of the high- and low-gamma nuclei, quantum–mechanical bounds on spin dynamics, and Hartmann–Hahn mismatches due to rf field inhomogeneity. Moreover, for fully hydrated membrane proteins refolded in magnetically oriented bicelles, short spin-lock relaxation times ($T_{1\rho}$) and rf heating can further decrease cross polarization efficiency. Here we show that multiple equilibrations-re-equilibrations of the high- and low-spin reservoirs during the preparation period yield an over twofold gain in the magnetization transfer as compared to a single-contact cross polarization (CP), and up to 45% enhancement as compared to the mismatch-optimized CP-MOIST scheme for bicelle-reconstituted membrane proteins. This enhancement is achieved by employing the differences between the spin–lattice relaxation times for the high- and low-gamma spins. The new technique is applicable to systems with short $T_{1\rho}$'s, and speeds up acquisition of the multidimensional solid-state NMR spectra of oriented membrane proteins for their subsequent structural and dynamic studies.

As for virtually every spectroscopic measurement, signal-to-noise ratio plays perhaps the most critical role in the acquisition and interpretation of solid-state NMR spectra of macroscopically aligned samples. This method has recently demonstrated the capability of providing remarkable detail about the conformations of membrane proteins in their native-like, fully hydrated lipid environment at nearly atomic resolution [1]. Magnetically aligned bicelles [2,3] have the potential of advancing this technique even further since they provide superior spectral resolution as compared to glass plates [4–6]. However, the necessity to detect dilute spins in such strongly proton-coupled systems is inherently connected with the problem of low sensitivity. As a result, the acquisition of multidimensional solid-state NMR spectra can take several days. Usually, magnetization enhancement for the dilute spins during the preparation period is achieved via the cross-polarization (CP) method [7] under the Hartmann-Hahn matching conditions [8]. Numerous improvements of the method have been proposed, including ramped CP [9], CP-MOIST [10,11], CP involving simultaneous phase inversion [12], variable-amplitude CP [13], selective-excitation RELOAD-CP technique [14], frequency-modulated CP [15], and CP-COMPOZER [16]. The latter two techniques have shown their robustness with respect to Hartmann–Hahn mismatches and the capability of improving the signal to noise ratio by up to 20–25% for aligned samples [15,17]. However, only a part of the overall magnetization is transferred from the abundant proton bath to the dilute spins in a single cross-polarization contact [18]. This can be due to the finite ratio of the total number of protons with respect to that of the low-gamma spins, as well as relaxation effects. In addition, universal or quantum–mechanical bounds on spins dynamics [19] may play a role, thus further limiting the amount of the maximum transferred quasi-stationary magnetization [10,20]. The classical multiple-contact scheme [7] can be employed to further enhance the magnetization transfer from the protons to the dilute spins in static and spinning solids. However, for membrane proteins reconstituted in magnetically aligned bicelles [4], this scheme may not be appropriate due to the relatively short $T_{1\rho}$ relaxation times [21] (typically up to several milliseconds) inherent to the liquid-like bilayers and uniaxially rotating membrane proteins [22,23]. In such samples, the proton spin-lock responsible for the successive enhancement of magnetization would be lost during the first acquisition period (typically 10 ms). Moreover, substantial heating of the sample would take place if the protons are irradiated for 30 ms or longer during the multiple contacts. Therefore, CP-MOIST and ramped CP currently remain the most widely used methods to enhance ^{15}N magnetization in uniaxially aligned membrane protein systems. Here we employ an alternative scheme [24] based on repetitive short CP contacts during the preparation period that circumvents the above thermodynamic

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and quantum–mechanical bounds and the issue of short $T_{1\rho}$'s. This pulse sequence (which we term here as REP-CP) yields more than a factor two enhancement of magnetization as compared to a single-contact CP for membrane proteins refolded in bicelles, and up to 45% on average improvement as compared to CP-MOIST. The REP-CP sequence is based on multiple equilibration-re-equilibrations of the two spin reservoirs, and is depicted in Fig. 1. Consider N_I abundant and N_S dilute spins (e.g. ^{15}N nitrogens) with the gyromagnetic ratios γ_I and γ_S, respectively. After each CP-contact followed by two flip-back pulses and the z-filter, the protons are re-equilibrated to the lattice temperature. After two simultaneous 90-degree pulses followed by the application of radiofrequency irradiation with the amplitude B_{1I} for the protons and B_{1S} for the nitrogens, the spin temperature of the latter equilibrates with that of the proton bath, initially at the temperature T_0 (in the tilted frame). Assuming that the two spin systems are at thermodynamic equilibrium at all times, we write for the conservation of energy in the doubly tilted rotating frame [18] after each contact:

$$-\beta_0 C_I B_{1I}^2 - \beta_{n-1} C_S B_{1S}^2 = -\beta_n (C_I B_{1I}^2 + C_S B_{1S}^2) \quad (1)$$

Here the symbol β_n denotes the inverse spin temperature in the tilted frame after the nth contact, $\beta_0 = \hbar/k_B T_0$, and $C_I = 1/3\gamma_I^2 \hbar I(I+1)N_I$ and $C_S = 1/3\gamma_S^2 \hbar S(S+1)N_S$ are the Curie constants for the I and S spins, respectively. In establishing the above relation, we have also assumed that the spin–lattice relaxation time of the dilute spins is much longer than that of the high-gamma abundant spins. Consequently, the inverse spin temperature of the low-gamma spins, β_{n-1}, remains constant after the previous contact if the z-filter is sufficiently short. At the exact Hartmann–Hahn match for $S = I = 1/2$, $\gamma_I B_{1I} = \gamma_S B_{1S}$, the above equation can be rewritten as:

$$\beta_n = \frac{\beta_0 + \varepsilon\beta_{n-1}}{1+\varepsilon} \quad (2)$$

where $\epsilon = N_S/N_I < 1$. The final spin temperature of the I–S system after n contacts can be obtained by summing up a geometric progression, which yields:

$$\beta_n = \frac{\beta_0}{1+\varepsilon}\sum_{k=0}^{n-1}\left(\frac{\varepsilon}{1+\varepsilon}\right)^k = \beta_0\left[1-\left(\frac{\varepsilon}{1+\varepsilon}\right)^n\right] \to \beta_0, n \to \infty \quad (3)$$

In contrast to the original multiple-contact experiment [7], the convergence (albeit a very fast one for small values of ϵ) is achieved to the inverse spin temperature β_0 instead of being proportional to β_0/ϵ (which would have been a much more desirable behavior since $\epsilon < 1$). This simplified thermodynamic treatment implies that even if the ratio between the number of protons and that of the dilute spins is finite, one can nevertheless equilibrate them to the same spin temperature corresponding to the intact proton bath. Having equal amounts of magnetization on both nitrogen spins and protons is advantageous for multidimensional separated local field (SLF) experiments in order to minimize the positive or negative zero-frequency peaks such as those observed in PISEMA [25] or SAMPI4 [26]. For instance, if $\epsilon = 0.13$ (7–8 protons per nitrogen spin, the ratio typical for proteins), after one CP contact resulting in the nitrogen spin-locking temperature $\beta_0(1+\epsilon)^{-1}$ only 88% of the intact proton bath temperature will be transferred. However, after just two re-equilibrations this number will increase to 99%. It should be noted that $T_{1\rho}$ relaxation and Hartmann–Hahn mismatches due to rf field inhomogeneity have not been explicitly taken into account by Eq. (1), which may yield even lower magnetization transferred in each contact. Moreover, there is an additional factor that may limit the final amount of magnetization. A recent many-body quantum–mechanical treatment [24] has shown that a single nitrogen spin never achieves full thermodynamic contact with the proton bath even under perfect Hartmann–Hahn matching conditions. Briefly, for a system consisting of a single nitrogen spin and N_I protons, the density matrix $\rho(t)$ obeys unitary evolution in the doubly tilted rotating frame:

$$\rho(t) = e^{-iH_T t} I_z e^{iH_T t} \quad (4)$$

Here the truncated CP Hamiltonian for the IS system, H_T, is given by:

$$H_T = \frac{1}{4}\sum_{n=1}^{N_I} a_n (S_+ I_-^{(n)} + S_- I_+^{(n)}) - \frac{1}{2}\sum_{i<j}^{N_I} b_{ij}\left[\frac{3}{2} I_z^{(i)} I_z^{(j)} - \frac{1}{2}\mathbf{I}^{(i)}\mathbf{I}^{(j)}\right] \quad (5)$$

where the a_n are the coupling constants describing the dipolar interactions between the S spin (nitrogen) and the I spins (protons), and b_{ij} are the coupling constants for the homonuclear interactions. The quasistationary amount of normalized transferred magnetization is calculated as [24]:

$$M_S(\infty) = \lim_{t\to\infty}\frac{Tr(S_z e^{-iH_T t} I_z e^{iH_T t})}{Tr(S_z^2)} = 1 - 2^{-N_I+1}\sum_k |\langle k'|S_z|k\rangle|^2 < 1 \quad (6)$$

where the summation is carried over the eigenvectors indexed by k and k' that correspond to the same degenerate eigenvalues of the Hamiltonian, H_T. The standard bra-ket notation is used to designate the matrix elements of the operator S_z. Many-spin simulations have shown that the quasi-stationary limit of the transferred magnetization, Eq. (6), converges to a value of around 0.84 [24]. Therefore, the nitrogen spin temperature as given by Eq. (2) should be corrected by this factor at each transfer step. The combination of the above quantum–mechanical (84%) and thermodynamic bounds (88%) would result in the single-step CP enhancement of around 74% relative to the maximum thermodynamic limit (i.e. γ_H/γ_N). Hartmann–Hahn mismatches and loss of spin-lock due to $T_{1\rho}$ relaxation could lower this amount even further. Disregarding the additional losses, the theoretical gain factor for REP-CP is thus estimated to be 1.35 relative to a single-contact CP.

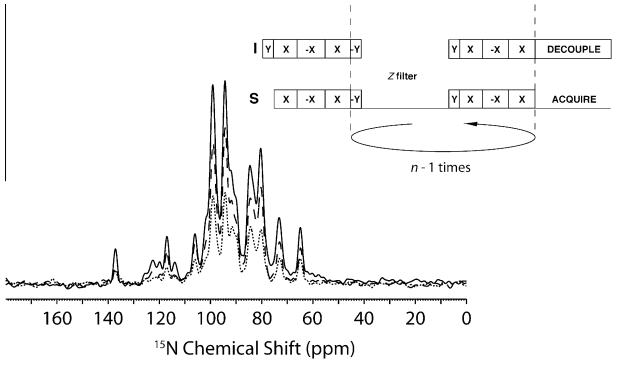

Fig. 1. The REP-CP pulse sequence employing repetitive CP contacts during the preparation period and experimentally observed enhancement for NMR spectra for Pf1 coat protein reconstituted in magnetically aligned bicelles. Single-contact CP (dotted line); CP-MOIST (dashed line); REP-CP (solid line). A twofold gain in the signal-to-noise ratio is obtained for the case of REP-CP as compared to the conventional CP, and a 45% gain over CP-MOIST. All experiments have equal total experimental times; 50 Hz exponential line broadening has been applied.

The method of multiple equilibrations of high- and low-gamma spins has been applied to Pf1 coat protein both in the phage form and reconstituted in magnetically aligned bicelles [5]. Uniformly ^{15}N-labeled Pf1 phage sample was purchased from Hyglos GmbH (Regensburg, Germany). To isolate the protein, the sample was dissolved in 1 ml of TFE (50%)/TFA (0.1%) in order to remove the DNA, and the soluble fraction was isolated and lyophilized. About 6 mg of pure lyophilized protein was reconstituted in DMPC/DHPC (at 3:1 M ratio) bicelles as previously described [27]. All experiments have been performed on a Bruker Avance II spectrometer operating at 500 MHz ^{1}H frequency with Topspin 2.0 software. A commercial Bruker 5 mm round low-E coil probe was used. For the bicelle-

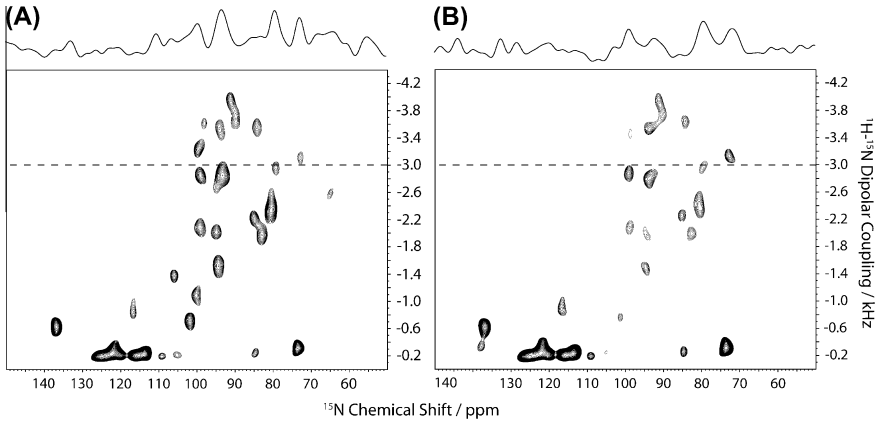

Fig. 2. SAMPI4 experiments for Pf1 coat protein reconstituted in magnetically aligned bicelles acquired at 500 MHz proton frequency. (A) REP-CP enhanced SAMPI4 (14 scans for each t_1 increment); (B) CP-MOIST enhanced SAMPI4 (16 scans). Representative slice along 3 kHz in the dipolar dimension shows that that superior sensitivity is obtained in part A (with 6 REP-CP contacts, 300 μs each, 0.15 s *z*-filter time) as compared to CP-MOIST with a single 1 ms contact (part B). The experimental time was 1 h and 42 min for each experiment.

reconstituted protein, the sample temperature was maintained at 38 °C, 6 s recycle delay and 40.3 kHz B_1 fields were used. The *z*-filter time was chosen as short as possible to shorten the overall length of the experiment, on the one hand, and to minimize the losses of ^{15}N magnetization due to proton-driven spin diffusion, on the other. At the same time, however, the *z*-filter should be sufficiently long to let the proton bath equilibrate to the lattice temperature after each flip-back pulse. Optimal *z*-filter times of less than 1 s have been found. Increasing the *z*-filter time to greater than 1 s yielded considerable loss in intensity due to proton-driven spin diffusion (results not shown).

As expected for biological samples having $T_{1\rho}$'s of several milliseconds [21], which is shorter than the acquisition period (typically about 10 ms), the original multiple-contact scheme [7] is not very efficient. Experiments on ^{15}N-labeled Pf1 phage (see Supplementary material) have demonstrated that for the same number of transients, the signal-to-noise ratio is even less than for the single-contact CP-MOIST experiment [10,11] since more noise than signal is acquired during the subsequent contacts. For Pf1 coat protein reconstituted in magnetically aligned bicelles, ^{15}N-detected proton $T_{1\rho}$ experiment (see Supplementary material) has yielded the $T_{1\rho}$ relaxation time of 4.3 ms, which would make the multiple contacts also prohibitive for such systems. The spin–lattice relaxation time for the protons has been determined by an ^{15}N-detected proton inversion recovery, which yielded T_{1Z} = 1.3 s. While the experimentally found optimal time of the *z*-filter for the bicelle-reconstituted protein (0.15 s) is much shorter than ^{1}H T_{1Z}, in combination with the flip-back pulses it appears to be sufficient to re-equilibrate the proton bath to the lattice temperature, and thus achieve the successive ^{15}N magnetization enhancement. This is due to the fact that the amide protons, which donate most of the magnetization to the nitrogen spins, are quickly re-equilibrated by the rest of the proton bath by spin diffusion. A very similar mechanism of an accelerated T_1 relaxation was observed for the carbonyl carbons in the RELOAD-CP experiment employing selective excitation pulses [14]. Fig. 1 shows an overlay of the REP-CP experiment (5 CP-MOIST contacts each having 300 μs contact time, 0.15 s z-filter time), CP-MOIST, and CP (with a 300 μs contact time). (The parameters for REP-CP may need to be optimized depending on the type of sample; fewer numbers of contacts can also be used since most of the magnetization is transferred from the protons to the nitrogen spins after as little as 3–4 contacts.) One thousand twenty-four transients were acquired in the conventional CP and CP-MOIST experiments; whereas 930 scans were acquired for the REP-CP experiment (resulting in equal total times for each experiment of 1 h and 42 min). It should be noted that the single-contact CP-MOIST sequence [10,11] already yields an over 50% enhancement as compared to the conventional CP (the integral ratio between the two spectra is 1.55). This observation may indicate that in magnetically aligned bicelles it is difficult to satisfy the exact Hartmann–Hahn match for every protein species present in the sample, possibly due to rf field inhomogeneity and/or sample heating [17]. Notably, more than a factor-two gain in intensity is achieved by the REP-CP experiment as compared to the single-contact CP experiment (the integral ratio between the two spectra is 2.2), and about a 45% gain in the signal-to-noise ratio when compared to the CP-MOIST. A comparison of the REP-CP spectrum with direct-excitation ^{15}N spectrum for the transmembrane helix region (see Supplementary material) has yielded from 9 to 13-fold intensity enhancement depending on the spectral position, with the integral ratio taken over the helical regions equal to 9.8. Taking into account the very low sensitivity obtainable by direct ^{15}N excitation, these gain values on average correspond to the ratio γ_H/γ_N. It should also be noted that for Pf1 phage the enhancement factor for the REP-CP was only 1.51 as compared to a single-contact CP, and 1.25 as compared to CP-MOIST (see Supplementary material). This would mean that, for the more rigid phage samples, a single-contact CP is more efficient than for the more dynamic bicelle-reconstituted proteins which are rapidly re-orienting about the axis perpendicular to the main magnetic field. An even lower (<20%) enhancement was previously observed for a single crystal of *n*-aceyl Leucine [24], which has a much higher proton-to-nitrogen ratio (15:1), on the one hand, and virtually no dynamics on the other, thus yielding greater single-contact CP efficiencies. This issue merits additional investigation as it may allow one to study semi-quantitatively the dynamics of membrane proteins by comparing the relative amounts of the transferred cross-polarization as compared to the single-contact CP and, whenever feasible, to direct excitation of the low-gamma nuclei.

Such an enhancement can appreciably speed up the acquisition of multidimensional NMR data. Fig. 2 shows two-dimensional SAMPI4 [26] spectra for the bicelle-reconstituted Pf1 coat protein acquired with only 14 transients per each of the 64 t_1 increments for the REP-CP and 16 scans for the CP-MOIST enhanced SAMPI4 (resulting in less than 2 h total time per experiment). It can be seen that the application of repetitive CP contacts during the preparation period is sufficient to detect all peaks in the spectrum; whereas if the initial enhancement is made via CP-MOIST more scans would be necessary. A comparison of the signal-to-noise ratios for 21 fully resolved resonances in the transmembrane α-helical region [5] of the Pf1 protein (performed at 128 scans; see Supplementary material) is shown in Fig. 3. As can be seen, the

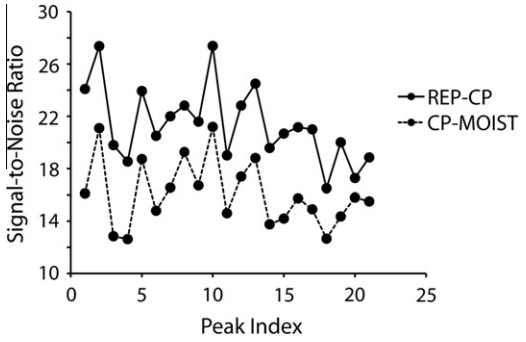

Fig. 3. Comparison of the signal-to-noise ratios for 21 resolved resonances in the α-helical transmembrane region of Pf1 coat protein. Solid-dotted line: REP-CP enhanced SAMPI4 (128 scans, other parameters are as in Fig. 2); dashed-dotted line: CP-MOIST enhanced SAMPI4 (128 scans). For select peaks the gain is up to 60% with an average gain of 35%.

use of the REP-CP scheme of Fig. 1 has yielded up to 60% intensity enhancement for some peaks as compared to CP-MOIST SAMPI4 (with an average enhancement of 35%). Such a gain in sensitivity would amount to reducing the overall time of the experiment which otherwise would have been required to achieve the same signal-to-noise ratio by a factor of 1.8. In combination with the recently developed selective evolution technique [28,29] even greater signal-to-noise enhancements can be expected in the SLF experiments. In addition, the use of the above methods in combination with paramagnetic T_1 relaxation enhancers [30] may considerably reduce the *z*-filter lengths and the overall acquisition time for NMR of oriented membrane proteins.

In conclusion, the REP-CP cross-polarization scheme based on multiple equilibration-re-equilibration of the high- and low-gamma reservoirs has yielded a significant gain in the signal-to-noise ratio as compared to conventional cross-polarization. Contrary to only a marginal (12%) enhancement expected from the conservation of energy argument under the assumption of thermodynamic equilibrium, and experimentally observed in single crystals [24], a twofold enhancement has been obtained for Pf1 coat protein reconstituted in magnetically aligned bicelles. This is primarily due to a combination of thermodynamic bounds (finite ratios of the numbers of high- and low-gamma spins in proteins) and quantum mechanical bounds on spin dynamics resulting in reduced quasi-equilibrium magnetization transferred to the low-gamma spins in a single CP contact. Furthermore, Hartmann–Hahn mismatches due to rf field inhomogeneity, protein and bilayer dynamics, rf heating, and $T_{1\rho}$ relaxation, can further considerably reduce the amount of the transferred magnetization for fully hydrated biological samples. Regardless of the type of losses, however, repetitive CP contacts during the preparation period would bring the two spin reservoirs to the spin temperature corresponding to the intact proton bath. The proposed REP-CP scheme can also be applied to other NMR samples where the $T_{1\rho}$ relaxation times are short.

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Appendix A. Supplementary material

Supplementary data associated with this article (additional experiments and the SAMPI4 pulse program incorporating repetitive CP contacts) can be found, in the online version.

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmr.2011.06.028.

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