Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Magnetic Resonance 164 (2003) 329–337
JMR Journal of Magnetic Resonance
www.elsevier.com/locate/jmr

Recoupling of residual dipolar couplings in single-domain polymer-stabilized liquid crystals undergoing magic-angle spinning

Jean-François Trempe,[1] Alexei Denisov, and Kalle Gehring*

Department of Biochemistry, McGill University, McIntyre Medical Sciences Building, 3655 Promenade Sir William Osler, Montréal, Que., Canada H3G 1Y6

Received 23 March 2003; revised 29 June 2003

Abstract

Measurement of dipolar couplings, chemical shift anisotropies, and quadrupole couplings in oriented media such as liquid crystals are of great importance for extraction of structural parameters in biological macromolecules. Here, we introduce a new technique, SAD-REDOR, that consists of recoupling heteronuclear dipolar couplings in molecules dissolved in a single-domain liquid crystal or other oriented medium through the combined use of magic-angle spinning and rotor-synchronized radiofrequency pulses. This application of the REDOR pulse sequence to oriented media offers several advantages such as selectivity over the type of coupling recovered and tunable scaling of the interaction. The effectiveness of the technique is demonstrated both theoretically and experimentally, using the recently developed polyacrylamide-stabilized Pf1 phage medium and ^{15}N-labeled benzamide as the aligned molecule.

Keywords: Polyacrylamide-stabilized liquid crystals; Pf1 phage; REDOR; Magic-angle spinning; Recoupling; Residual dipolar coupling

1. Introduction

Liquid crystals have been extensively used in NMR spectroscopy for the study of anisotropic interactions and to characterize structural and dynamic properties of aligned molecules [1,2]. Averaged anisotropic interactions in liquid crystals depend not only on the distance between interacting nuclei, but also on the angle of the internuclear vector (or CSA tensor) in the principal alignment system of the molecule. A recent and important advance in that field is the measurement of residual dipolar couplings and chemical shift anisotropies (CSA) in biological macromolecules [3,4]. An alignment tensor of low magnitude (typically below 10^{-3}) is obtained by dissolution of the macromolecule of interest in a dilute liquid crystal [5,6] or in an anisotropic medium such as strained polyacrylamide gel (SAG) [7,8]. The low degree of alignment enables measurement of strong dipolar couplings and chemical shift anisotropies, while keeping favorable characteristics of liquid state spectra such as sharp resonances and long relaxation times.

Although the methodology has been successfully applied in a number of studies, there are some limitations. In the alignment medium, the anisotropic and isotropic interactions are expressed simultaneously and it is necessary to measure the isotropic component in a second medium where the molecule is tumbling isotropically. The isotropic value is assumed to be the same in both media and the desired anisotropic interactions can then be extracted. This assumption is generally true if there are no conformational or chemical changes in the macromolecule dissolved in the aligning medium. The pH, temperature, and ionic strength of the buffer also need to remain the same. Scalar couplings are relatively insensitive to these parameters, but the isotropic chemical shifts are more sensitive to the chemical environment and temperature. This renders

* Corresponding author. Fax: +1-514-847-0220.
E-mail addresses: jean-francois.trempe@bioch.ox.ac.uk (J.-F. Trempe), kalle.gehring@mcgill.ca (K. Gehring).
[1] Present address: Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK.

1090-7807/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/S1090-7807(03)00247-7

difficult the accurate measurement of the anisotropic component of the chemical shift in liquid crystals [9]. Another difficult case is when the macromolecule interacts strongly with a particular medium; for example, a peptide associated to magnetically aligned bicelles [10,11] or a basic protein binding to negatively charged Pf1 phage particles. The alignment magnitude is then typically greater than 10^{-3}, resulting in shorter relaxation times and multiple unresolved dipolar couplings that increase the apparent resonance linewidth. Finally, even if the alignment magnitude is tuned to desired levels, intrinsically small long-range dipolar couplings remain small and the precision at which they can be measured is limited.

Methods have been developed to overcome some of the difficulties mentioned above. One promising approach is to manipulate the director of bicellar liquid crystalline phase by variable angle sample spinning (VASS) [12,13]. This method enables the scaling down and sign-determination of residual dipolar couplings. This is made possible by the fast reorientation rate of the bicellar mesophase. Consequently, it cannot be applied to fixed orientation media such as strained polyacrylamide gels [7,8] and polymer-stabilized liquid crystals [8,14], whose principal alignment axes do not reorient with the magnetic field. Albeit, magic-angle spinning (MAS) is a simple and powerful mean of obtaining the isotropic component of a sample in an anisotropic medium. Recently, switched-angle spinning (SAS) experiments, which correlate the isotropic MAS spectrum with its anisotropic component in a two-dimensional NMR spectrum, have also been introduced [11,13,15]. Finally, unresolved homonuclear ^{1}H–^{1}H dipolar couplings that broaden lineshapes can be removed by selective RF decoupling [16].

Here, we propose a new alternative to measure dipolar couplings and CSA in biological macromolecules that complements the methods previously mentioned. It consists in recoupling anisotropic interactions in a MAS single-domain polymer-stabilized uniaxial liquid crystal [14]. Using rotor-synchronized pulse sequences, we show that it is possible to recover residual dipolar couplings, which are otherwise eliminated by sample rotation. The method combines MAS with rotor phase synchronization, previously studied in single-crystals [17] and partially oriented solids [18], with solid-state recoupling techniques such as REDOR [19] or two-dimensional spin-echo spectroscopy [20]. We refer to the application of the REDOR methodology as SAD-REDOR, for single-alignment domain REDOR. In the experiment, specific dipolar couplings are reintroduced through a combination of spatial reorientation (magic-angle spinning) of the liquid crystal director and synchronized radiofrequency (RF) pulse spin manipulation. Because the technique is equivalent to spinning a single crystal, the *rotor phase* when the RF pulse is applied is of the utmost importance in determining the size and sign of the recoupled interaction. This feature is used advantageously to scale isotropic and anisotropic quantities by varying the rotor phase of pulse application. We describe theoretically the pulse sequence and then demonstrate its applicability in a model system of ^{15}N-labeled benzamide dissolved in a liquid crystal of polyacrylamide-stabilized Pf1 phage. Potential biomolecular applications, advantages, and limitations of the method are discussed.

2. Theory

The anisotropic contribution of a second-order tensor interaction λ in the laboratory frame is related to the component in a uniaxially symmetric alignment medium by:

$$T_{\lambda}^{(2,0)} = \frac{1}{2}(3\cos^2\theta - 1)T_{\lambda,d}^{(2,0)}, \tag{1}$$

where θ is the angle between $\mathbf{B}_0$ and the liquid crystal director [2,21]. The term $T_{\lambda,d}^{(2,0)}$ is itself related to spherical tensor quantities in the molecular frame, as well as to the average of Wigner D-functions describing molecular frame rotation into the director frame

$$T_{\lambda,d}^{(2,0)} = \sum_{m=-2}^{2} T_{\lambda,\mathrm{mol}}^{(2,m)} \langle D_{m,0}^{2*} \rangle. \tag{2}$$

There are five independent terms in the set of $\langle D_{m,0}^{2*} \rangle$, which are related to the five independent elements of the Saupe order matrix [22]. The term $T_{\lambda,d}^{(2,0)}$ is the residual anisotropic interaction observed when the aligned uniaxial phase director is parallel to the magnetic field.

Considering the evolution of anisotropic interactions under sample rotation, we can conveniently decompose $\cos\theta$ in Eq. (1) as a function of α, β, and γ (Fig. 1a):

$$\cos\theta(t) = \cos\beta\cos\gamma + \cos\alpha(t)\sin\beta\sin\gamma. \tag{3}$$

The angle β is constant and unique in a single-domain PSLC sample, and constant rate sample rotation makes α the only dynamic variable. The variable γ is the fixed angle of the rotor axis. Time-averaging of the interaction can be described by integrating the term in Eq. (1) over the variable α:

$$\overline{T}_{\lambda}^{(2,0)} = \frac{T_{\lambda,d}^{(2,0)}}{4\pi} \int_{\alpha_0}^{\alpha_0+\omega_r t} [3(\cos\beta\cos\gamma + \cos\alpha(t) \times \sin\beta\sin\gamma)^2 - 1]\,\mathrm{d}\alpha, \tag{4}$$

where ω_r is the sample rotational velocity (rad s^{-1}) and α_0 is the rotor phase at the beginning of the pulse sequence. It should be noticed that this latter calculation is equivalent to an integration over the variable t, after replacing α by the term $\alpha_0 + \omega_r t$. The final result of this

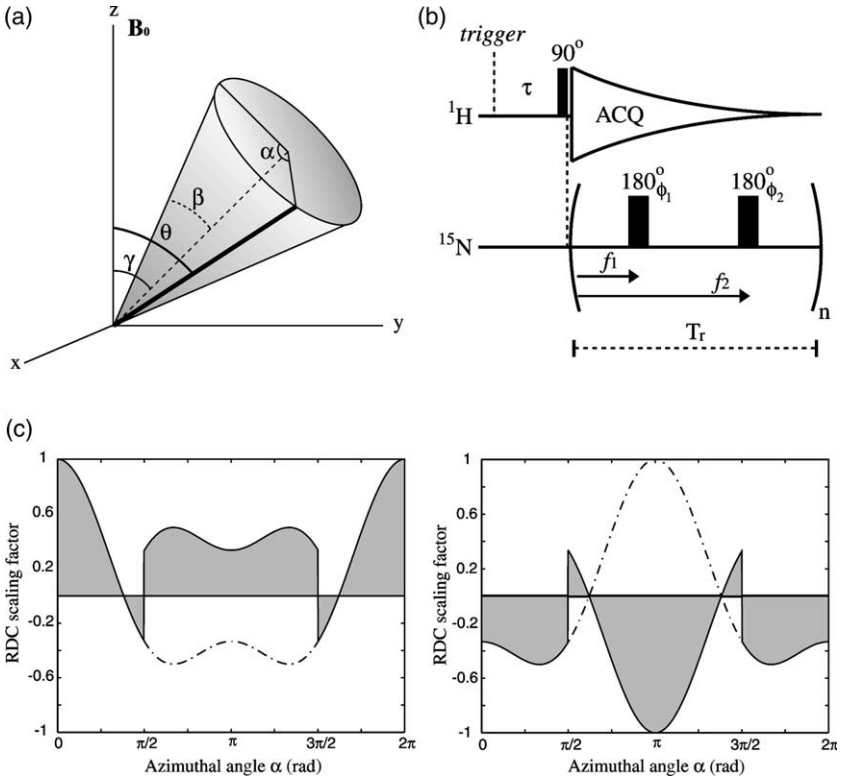

Fig. 1. Schematic illustration of the phase-synchronized SAD-REDOR experiment. (a) Geometry of the various angles involved in description of the experiment. The PSLC director is the thick line lying on the surface of the cone, which represents the trajectory of the director under sample rotation, and the dashed line is the rotor axis. The angle θ between the magnetic field $\mathbf{B}_0$ and the PSLC director is related to other angles in Eq. (3). (b) One-dimensional SAD-REDOR rotor-synchronized pulse sequence. The trigger pulse is a command in the pulse program that enables phase synchronization with the rotor azimuthal angular position. After the ^{1}H 90° pulse, the recoupling pulse train starts on the ^{15}N channel, synchronously with the acquisition period. T_r is the MAS rotation period, and f_1, f_2 are the fractions of T_r at which recoupling pulses are applied. The phase cycle for ^{15}N inversion pulses is $\phi_1 = +x$, $\phi_2 = -x$. (c) Principle of heteronuclear recoupling under PSLC director rotation, using $\beta = \gamma = 54.7°$ and $\alpha_0 = 0°$ (left) or π (right). Inversion pulses are applied at $\pi/2$ and $3\pi/2$ of the rotor cycle. When no recoupling pulse is applied (dashed line), the integral from 0 to 2π is zero and there is no average dipolar coupling evolution. Application of a π pulse synchronized with the rotation of the MAS rotor reverses the sign of dipolar coupling dephasing and makes the integral non-zero over a complete rotor cycle (gray area). Note that the sign of dephasing depends on the initial orientation of the director and the position of the inversion pulses.

calculation is similar to that obtained in previous theoretical analysis of the REDOR experiment [23]. Integration of Eq. (4) gives:

$$\overline{T}_{\lambda}^{(2,0)} = \frac{T_{\lambda,d}^{(2,0)}}{4\pi}\Big[\alpha(3\cos^2\beta\cos^2\gamma - 1) + 6\sin\alpha\cos\beta\sin\beta\cos\gamma\sin\gamma + 3\sin^2\beta\sin^2\gamma\left(\frac{\alpha}{2} - \frac{\sin 2\alpha}{4}\right)\Big]_{\alpha_0}^{\alpha_0+\omega_r t}. \quad (5)$$

If γ is set to the magic-angle, $\cos^{-1}(\sqrt{1/3})$, the integral over a complete rotor cycle is equal to zero, irrespective of the value of β or α_0. Consequently, all second-order interactions are averaged to zero under MAS.

It is possible to interfere with the averaging process by application of rotor-synchronized RF pulses . Let us consider a coupled I–S spin system, with antiphase magnetization of the type $2I_{x,y}S_z$. To simplify calculations, we will abbreviate Eq. (5) by:

$$\overline{T}_{\lambda}^{(2,0)} = \frac{T_{\lambda,d}^{(2,0)}}{4\pi} I_{\alpha_0}^{\alpha_0+\omega_r t}. \quad (6)$$

The effect of a π pulse on the S nucleus is to invert the sign of heteronuclear coupling, while a π pulse on the I nucleus will invert the evolution of both heteronuclear coupling and chemical shift interactions. Application of a pair of π pulses at fractions f_1 and f_2 of the rotor cycle generally yields non-zero residual anisotropic interaction over a complete rotor cycle [23]. Assuming the recoupling pulse lengths are a negligible fraction of the rotor period, the latter situation can be formulated as

$$\overline{T}_{\lambda}^{(2,0)} = \frac{T_{\lambda,d}^{(2,0)}}{4\pi}\left[I_{\alpha_0}^{\alpha_0+2\pi f_1} - I_{\alpha_0+2\pi f_1}^{\alpha_0+2\pi f_2} + I_{\alpha_0+2\pi f_2}^{\alpha_0+2\pi}\right] \quad (7)$$

as graphically depicted in Fig. 1. The magnitude and sign of the recoupled anisotropic interaction not only depend on the position of pulses in the rotor cycle, but also on the initial director orientation (α_0) at the beginning of the recoupling scheme [17,18].

Inversion π pulses also refocus isotropic interactions such as the heteronuclear scalar coupling and the isotropic component of the chemical shift. The time-average of these interactions over a period equivalent to a complete rotor cycle with REDOR pulses is given by:

$$\overline{T}_{\mathrm{iso}} = T_{\mathrm{iso}}(1 + 2f_1 - 2f_2), \tag{8}$$

where T_{iso} is equal to one-third of the trace of the chemical shift tensor in the case of the CSA, or equal to the isotropic J-coupling for the corresponding pair of nuclei in the case of the dipolar coupling. The aforementioned interactions are invariant under sample rotation. The anisotropic component of the J-coupling is negligible for nuclei encountered in biomolecular NMR, and is thus safely ignored. The observed total interaction along the magnetic field z is thus simply the sum of the anisotropic and isotropic contributions [21]:

$$\overline{T}_{zz} = \overline{T}_{\lambda}^{(2,0)} + \overline{T}_{\mathrm{iso}}. \tag{9}$$

The total interaction can be measured either directly during the acquisition period, or indirectly as part of a two-dimensional experiment.

Differential scaling of the isotropic and anisotropic interactions can be achieved since $\overline{T}_{\lambda}^{(2,0)}$ is a function of α_0, f_1, and f_2, while $\overline{T}_{\mathrm{iso}}$ is a function of f_1 and f_2 only. The scaling of the isotropic interaction is tuned first by adjusting f_1 and f_2. The anisotropic interaction is then optimized by adjusting α_0, within the limits imposed by the choice of f_1 and f_2.

For a MAS rotor, where γ is set to the magic-angle, we can calculate the scaling factor of the recoupled residual dipolar coupling for different values of f_1 and β (Fig. 2). This scaling factor, which we will represent by the symbol ζ, is defined as the ratio of the dipolar couplings measured with SAD-REDOR, over that in a fully aligned static sample. One advantage of SAD-REDOR is that it is possible to recouple the dipolar coupling with the initial orientation of the director either parallel ($\alpha_0 = 0$) or nearly perpendicular ($\alpha_0 = \pi$) to the magnetic field. This inverts the sign of recoupling evolution so that subtraction of the two couplings, measured at $\alpha_0 = 0$ and π, doubles the size of the dipolar coupling component, while canceling the invariant scalar coupling. In a static sample, this would be analogous to measuring the dipolar coupling by comparing $D_{\parallel}$ and $D_{\perp}$.

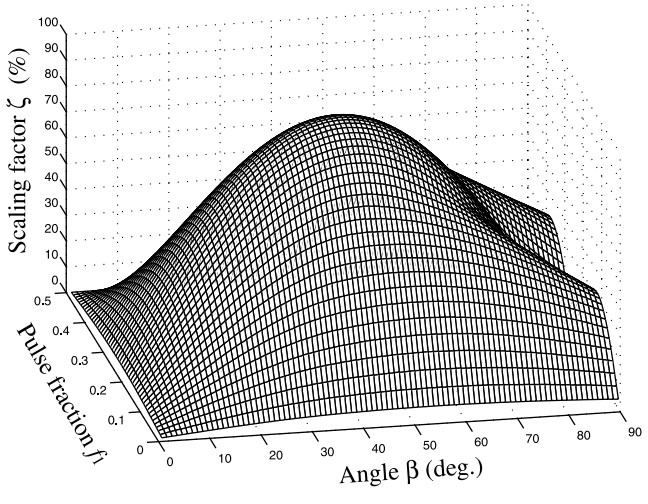

Fig. 2. The recoupling scaling factor ζ as a function of the angle β between the director and the rotor axis, as well as on the fraction f_1 of inversion pulse application. Two pulses are applied symmetrically with respect to the middle of the rotor cycle, which makes $f_2 = (1 - f_1)$. The recoupling efficiency is defined as the ratio of the difference between the largest positive and negative recoupled dipolar couplings obtained with α_0 of 0 and π, respectively, and the full static dipolar coupling. The maximum efficiency of 90.0% is found at $\beta = 45°$, $f_1 = 0.25$, and $f_2 = 0.75$.

The optimal recoupling sequence is 0.25 and 0.75 for f_1 and f_2, respectively, and β equal to 45°, which make ζ equal to 90.0%. However, this pulse scheme cancels the contribution of the scalar coupling to the observed splitting, making the dipolar sign discrimination difficult. In other words, the sign of the residual scalar contributions enables the determination of the sign of the residual dipolar coupling. Therefore, it is more convenient to set f_1 and f_2 to 0.2 and 0.8, and β to the magic-angle. In this case, ζ is equal to 80.4% and the scalar coupling contribution is reduced to 20% of its full value, which permits measurement of the sign of the dipolar coupling. For CSA measurements, f_1 and f_2 values of 0.25 and 0.75 is preferable since the isotropic chemical shift is completely eliminated and the sign of the CSA can be measured without it.

For angles β greater than 70.6° (180°–54.7°–γ(54.7°)), the RDC scaling factor crosses zero four times and optimal recoupling pulse sequences contain four π pulses per rotor cycle. While less useful for polymer-stabilized phage liquid crystals, four pulse recoupling schemes might be applicable to other oriented media such as compressed polyacrylamide where β, the angle between the director and the spinning axis, might naturally be 90°.

3. Experimental

Pf1 bacteriophages were grown and purified according to the literature [24]. ^{15}N-labeled benzamide (CAS 31656-62-9) was obtained from Cambridge Isotope Laboratories (MA). The amide proton resonances of benzamide (0.1 M) dissolved in 70/5/25% v/v H_2O/D_2O/DMSO-d_6 were assigned using a 2D-NOESY spectrum at 10 °C recorded on a Bruker DRX-600 spectrometer. A positive NOE (2%) was found between *ortho* protons of the phenyl ring and the amide proton at 8.0 ppm. Therefore, amide protons at 8.0 and 7.2 ppm, respectively, correspond to *cis* and *trans* positions with respect to the *ipso* carbon. This was confirmed by the vicinal

couplings J(C*ipso*–H*trans*) = 7.5 Hz and J(C*ipso*–H*cis*) <4 Hz, measured in a proton-coupled ^{13}C NMR spectrum. Residual dipolar couplings were measured in solutions of 20 and 25 mg/ml Pf1 with 25/5% DMSO/D_2O.

Polyacrylamide-stabilized samples were prepared as recently described [14] with 7.0% w/v acrylamide, 0.25% w/v 1,9-bisacrylamide, 0.1% v/v *N*,*N*,*N*′,*N*′-tetramethyl-ethylene-diamine, 0.2% w/v ammonium persulfate, 30 mg/ml w/v Pf1 phage, 0.15 M ^{15}N-labeled benzamide, 25% v/v DMSO-d_6, and 5% v/v D_2O. Recoupling NMR experiments were carried out on a Bruker DRX-500 spectrometer equipped with a triple-resonance ^{1}H/^{15}N/^{13}C high-resolution air-driven MAS probe (Bruker Biospin). Samples were polymerized for 1 h with the static rotor at the magic-angle, so that the angle β is equal to the magic-angle (Fig. 1a). Static ^{2}H spectra acquired at different rotor azimuths confirmed the stability of the liquid crystal director, before and after MAS.

The REDOR pulse sequence used to recover ^{1}H–^{15}N residual dipolar couplings is shown in Fig. 1b. The ^{15}N RF π pulse train was written as a composite decoupling pulse program which starts synchronously with the ^{1}H acquisition period, with a field strength of 5.8 kHz. Rotor speeds varied between 250 and 1000 Hz. The phase of recoupling pulses followed a $(x)(-x)$ scheme to compensate for errors in the ^{15}N pulse length. The dwell time during acquisition was set independently of the rotor period. Rotor phase synchronization was achieved using the tachometer signal from the MAS pneumatic unit. The optical signal from the rotor was used not only to monitor the angular velocity, but also to synchronize the pulse program with the rotor phase. The Bruker pulse program contained the *trigpe1* command, which waits for a positive edge signal before resuming the acquisition. The delay τ was varied to change the rotor phase α_0 at the beginning of the acquisition period. The synchronization delay varied from 1/20th to one rotor cycle by fractional increments of 1/20, for a total of 20 experiments per pulse scheme. Four different pulse schemes were tested with $f_1 = 0.1, 0.2, 0.3, 0.4$ and $f_2 = (1 - f_1)$. A MAS spinning rate of 302 Hz was chosen so that the sidebands originating from the water signal did not overlap with the benzamide amide protons resonances, and to minimize centrifugal forces on the sample. Recoupling experiments were conducted at 20 °C.

4. Results

4.1. Alignment of benzamide in Pf1 phage liquid crystalline phase

The alignment tensor of ^{15}N-labeled benzamide in liquid crystalline Pf1 phage was determined from ^{1}H–^{15}N and natural abundance ^{1}H–^{13}C couplings. At 20 mg/ml phage, ^{1}H–^{15}N dipolar couplings were 25 and −3 Hz for the downfield and upfield amide proton resonances, respectively. ^{1}H–^{13}C dipolar couplings were −37, −37, and −5 Hz, for the *meta*, *ortho*, and *para* positions at 25 mg/ml phage. The couplings were scaled for differences in phage concentration and the effective interatomic distances of 1.04 and 1.12 for the N–H and C–H bonds. Since all atoms of the amide group are in a single plane, the alignment tensor of this group has only three independent elements: the axial and rhombic components and a single angle to describe the orientation of the alignment tensor, due to the planar symmetry of the amide group [2]. Due to the rotation of the phenyl group, the *meta* and *ortho* C–H couplings were not used to calculate the alignment tensor. The couplings were fitted to an atomic model of benzamide with regular covalent geometry using an in-house algorithm written in MATLAB. The element S_{zz} of the diagonalized Saupe alignment tensor was 9.7×10^{-4} (D_a(NH) = 12.6 Hz). The rhombicity was found to be 0.05, but due to poor angular sampling in the plane perpendicular to S_{zz}, this variable was ill-defined. The principal axis of the alignment tensor was in the plane of the amide group and oriented between the *trans* and *cis* N–H bonds, 2.5° from the latter. We speculate that this axis must be nearly parallel to the carbon–oxygen electric dipole, which seems likely considering that phages themselves have a strong electric dipole due to multiple α-helices along their main axis. A very small (0.2 ppm) ^{13}C CSA measured for the carbonyl resonance confirmed the alignment tensor determination.

4.2. Measurement of couplings in benzamide

A 30 mg/ml Pf1 phage PSLC sample was subjected to slow MAS and the REDOR pulse sequence applied with τ delays ranging from $(1/20)T_r$ to T_r, using four different recoupling schemes (Fig. 3). The schemes varied in the interpulse spacing and the phase of their application with respect to the rotor position. ^{15}N–^{1}H couplings were measured in the one-dimensional ^{1}H spectra by fitting the coupled spectrum with the corresponding lineshape of each doublet components, derived from a control experiment with no recoupling pulses and well-resolved doublets separated by ca. 90 Hz. This procedure corrects for the linewidth effect that reduces the apparent splitting in overlapping doublets. The error in the coupling measurement using this method is less than ±0.4 Hz.

Given a set of experimental couplings acquired using different phase delays and pulse schemes, it is possible to extract the scalar and the dipolar couplings using Eqs. (7)–(9). The pulse positions f_1 and f_2 and the rotor angular velocity are preset experimental parameters and,

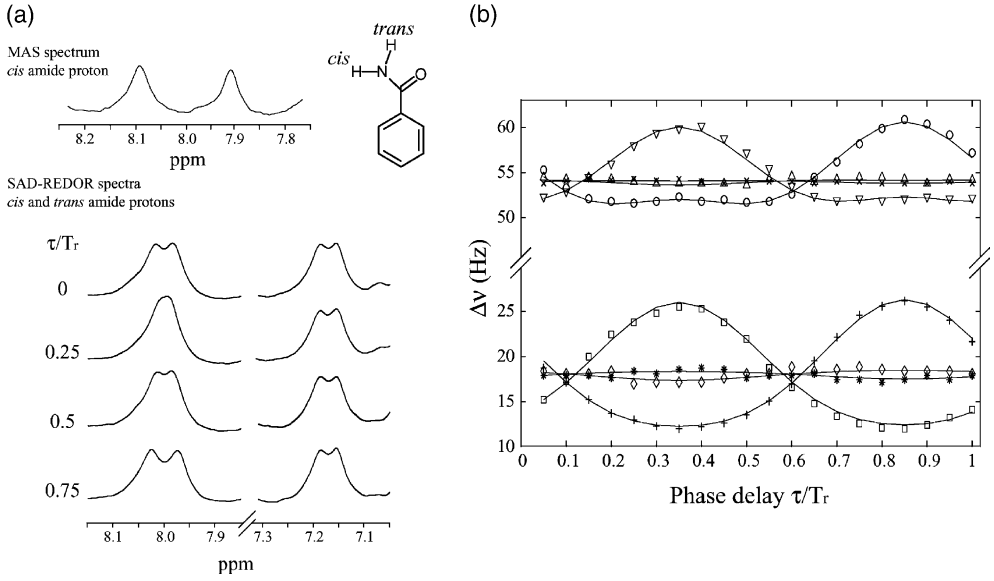

Fig. 3. Dependence of the experimental ^{15}N splitting of the two amide protons in ^{15}N-labeled benzamide on the synchronization delay τ. (a) Amide proton resonances showing the dependence of the observed coupling on the rotor phase (orientation of the PSLC director) at the beginning of the recoupling sequence. A control MAS spectrum of the downfield amide resonance is shown at the top, along with the chemical structure of benzamide. The fraction f_1 as defined in Fig. 1 is equal to 0.2. The upfield amide proton has a very small RDC and shows almost no variation of the coupling, as opposed to the downfield proton. (b) Different pulse schemes have been tested, with $f_1 = 0.1, 0.2, 0.3, 0.4$ and $f_2 = (1 - f_1)$. Experimental data points for these four schemes are, respectively, represented with symbols (○, +, □, ▽) and (×, ◇, ∗, △) for the *cis* and *trans* amide protons with respect to the *ipso* carbon. Simulated data are shown as black lines. The rmsd between predicted and experimental data is 0.3 Hz.

therefore, there are only three experimental parameters to extract: the static residual dipolar coupling, the scalar coupling, and the phase factor α_0, which are all unknown a priori. In the present case, the problem is clearly overdetermined and simple multivariable optimization was used to find the values that best fit the experimental data. The results are summarized in Table 1. The overall rmsd between experimental and estimated total couplings is ca. 0.3 Hz for both resonances. The phase factor α_0, estimated from the *cis* proton only, is found to be 5.34 ± 0.06 rad or $85 \pm 1\%$ of a rotor period.

Table 1
Least-square fit of benzamide ^{15}N–^{1}H couplings and PSLC director angular phase from SAD-REDOR measurements

Amide proton	f_1–f_2[a]	Phase[b]	J[c]	D[d]	rmsd[e]
cis	0.1–0.9	0.861	−91.2	17.3	0.14
	0.2–0.8	0.846	−87.7	18.8	0.31
	0.3–0.7	0.841	−87.3	18.3	0.44
	0.4–0.6	0.863	−91.3	15.7	0.18
	Average (SD)	0.85 (0.01)	−89.4 (2.2)	17.5 (1.4)	0.30
	In solution[f]	n.a.[g]	−89.7	25.0	n.a.
trans	0.1–0.9	0.863	−90.0	−0.6	0.14
	0.2–0.8	0.828	−88.0	−1.1	0.31
	0.3–0.7	0.814	−89.2	−1.5	0.35
	0.4–0.6	0.251[h]	−90.3	−0.6	0.28
	Average (SD)	0.83 (0.03)	−89.4 (1.0)	−0.9 (0.4)	0.28
	In solution[f]	n.a.	−88.3	−3.0	n.a.

[a] Phases of recoupling pulses application (see Fig. 1).
[b] PSLC director phase at triggering signal (fraction of T_r).
[c] Scalar coupling (Hz).
[d] Residual dipolar coupling (Hz).
[e] Root mean square deviation between predicted and experimental data (Hz).
[f] In water/DMSO and 25 mg/ml Pf1 phage.
[g] Not applicable.
[h] Not included in the average calculation.

The fitted scalar couplings were estimated to −89.4 Hz for both protons. This is close to the isotropic values measured in solution but there was considerable variation from one REDOR pulse scheme to another. One source of error that could account for these differences is the scaling of the coupling, which amplifies measurement errors. When the fitting procedure was done with the fixed scalar coupling measured in isotropic solution, there was a general increase in the rmsd by approximately 0.7 Hz. This indicates that the effective, or τ-invariant, isotropic component is very well determined from the data and that the difference observed is due to a systematic error arising from the pulse sequence. One possible cause is the propagation of errors due to slight inaccuracy in the determination of the ^{15}N π pulse length. The phase cycle $x(-x)$ was used to correct π pulse imperfections and inability to refocus completely magnetization. Although the phase cycle partially compensates for pulse errors, it does not take into account chemical shift evolution of x–y plane residual magnetization, which depends on the frequency difference between a particular resonance and the carrier frequency. Phase cycles like XY8 should compensate for such artifacts, but their application did not make a noticeable difference in the measured couplings (data not shown). This is probably because the pulse error was negligible and errors propagate only after a large number of pulses. Since most of the signals were detected in the first 100 ms or 30 rotor cycles of the acquisition period, equivalent to 60 ^{15}N pulses, this was not enough to bring the ^{15}N magnetization away from the z axis and to interfere with couplings evolution. This issue needs to be addressed further both experimentally and theoretically.

The experimentally determined recoupling scaling factor values are 56% smaller than those extrapolated from the static liquid crystalline solutions of 30 mg/ml Pf1 phage. However, the relative strengths and signs of the residual dipolar couplings have been preserved. We attribute the smaller alignment magnitude to a global loss of order during spinning caused by gel distortion from centrifugal forces and the presence of bubbles in the PSLC gel. It was not caused by disordering of the phage particles by the polymerization reaction since an initial deuterium quadrupolar splitting of ca. 30 Hz was observed before spinning, corresponding to the expected splitting. Nonetheless, sufficient order was maintained to ensure efficient and predictable phase-synchronized recoupling, as demonstrated by the goodness of the fit between the experimental and theoretical curves. For structural analysis, it is the accurate determination of relative dipolar couplings and CSA that is important.

The theoretical assumption that the pulses were small with respect to the rotor cycle, was well fulfilled since a ^{15}N π pulse at a field strength of 5.8 kHz is just 2.6% of a rotor cycle.

5. Discussion

In practice, it is not necessary to use the multivariable fitting procedure described in the preceding section. By subtracting the total splittings from data points acquired using the same recoupling scheme but with different τ delays, the scalar coupling component is exactly cancelled and a value proportional to the dipolar coupling is left. As discussed in the theory section, this process is optimal when data are acquired at $\alpha_0 = 0$ and $\alpha_0 = \pi$. For example, in the case of benzamide, we find signed values of 15.6 and −1.7 Hz for the *cis* and *trans* amide protons by subtracting observed splittings at τ/T_r 0.35 and 0.85, for $f_1 = 0.2$ (Fig. 3b). These quantities are directly proportional to the dipolar couplings measured in Pf1 phage liquid crystal. Hence, the value of the scalar coupling does not need to be explicitly determined to calculate the dipolar contribution from two SAD-REDOR measurements. However, knowledge of its sign is required to determine the absolute sign of the residual dipolar couplings using one-dimensional spectra.

The effect of varying the phage concentration in the SAD-REDOR experiment would certainly affect the resolution of the NMR spectrum. The alignment magnitude increases roughly linearly with the nematogen concentration, but the relaxation time T_2 decreases. Dipolar couplings are eliminated by the sample rotation, and thus it is the effect on relaxation that will effectively limit the use of this technique for high-resolution studies. Below alignment magnitudes of 10^{-3}, the effect of Pf1 phages on T_2 is likely to be negligible, but it could become substantial in strongly aligned systems. This will be the primary factor in determining the precision of the measurement of long-range dipolar couplings.

A striking advantage of REDOR is the selectivity over the type of interaction to be reintroduced. The MAS frees the spectrum of dipolar couplings (including ^{1}H–^{1}H homonuclear dipolar couplings) and CSA terms. RF pulses and delays are used to reintroduce a particular interaction. Conversely, a disadvantage of VASS and SAS experiments is that all interactions are scaled down by a unique, non-selective, scaling factor, determined by the angle between the spinning axis and the magnetic field [11,13,15].

The SAD-REDOR method also provides an unambiguous and accurate way to measure the anisotropic component of the chemical shift, as opposed to current methods which are based on comparison of different (isotropic and anisotropic) samples that vary in temperature, salt or buffer composition [9]. By using homonuclear pulse sequences very similar to those described here, it is possible to re-introduce the anisotropic term of the chemical shift Hamiltonian [20]. For benzamide in Pf1 phage, the residual CSA of the ^{15}N resonance was less than 0.06 ppm and too small to be measured.

Although the technique introduced here is mostly aimed at measuring residual anisotropic interactions in weakly aligned biological macromolecules, it could be adapted to study more strongly aligned systems. Various coherent averaging techniques have been developed to simplify spectral analysis in strongly aligned liquid crystals. Homonuclear and heteronuclear decoupling using RF irradiation or composite pulse sequences have been used to simplify measurement of chemical shifts or dipolar couplings in complex spin systems [25,26]. Spatial reorientation using variable or near MAS has been explored for measurement of dipolar couplings and chemical shift anisotropies [21,27] and quadrupole splittings [13]. Dynamic-director and switched-angle spinning techniques are also interesting alternatives for scaling and separating the isotropic and anisotropic interactions that occur in strongly aligned liquid crystal [15,28,29]. In the SAS experiment, the strongly coupled anisotropic spectrum is correlated with the isotropic spectrum, which allows assignment of the chemical shifts and the dipolar couplings [11,15]. Similarly, the SAD-REDOR method could be extended to study strongly aligned biomolecules, providing the extra advantage of selectivity.

Magic-angle spinning of a fixed-director liquid crystal is reminiscent of early studies by Maricq and Waugh on single-crystals and Harbison et al. on partially oriented solids [17,18]. The current study is distinguished by three major differences. The first is that the size of anisotropic interactions in dilute liquid crystals is very small. Thus, the spinning frequency need not be very high to average out completely residual CSA and dipolar couplings. This eliminates the phase distortions and spinning sidebands observed in strongly ordered systems. The low spinning rate requirement is fortunate since the PSLC hydrogel used here is soft and subject to distortion by centrifugal forces. Secondly, the uniaxial symmetry of the liquid crystal scales the anisotropic interactions according to elements of the Saupe order matrix. Couplings which are small in the liquid crystal will always remain so regardless of the angle of the MAS rotor or director. Finally, we apply REDOR [19] pulse sequences to extract dipolar information from the partially ordered system instead of analyzing sideband intensities.

The technique is still in an early stage and some technical obstacles remain. Ideally, the sample needs to be prepared without bubbles. Gel distortion from bubbles makes linewidths larger and the apparent residual dipolar couplings smaller. These bubbles arise because the cap of the rotor is empty inside and inevitably leaves some air between the cap and the meniscus of the acrylamide solution undergoing polymerization in the magnet. This could be overcome by designing a rotor cap that would extrude some of the solution such as to leave no air between the cap material and the bulk of the gel. Also, multiple spinning sidebands arise from the water signal and the unpolymerized acrylamide signals. These seriously distort the baseline and hinder the measurement process. This problem could be partially solved by using new media such as the recently reported polymer Pluronic F-127 that can capture the orientation of Pf1 phage, in the same way as polyacrylamide [30].

It is perfectly conceivable to design double- or triple-resonance multidimensional SAD-REDOR experiment that would encode the sign and size of the residual dipolar coupling (or CSA) into peak intensities using a constant-time evolution period with rotor-synchronized REDOR pulses. Such NMR spectra could be used to simultaneously assign backbone protein resonances and measure a number of residual dipolar couplings.

6. Conclusion

In conclusion, the SAD-REDOR experiment allows tunable scaling of the recoupled dipolar coupling by the application of rotor-synchronized RF pulses. *J*- and RDC-couplings scale differently with the pulse sequence parameters enabling their accurate determination. Relative to measurements made in isotropic and aligned media, the theoretical scaling factor of recoupling of SAD-REDOR is over 80% and yields both the isotropic and anisotropic components of the spin Hamiltonian in a single sample. The technique has exciting potential for improving the measurement of residual CSAs and dipolar couplings in weakly aligned biomolecules.

Acknowledgments

We thank Bruker USA for the loan of an HR-MAS probe and M. Ditty, M. Monette, T. Buser, and H. Stronks from Bruker Canada for their precious help and support. We also thank H. Jarrell, L. Reven, and R. Tycko for ideas and discussions. This work was supported by a Natural Sciences and Engineering Research Council of Canada grant to K.G.

References

[1] R.Y. Dong, Nuclear Magnetic Resonance of Liquid Crystals, Springer, New York, 1994.
[2] J.W. Emsley, in: D.M Grant, R.K. Harris (Eds.), Encyclopedia of Nuclear Magnetic Resonance, Wiley, London, 1996, pp. 2788–2799.
[3] J.H. Prestegard, H.M. Al-Hashimi, J.R. Tolman, NMR structures of biomolecules using field oriented media and residual dipolar couplings, Q. Rev. Biophys. 33 (2000) 371–424.
[4] A. Bax, G. Kontaxis, N. Tjandra, Dipolar couplings in macromolecular structure determination, Methods Enzymol. 339 (2001) 127–174.

[5] N. Tjandra, A. Bax, Direct measurement of distances and angles in biomolecules by NMR in a dilute liquid crystalline medium, Science 278 (1997) 1111–1114.
[6] M.R. Hansen, L. Mueller, A. Pardi, Tunable alignment of macromolecules by filamentous phage yields dipolar coupling interactions, Nat. Struct. Biol. 5 (1998) 1065–1074.
[7] R. Tycko, F.J. Blanco, Y. Ishii, Alignment of biopolymers in strained gels: a new way to create detectable dipole–dipole couplings in high-resolution biomolecular NMR, J. Am. Chem. Soc. 122 (2000) 9340–9341.
[8] H.-J. Sass, G. Musco, S.J. Stahl, P.T. Wingfield, S. Grzesiek, Solution NMR of proteins within polyacrylamide gels: diffusional properties and residual alignment by mechanical stress or embedding of oriented purple membranes, J. Biomol. NMR 18 (2000) 303–309.
[9] G. Cornilescu, A. Bax, Measurement of proton, nitrogen, and carbonyl chemical shielding anisotropies in a protein dissolved in a dilute liquid crystalline phase, J. Am. Chem. Soc. 122 (2000) 10143–10154.
[10] C.R. Sanders, G.C. Landis, Reconstitution of membrane proteins into lipid-rich bilayered mixed micelles for NMR studies, Biochemistry 34 (1995) 4030–4040.
[11] G. Zandomeneghi, P.T.F. Williamson, A. Hunkeler, B.H. Meier, Switched-angle spinning applied to bicelles containing phospholipid-associated peptides, J. Biomol. NMR 25 (2003) 125–132.
[12] F. Tian, J.A. Losonczi, M.W.F. Fischer, J.H. Prestegard, Sign determination of dipolar couplings in field-oriented bicelles by variable angle sample spinning (VASS), J. Biomol. NMR 15 (1999) 145–150.
[13] G. Zandomeneghi, M. Tomaselli, J.D. van Beek, B.H. Meier, Manipulation of the director in bicellar mesophases by sample spinning: a new tool for NMR spectroscopy, J. Am. Chem. Soc. 123 (2001) 910–913.
[14] J.-F. Trempe, F.G. Morin, Z. Xia, R.H. Marchessault, K. Gehring, Characterization of polyacrylamide-stabilized Pf1 phage liquid crystals for protein NMR spectroscopy, J. Biomol. NMR 22 (2002) 83–87.
[15] R.H. Havlin, G.H. Park, A. Pines, Two-dimensional NMR correlations of liquid crystals using switched angle spinning, J. Magn. Reson. 157 (2002) 163–169.
[16] C.W.V. Kooi, Ē. Kupče, E.R.P. Zuiderweg, M. Pellecchia, Line narrowing in spectra of proteins dissolved in a dilute liquid crystalline phase by band-selective adiabatic decoupling: application to $^{1}H^{N}$–^{15}N residual dipolar coupling measurements, J. Biomol. NMR 15 (1999) 335–338.
[17] M.M. Maricq, J.S. Waugh, NMR in rotating solids, J. Chem. Phys. 70 (1979) 3300–3316.
[18] G.S. Harbison, V.-D. Vogt, H.W. Spiess, Structure and order in partially oriented solids: characterization by 2D-magic-angle-spinning NMR, J. Chem. Phys. 86 (1987) 1206–1218.
[19] T. Gullion, J. Schaefer, Rotational-echo double-resonance NMR, J. Magn. Reson. 81 (1989) 196–200.
[20] M.G. Munowitz, R.G. Griffin, G. Bodenhausen, T.H. Huang, Two-dimensional rotational spin-echo nuclear magnetic resonance in solids: correlation of chemical shift and dipolar interactions, J. Am. Chem. Soc. 103 (1981) 2529–2533.
[21] J. Courtieu, J.P. Bayle, B.M. Fung, Variable angle sample spinning NMR in liquid crystals, Prog. Nucl. Magn. Reson. Spectrosc. 26 (1994) 141–169.
[22] A. Saupe, G. Englert, High-resolution nuclear magnetic resonance spectra of oriented molecules, Phys. Rev. Lett. 11 (1963) 462–465.
[23] T. Gullion, J. Schaefer, Detection of weak heteronuclear dipolar coupling by rotational-echo double-resonance nuclear magnetic resonance, in: W.S. Warren (Ed.), Advances in Magnetic Resonance, vol. 13, Academic Press, New York, 1989, pp. 57–83.
[24] M.R. Hansen, P. Hanson, A. Pardi, Filamentous bacteriophage for aligning RNA, DNA, and proteins for measurement of nuclear magnetic resonance dipolar coupling interactions, Methods Enzymol. 317 (2000) 220–240.
[25] B.M. Fung, D.S. Mui, I.R. Bonnel, E.L. Enwall, Evaluation of broadband decoupling sequences applied to liquid crystal solutions, J. Magn. Reson. 58 (1984) 254–260.
[26] B.M. Fung, A comparison of homonuclear dipolar-decoupling techniques in heteronuclear experiments, J. Magn. Reson. 72 (1987) 353–357.
[27] J. Courtieu, D.W. Alderman, D.M. Grant, J.P. Bayles, Director dynamics and NMR applications of nematic liquid crystals spinning at various angles from the magnetic field, J. Chem. Phys. 77 (1982) 723–730.
[28] M. Zhou, V. Frydman, L. Frydman, Order determination in liquid crystals by dynamic director NMR spectroscopy, J. Am. Chem. Soc. 120 (1998) 2178–2179.
[29] D. McElheny, M. Zhou, L. Frydman, Two-dimensional dynamic-director ^{13}C NMR in liquid crystals, J. Magn. Reson. 148 (2001) 436–441.
[30] S.A. Riley, J.R. Giuliani, M.P. Augustine, Capture and manipulation of magnetically aligned Pf1 with an aqueous polymer gel, J. Magn. Reson. 159 (2002) 82–86.