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# Anisotropy of collagen fiber orientation in sheep tendon by $^1\text{H}$ double-quantum-filtered NMR signals

R. Fechete,<sup>a</sup> D.E. Demco,<sup>a</sup> B. Blümich,<sup>a,\*</sup> U. Eliav,<sup>b</sup> and G. Navon<sup>b</sup>

<sup>a</sup> *Institut für Technische Chemie und Makromolekulare Chemie, Rheinisch-Westfälische Technische Hochschule, Worringerweg 1, D-52056 Aachen, Germany*

<sup>b</sup> *School of Chemistry, Tel Aviv University, Ramat Aviv, Tel Aviv 69978, Israel*

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## Abstract

The anisotropy of the angular distribution of collagen fibrils in a sheep tendon was investigated by  $^1\text{H}$  double-quantum (DQ) filtered NMR signals. Double-quantum build-up curves generated by the five-pulse sequence were measured for different angles between the direction of the static magnetic field and the axis of the tendon plug. Proton residual dipolar couplings determined from the DQ build-up curves in the initial excitation/reconversion time regime which mainly represent the bound water are interpreted in terms of a model of spin-1/2 pairs with their internuclear axes oriented on average along the fibril direction in the presence of proton exchange. The angular distribution of collagen fibrils around the symmetry axis of the tendon measured by the anisotropy of the residual dipolar couplings was described by a Gaussian function with a standard deviation of  $12^\circ \pm 1^\circ$  and with the center of the distribution at  $4^\circ \pm 1^\circ$ . The existence of this distribution is directly reflected in the finite value of the residual dipolar couplings at the magic angle, the value of the angular contrast, and the oscillatory behavior of the DQ build-up curves. The  $^1\text{H}$  residual dipolar couplings were also measured from the doublets recorded by the DQ-filtered signals. From the angular dependence of the normalized splitting the angular distribution of the collagen fibrils was evaluated using a Gaussian function with a standard deviation of  $19^\circ \pm 1^\circ$  and with the center of distribution at  $2^\circ \pm 1^\circ$ . The advantages and disadvantages of these approaches are discussed.

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## 1. Introduction

Most biological tissues possess some degree of order. In ordered tissues, where the molecular motion is anisotropic, dipolar and quadrupolar interactions are not averaged to zero. In such cases homonuclear and heteronuclear multiple-quantum (MQ) coherences can be formed. These coherences give information about the effect of the anisotropic motion of the water molecules and sodium ions in intact biological tissues (for a review see [1]). In collagen-containing tissues such as tendons, ligaments, cartilage skin, blood vessels, and nerves DQ coherences are formed as a result of the interaction of water molecules with the collagen fibers. Therefore, the orientation of the macromolecules induces an anisot-

ropy in the residual dipolar couplings. Moreover, such anisotropy can also be observed by the  $^1\text{H}$  residual dipolar couplings of the macromolecules in another class of soft solids, that is in strained elastomers [2,3].

The anisotropy of the residual dipolar couplings in tendon is related to the tightly bound water molecules which penetrate the interstitial volume of the triple-helix and are fixed by two hydrogen bonds. These water molecules can rotate about the axis defined by the two bonds so that the time average of the internuclear vector and with it the principal axis of the dipole-dipole coupling tensor is aligned along the rotation axis. There are a number of dynamic processes that result in the observed residual dipolar interaction [1,4]: (i) Anisotropic reorientation of the water molecules bound to the protein. The time scale of this motion is typically  $10^{-10}$ – $10^{-7}$  s. (ii) Fast chemical exchange between the molecules of bound water and free water on a time scale

\* Corresponding author. Fax: +49-241-8888-185.

E-mail address: [bluemich@mc.rwth-aachen.de](mailto:bluemich@mc.rwth-aachen.de) (B. Blümich).

shorter than  $10^{-9}$  s. This process scales down the residual dipolar interactions from their values in the bound states. (iii) Proton exchange between water molecules on time scale between  $10^{-4}$  and  $10^{-3}$  s. As the water molecules move from between the bonded to and the free state, it can exchange proton with the other water molecules, thus replacing the proton which has been in an up (or down) spin state with another proton in the opposite spin state. This process also averages the intraproton dipolar coupling.

The investigation of tendon anisotropy by various NMR methods is a topic of long standing interest. The first NMR study by Berendsen [5] used the  $^1\text{H}$  van Vleck second moment to measure the orientation distribution of the collagen fibrils in tendon. Unfortunately, signals from the free water and proteins are interfering with the signal from the bound water, and also the magnetic field inhomogeneities present made the measurements of second moment inaccurate. Fullerton et al. [6] provided the first observation of the contrast anisotropy in magnetic resonance imaging (MRI) which was identified as the same effect reported in hydrated collagen [5]. The orientational anisotropy of  $T_2$  and other NMR parameters was shown to be correlated with the underlying geometrical structure of the ordered tissues [7–9]. Recently it was demonstrated that the NMR-MOUSE (mobile universal surface explorer), a hand-held NMR sensor, can be employed to investigate the anisotropy of  $T_2$  in Achilles tendon in vivo [10].

The presence of residual dipolar couplings allows MQ coherences to be generated in ordered tissues (see [1] and references therein). These MQ coherences, in particular double-quantum (DQ) coherences, have the advantage of suppressing the intense signal of free water and to a high degree the signals originating from the macromolecules. Therefore, the observation of MQ coherences represents a convenient approach to investigate local and macroscopic order in a variety of tissues. Recently, Tsoref et al. [11] reported the dependence of the width of the  $^1\text{H}$  DQ-filtered NMR signals of rat tail tendon on the tendon orientation, indicating that the effective director of the residual dipolar interaction is parallel to the collagen fibers. Double-quantum filtered  $^1\text{H}$  NMR spectra from human and bovine skeletal muscle showed frequency splittings of creatine and lactate protons that varied with the orientation of the muscle fibers relative to the magnetic field [12–14]. In all of the studies mentioned above, the distribution of the collagen fiber orientation was not investigated quantitatively.

The residual quadrupolar interaction of  $^2\text{H}$  and  $^{23}\text{Na}$  nuclei was also employed by measurements of spectra, relaxation rates, and MQ coherences to characterize the anisotropy in macroscopically ordered and disordered biological materials ([1,4,15–17], and references therein).

The goal of this paper was to investigate quantitatively the anisotropy of the collagen fibril orientation in tendon using  $^1\text{H}$  residual dipolar couplings. These residual dipolar couplings were determined from DQ build-up curves and DQ-filtered doublets for water interacting with the collagen fibers. The DQ-filtered NMR signals reject the  $^1\text{H}$  signals of free water and show only a small contribution from the collagen fibers. The orientation distribution of the collagen fibrils in tendon was described by the heuristic approach of a Gaussian distribution function. The parameters of this angular distribution function were obtained from the anisotropy of the slopes of the experimentally determined DQ build-up curves in the initial excitation/reconversion regime as well as the splitting of the DQ-filtered spectra. The advantages and disadvantages of these approaches are also discussed.

## 2. Experimental

### 2.1. Materials

Samples of sheep Achilles tendon were obtained from the butcher. The samples were kept at  $-18^\circ\text{C}$  after excision until the time of measurements which were performed at room temperature  $20 \pm 1^\circ\text{C}$ . A plug was cut from the middle of the tendon and was wrapped with teflon tape in order to avoid dehydration. A new tendon plug was used after about 12 h of measurements to preclude tissue degradation. The dominant orientation of plug was parallel to the collagen fibers and along the macroscopic axis of the tendon. Because the tendon plugs were not immersed in any liquid to match susceptibility, the reoriented samples were subject to slight susceptibility inhomogeneities. NMR measurements for each set of orientation angles were made on at least three different tendon plugs to account for biological heterogeneity.

### 2.2. NMR experiment

The NMR experiments were performed at a  $^1\text{H}$  frequency of 200.025 MHz on a Bruker DSX-200 spectrometer. The DQ build-up curves were recorded with the five-pulse sequence [18] which is discussed below. The applicable phase cycling scheme is described in [18]. The  $90^\circ$  pulse length was  $13.5 \mu\text{s}$  and recycle delays of 1 s were used. The evolution time and the  $z$ -filter delay were fixed to  $t_1 = 10 \mu\text{s}$  and  $\tau_f = 10 \mu\text{s}$ , respectively. In order to measure the DQ build-up curves the DQ excitation and reconversion periods were varied in the range of  $4 \mu\text{s} \leq \tau \leq 800 \mu\text{s}$ . A special Bruker NMR probe with a tilt coil was used which allows the orientation of the resonator axis relative to the direction of the static magnetic field in the range  $0^\circ \leq \theta \leq 90^\circ$ .

### 3. Theory

#### 3.1. Double-quantum build-up curves

##### 3.1.1. Slow proton exchange

In the secular approximation, the dipolar Hamiltonian describing the intramolecular interaction of rigid two spin-1/2 pairs is given by

$$H_d = (-\sqrt{6})DP_2(\cos \beta^{ij})T_{2,0}^{ij}, \quad (1)$$

where the dipolar coupling constant  $D = (\mu_0/4\pi)\gamma^2 \hbar(1/r_{ij}^3)$  of the spin pairs  $ij$  depends on the internuclear distance  $r_{ij}$ . The angle  $\beta_{ij}$  is the angle between the internuclear vector  $\vec{r}_{ij}$  and the direction of the main magnetic field  $\vec{B}_0$  (cf. Fig. 1). The second-order Legendre polynomial is denoted by  $P_2(\cos \beta_{ij})$ , and  $T_{2,0}^{ij}$  is an irreducible tensor operator. The equivalent spin operator for a quasiparticle with spin quantum number  $F = 1$  is given by  $T_{2,0} = 2T_{2,0}^{ij}$  [19,20].

The dipolar coupling between spin pairs is averaged on the time scale of the NMR experiment which results from the reorientation motion of the bound water as

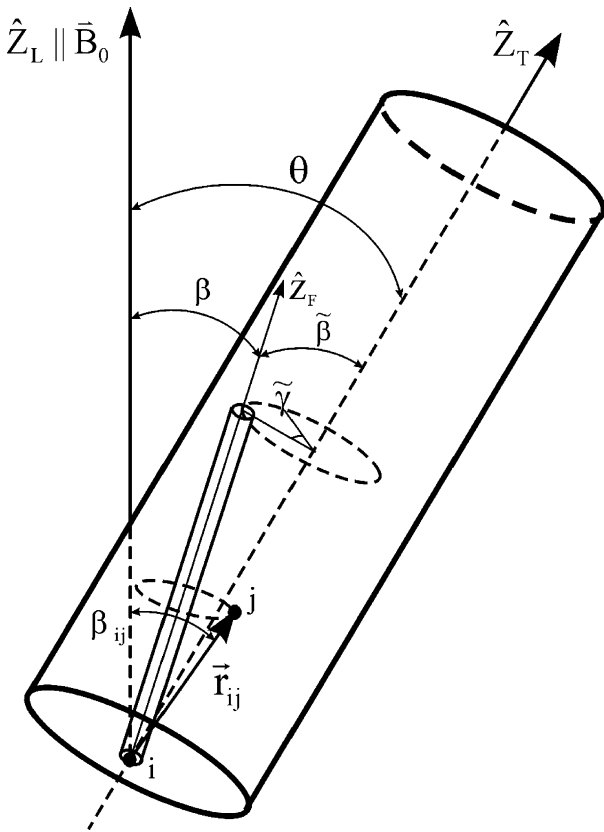


Fig. 1. Angles and reference frames used for describing the anisotropy of the residual dipolar couplings. The  $z$ -axis of the laboratory reference frame (denoted by the unity vector  $\hat{Z}_L$ ) is parallel to the direction of the static magnetic field  $\vec{B}_0$ . The  $z$ -axes of the tendon plug and collagen fibril are denoted by  $\hat{Z}_T$  and  $\hat{Z}_F$ , respectively. The spin pair  $ij$  is characterized by the internuclear distance  $r_{ij}$  and the polar angle  $\beta_{ij}$ .

well as from the chemical exchange between free and bound water molecules [1,4]. The time scale of our NMR experiments is of the order of excitation/reconversion times, i.e., of about 0.1–1 ms. As a result of this partial motional averaging, the axis of the dipolar tensor is oriented on average along the collagen fibril direction, which defines the local director having the angle  $\beta$  with the direction of  $\vec{B}_0$  and the angle  $\tilde{\beta}$  with the axis of symmetry of the tendon plug (cf. Fig. 1). It is assumed that the distribution of collagen fibrils around the axis of tendon is uniaxial, so that the orientation distribution can be specified in terms of a single angular variable  $\tilde{\beta}$ . Using Eq. (1) the averaged Hamiltonian of the spin pair is given by

$$\bar{H}_d = \left( -\sqrt{\frac{3}{2}} \right) DSP_2(\cos \beta)T_{2,0}. \quad (2)$$

This residual dipolar Hamiltonian depends on the dynamic order parameter  $S = \bar{P}_2(\cos \beta^{ij}(t))$ , where the bar denotes an ensemble average over the instantaneous angle  $\beta^{ij}(t)$  (see Fig. 1) [4,21,22]. To simplify the notation Eq. (2) is rewritten as

$$\bar{H}_d = \varpi_d T_{2,0}, \quad (3)$$

where  $\varpi_d$  is the residual dipolar coupling which includes the angular anisotropy. The effect of very slow hydrogen exchange can be considered to be present in the value of the dynamic order parameter.

The spin system response to the five-pulse sequence used for DQ excitation (Fig. 2) can easily be evaluated in the density matrix formalism. The initial state of the spin system is described by the thermodynamic equilibrium density operator  $\rho(0) \propto CT_{1,0}$ , where  $C$  is the Curie constant and  $T_{1,0} = F_z$ , is the irreducible tensor operator given by the  $z$ -component  $F_z$  of the total spin of the spin-1/2 pair. Finally, by employing the specific irreducible tensor operator algebra [20] for the density operator

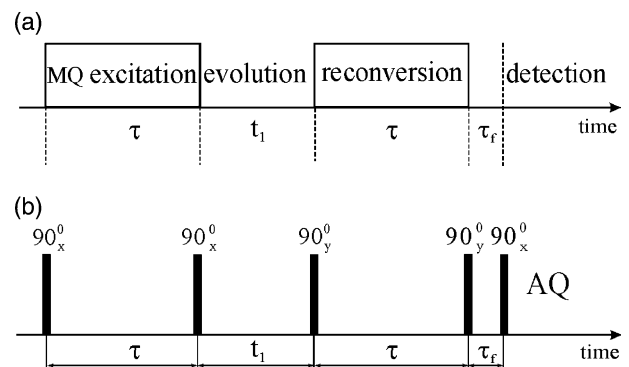


Fig. 2. The five-pulse sequence used for measuring double-quantum build-up curves. The multiple-quantum (MQ) excitation, evolution, reconversion,  $z$ -filter, and detection periods are denoted by  $\tau$ ,  $t_1$ ,  $\tau$ ,  $\tau_r$  and  $t_2$ , respectively.

describing the spin pairs at the end of the DQ excitation period (see Fig. 2) we can write

$$\rho(\tau) \propto C \left\{ -\cos\left(\sqrt{\frac{3}{2}}\varpi_d\tau\right) T_{1,0} + i \sin\left(\sqrt{\frac{3}{2}}\varpi_d\tau\right) T_{2,2}(a) \right\}, \quad (4)$$

where the last term describes the DQ coherences represented by the antisymmetric irreducible tensor operator  $T_{2,2}(a) = (1/\sqrt{2})(T_{2,2} - T_{2,-2})$  [20]. The selection of the DQ coherences by phase cycling [20] and considering a short evolution of these coherences during the period  $t_1$  lead to the DQ density operator

$$\rho_{\text{DQ}}(t_1 + \tau) \propto C i \sin\left(\sqrt{\frac{3}{2}}\varpi_d\tau\right) T_{2,2}(a). \quad (5)$$

At the end of the reconversion period the DQ term of the density operator is converted to  $z$  polarization and single-quantum coherences. For a  $z$ -filter with duration large enough to dephase single-quantum coherences and short enough to avoid magnetization transfer to free water and protein [21], the normalized NMR signal is given by

$$\frac{S_{\text{DQ}}(\tau_f + \tau + t_1 + \tau)}{S_{\text{SQ}}(0)} = \left\langle \sin^2\left(\sqrt{\frac{3}{2}}\varpi_d\tau\right) \exp\left(-\frac{2\tau}{T_2^*}\right) \right\rangle_{\Omega}. \quad (6)$$

where the integral intensity of the single-quantum (SQ) spectrum corresponding to bound water is denoted by  $S_{\text{SQ}}(0)$ . The effective decay time of SQ coherences which are present during the excitation and reconversion periods is  $T_2^*$ . This quantity is also orientation dependent [6,8]. Their orientation average over the collagen fibers is described by  $\langle(\dots)\rangle_{\Omega}$  and is discussed in detail below.

For an arbitrary radio-frequency tilt angle  $\theta_{\text{rf}}$ , the right-hand side of the Eq. (6) has to be multiplied by the function  $f(\theta_{\text{rf}}) = (d_{1,2}^2(\theta_{\text{rf}}) + d_{-1,2}^2(\theta_{\text{rf}}))^2$ , with  $d_{m,m'}^l(\theta_{\text{rf}})$  being the reduced Wigner matrix element. For  $\theta_{\text{rf}} = 90^\circ$  we get  $f(\theta_{\text{rf}}) = 1$ .

The DQ build-up curve described by Eq. (6) can be easily evaluated in the initial DQ excitation regime, i.e., for  $\varpi_d\tau \ll 1$  and  $2\tau \ll T_2^*$ . From Eq. (6) we get

$$\frac{S_{\text{DQ}}(\tau_f + \tau + t_1 + \tau)}{S_{\text{SQ}}(0)} \approx \frac{3}{2} \langle \varpi_d^2 \rangle_{\Omega} \tau^2 + \dots \quad (7)$$

The above equation can also be obtained if the proton exchange is taken explicitly into account in the process of DQ coherences excitation and reconversion in the limit of very slow proton exchange, i.e. for  $k \ll \langle \varpi_d^2 \rangle_{\Omega}^{1/2}$ , where  $k$  is the sum of the forward and backward proton exchange rates [4]. For a less restrictive condition, i.e.  $(k/\langle \varpi_d^2 \rangle_{\Omega}^{1/2}) < \sqrt{6}$ , the dependence of the integrals of the measured spectrum in a DQ-filtered experiment on the excitation/reconversion time  $\tau$  is given by [4]

$$\frac{S_{\text{DQ}}(\tau_f + \tau + t_1 + \tau)}{S_{\text{SQ}}(0)} \propto \left\langle \frac{\varpi_d^2}{r^2} e^{-\tau/T_2^*} \sin^2(r\tau) \right\rangle_{\Omega} e^{-k\tau}, \quad (8)$$

where  $r = (1/2)\sqrt{6\varpi_d^2 - k^2}$ . For connective and soft tissues the condition  $\tau \ll T_2^*$  is easily fulfilled because  $T_2^*$  is typically larger than 10 ms [21]. Moreover, for very slow exchange, i.e.  $k \ll \langle \varpi_d^2 \rangle_{\Omega}^{1/2}$ , the condition  $r\tau \ll 1$  is fulfilled in the initial excitation/reconversion regime of the DQ build-up curves. Under these conditions Eq. (8) is becoming identical with Eq. (7).

### 3.1.2. Fast proton exchange

Exchange of protons between water molecules is causing a modulation of the dipolar interaction, leading to a scaling down of the residual dipolar interaction and a reduction of the intensity of the multiple-quantum signals. When taking into consideration the proton exchange, one obtains the following expression for the integrals of the measured spectrum in DQ-filtered experiment [4] valid for

$$\frac{S_{\text{DQ}}(\tau_f + \tau + t_1 + \tau)}{S_{\text{SQ}}(0)} \propto \left\langle \frac{\varpi_d^2}{\rho^2} e^{-\tau/T_2^*} \sinh^2(\rho\tau) \right\rangle_{\Omega} e^{-k\tau}, \quad (9)$$

where  $\rho = (1/2)\sqrt{k^2 - 6\varpi_d^2}$ .

For fast proton exchange the condition  $k > \langle \varpi_d^2 \rangle_{\Omega}^{1/2}$  is fulfilled and  $\rho \approx k/2$ . Moreover, for  $\tau \ll k^{-1}$  and  $\tau < T_2^*$  from Eq. (9) we get

$$\frac{S_{\text{DQ}}(\tau_f + \tau + t_1 + \tau)}{S_{\text{SQ}}(0)} \propto \langle \varpi_d^2 \rangle_{\Omega} \tau^2. \quad (10)$$

It is worth noting that under these conditions the analysis of the experimental results becomes somewhat more demanding due to decline in signal-to-noise ratio. For instance, at 24 and 41 °C the exchange rate constant  $k$  is about 3500 and 6000 s<sup>-1</sup> [4] requiring  $\tau < 100 \mu\text{s}$  and  $\tau < 30 \mu\text{s}$ , respectively.

In ordered tissues like tendon the <sup>1</sup>H DQ-filtered NMR signals in general consist of two main components, one characteristic for the collagen fibers and the other originating from the bound water. The former signal is difficult to be detected as a result of the large value of the dipolar coupling and very short value of the transverse relaxation time (vide infra). Actually, the pulse sequence used for detecting DQ-filtered NMR signals is suppressing not only the signal of the free water but also the signal of proteins. Therefore, it is sufficient to discuss only the anisotropy of the <sup>1</sup>H residual dipolar coupling of bound water in the following.

### 3.2. Anisotropy of the <sup>1</sup>H double-quantum-filtered NMR signals

The experimental parameter describing the anisotropy of the residual dipolar couplings is given by the angle  $\theta$  between the direction of the tendon plug and the direction of  $\vec{B}_0$ . This angle and other relevant angles for

the description of anisotropy are shown in Fig. 1. Using simple geometrical arguments which describe the change of the cosine function of the polar angles between different reference frames we can write

$$\cos \beta = \cos \tilde{\beta} \cos \theta - \sin \tilde{\beta} \sin \theta \cos \tilde{\gamma}. \quad (11)$$

Consequently, the square of the Legendre polynomial in the expression of the slope of the DQ build-up curve (see Eqs. (2),(3),(7)–(10)) is given by

$$\begin{aligned} (P_2(\cos \beta))^2 &= \frac{1}{4}(3 \cos^2 \beta - 1)^2 \\ &= \frac{1}{4}[3(\cos \tilde{\beta} \cos \theta - \sin \tilde{\beta} \sin \theta \cos \tilde{\gamma})^2 - 1]^2. \end{aligned} \quad (12)$$

The anisotropy of the square of the residual dipolar couplings (cf. Eq. (7) or Eq. (10)) can be evaluated by considering an isotropic distribution of the azimuthal angle  $\tilde{\gamma}$  of the collagen fibrils or local average directors around the tendon axis of symmetry. Therefore, an axial distribution function was assumed to describe the distribution of the angle  $\tilde{\beta}$ . The angular average  $\langle \dots \rangle_{\Omega}$  has to be performed over two independent angular averages, i.e.,  $\langle \dots \rangle_{\tilde{\beta}, \tilde{\gamma}}$ . For the initial slope of the DQ build-up curve, i.e. for

$$\frac{3}{2} \langle \varpi_d^2(\theta) \rangle_{\tilde{\beta}, \tilde{\gamma}} = \frac{9}{4} D^2 S^2 \langle P_2^2(\cos \beta) \rangle_{\tilde{\beta}, \tilde{\gamma}}, \quad (13)$$

we finally get

$$\begin{aligned} \langle \varpi_d^2(\theta) \rangle_{\tilde{\beta}, \tilde{\gamma}} &= \frac{3}{4\pi} D^2 S^2 \int_0^{2\pi} d\tilde{\gamma} \int_0^{\pi/2} \sin \tilde{\beta} d\tilde{\beta} R(\tilde{\beta} - \tilde{\beta}_0) \\ &\quad \times P_2^2(\cos \tilde{\beta} \cos \theta - \sin \tilde{\beta} \sin \theta \cos \tilde{\gamma}), \end{aligned} \quad (14)$$

where  $R(\tilde{\beta} - \tilde{\beta}_0)$  is the uniaxial orientation distribution function of the collagen fibrils around the axis of the tendon plug. The center of the distribution corresponds to the angle  $\tilde{\beta}_0$ .

The  $\tilde{\beta}$  distribution can be approximated by the normalized Gaussian distribution, i.e. by

$$\begin{aligned} R(\tilde{\beta} - \tilde{\beta}_0) &= \left[ 1 / \left( \int_0^{\pi/2} \exp \left\{ -\frac{(\tilde{\beta} - \tilde{\beta}_0)^2}{2\sigma_{\tilde{\beta}}^2} \right\} d\tilde{\beta} \right) \right] \\ &\quad \times \exp \left\{ -\frac{(\tilde{\beta} - \tilde{\beta}_0)^2}{2\sigma_{\tilde{\beta}}^2} \right\}, \end{aligned} \quad (15)$$

where  $\sigma_{\tilde{\beta}}$  is the standard angular deviation.

The normalized anisotropy of the square of the residual dipolar couplings measured from the initial region of the DQ build-up curves can be obtained from the above equations

$$\frac{\langle \varpi_d^2(\theta) \rangle_{\tilde{\beta}, \tilde{\gamma}}}{\langle \varpi_d^2(0) \rangle_{\tilde{\beta}, \tilde{\gamma}}} = \frac{\int_0^{\pi/2} R(\tilde{\beta} - \tilde{\beta}_0) f(\tilde{\beta}, \theta) \sin \tilde{\beta} d\tilde{\beta}}{\int_0^{\pi/2} R(\tilde{\beta} - \tilde{\beta}_0) f(\tilde{\beta}, 0) \sin \tilde{\beta} d\tilde{\beta}}, \quad (16)$$

where the angular function  $f(\tilde{\beta}, \theta)$  is given by

$$\begin{aligned} f(\tilde{\beta}, \theta) &= \frac{9}{8\pi} \left\{ \cos^4 \tilde{\beta} \cos^4 \theta + \frac{3}{8} \sin^4 \tilde{\beta} \sin^2 \theta \right. \\ &\quad + \frac{1}{8} \sin^2 2\tilde{\beta} \sin^2 2\theta + \frac{1}{9} \\ &\quad + \cos^2 \tilde{\beta} \sin^2 \tilde{\beta} \cos^2 \theta \sin^2 \theta \\ &\quad \left. - \frac{2}{3} \cos^2 \tilde{\beta} \cos^2 \theta - \frac{1}{3} \sin^2 \tilde{\beta} \sin^2 \theta \right\}. \end{aligned} \quad (17)$$

From the measured angular dependence of the normalized slopes of the DQ build-up curves described by Eqs. (16) and (17) the parameters of the angular distribution function, i.e.,  $\sigma_{\tilde{\beta}}$  and  $\tilde{\beta}_0$  can be determined (see below). The normalized angular dependence of the square of the residual dipolar interactions given by Eq. (16) is independent of the numerical factor present in the slope measured in the initial time regime of the DQ build-up curve (cf. Eq. (7)).

## 4. Results and discussions

### 4.1. Proton DQ-filtered spectra

Single-quantum (SQ)  $^1\text{H}$  spectrum recorded with a  $90^\circ$  radio-frequency pulse for sheep tendon oriented at  $\theta = 0^\circ$  at room temperature is shown in Fig. 3a. It consists of a doublet and a central line corresponding to the bound water and free water, respectively. The DQ-filtered spectrum measured in the same conditions as above at excitation/reconversion time of  $\tau = 50 \mu\text{s}$  is shown in Fig. 3b. A well resolved splitting is evident apart from the resonance coming from free water that does not exchange with those that interact with the collagen fibers. The splitting of  $2\Delta \approx 4400 \text{ Hz}$  is the same for both spectra. These spectra show that for the sheep tendon at room temperature the approximation of spin-1/2 pairs is valid and that the residual dipolar interaction is larger than the proton exchange rate (for more details see [1] and [4]).

The dependence of the  $^1\text{H}$  DQ-filtered spectra on the  $\tau$  pulse sequence parameter (cf. Fig. 2) is shown in Fig. 4. The maximum strength of the DQ-filtered signal occurs at  $\tau = 100 \mu\text{s}$  (see below) and in Fig. 4a various spectra are shown before and after this maximum. The intensity of the DQ-filtered spectra follows the strengths of the DQ build-up integrated signal without any change in the parameter  $2\Delta$ , indicating a high macroscopic order. Furthermore, the clear splitting also points out that for this particular orientation and temperature that the proton exchange process is slow in comparison with  $2\Delta$ . Moreover, for  $\tau \geq 50 \mu\text{s}$  a weak resonance becomes evident at the resonance frequency of free water. The relative intensity of this resonance is increasing for longer  $\tau$  values (see Fig. 4b). This result may be interpreted by a transfer of magnetization from the bound water to the

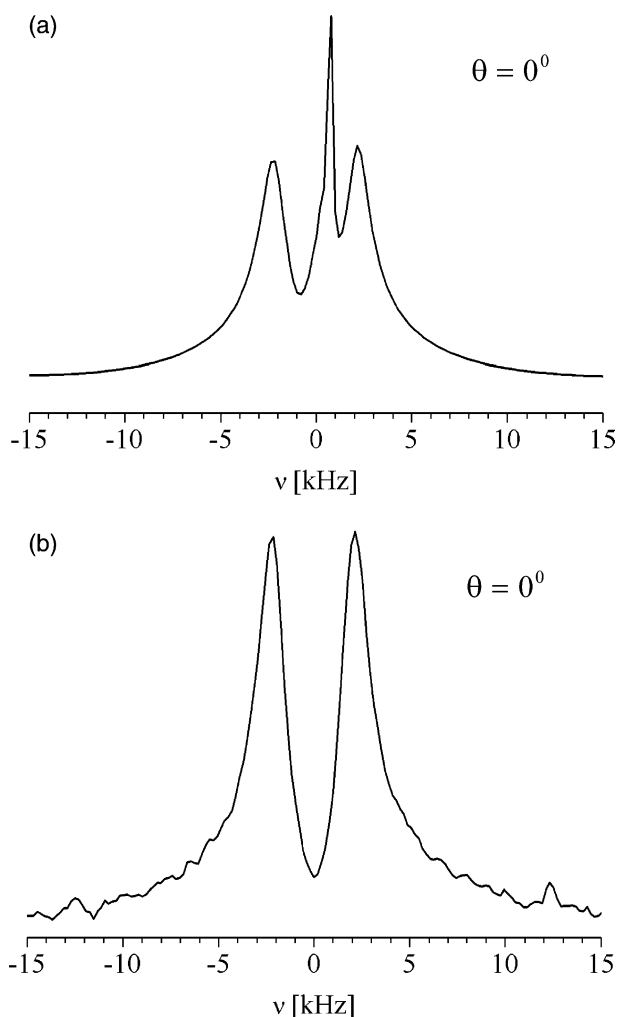


Fig. 3. Proton single-quantum (SQ) (a) and DQ-filtered spectra (b) of sheep tendon oriented at the angle  $\theta = 0^\circ$ . The SQ spectrum as well as DQ-filtered spectrum recorded with the excitation/reconversion time of  $\tau = 50 \mu\text{s}$  show a doublet. The NMR signal from the free water is present in the SQ spectrum (a) but filtered in the DQ spectrum (b). The measurements were performed at room temperature  $20 \pm 1^\circ\text{C}$ .

free water via chemical or proton exchange or tentatively by the presence of additional site with smaller residual dipolar interaction. In Fig. 4b the  $^1\text{H}$  DQ-filtered spectra corresponding to the maxima of the DQ build-up curve (cf. Fig. 5a) are shown. For  $\tau = 780 \mu\text{s}$  (see Fig. 4b) the five pulse sequence acts as a dipolar filter and only the signal from free water molecules is detected.

#### 4.2. Anisotropy of the $^1\text{H}$ residual dipolar couplings by DQ build-up curves

The values of the  $^1\text{H}$  residual dipolar coupling  $\langle \overline{\omega_d^2}(\theta) \rangle^{1/2}$  (where we have dropped here and in the following the indices indicating the angular averages) were measured from the initial slopes of the DQ build-up curves. These curves were recorded using the pulse se-

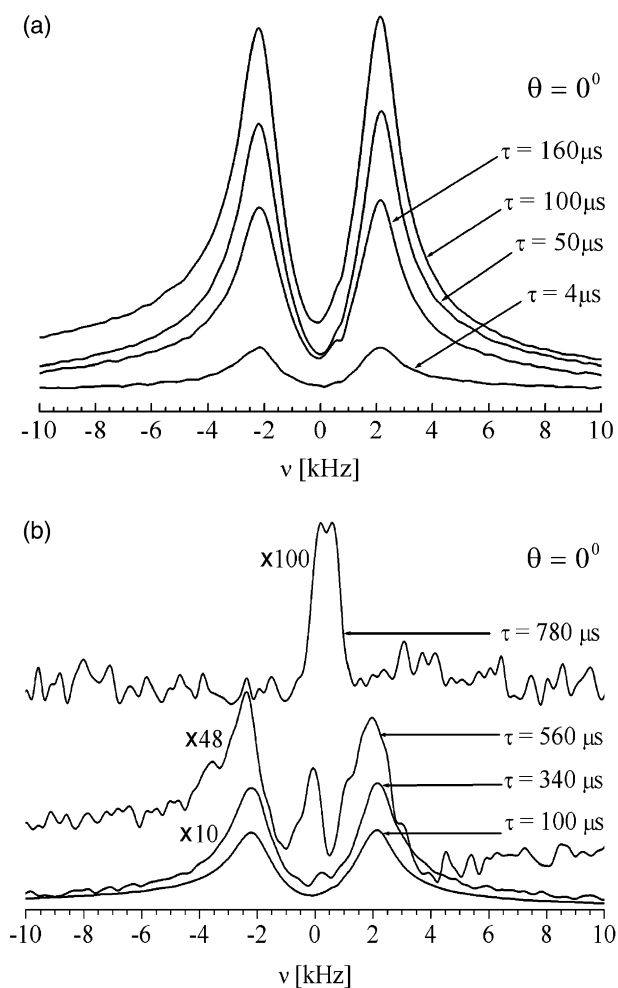


Fig. 4. Proton DQ-filtered spectra of the sheep tendon oriented at the angle  $\theta = 0^\circ$ . The spectra measured in the initial regime of DQ build-up curves of  $\tau = 4 \mu\text{s}$  and  $\tau = 50 \mu\text{s}$ , as well as the maximum and over the maximum for  $\tau = 100 \mu\text{s}$  and  $\tau = 160 \mu\text{s}$ , respectively, are shown in (a). The  $^1\text{H}$  DQ-filtered spectra at the excitation/reconversion times  $\tau$  corresponding to the four attenuated amplitudes of the DQ build-up curve oscillations are shown in figure (b). The measurements were performed at room temperature  $20 \pm 1^\circ\text{C}$ .

quence shown in Fig. 2. A normalized DQ build-up curve for orientation  $\theta = 0^\circ$  and that for an extended range of excitation/reconversion times are shown in Fig. 5. The integrated DQ-filtered signal oscillates with an attenuated amplitude. The period of oscillation is about  $T = 500 \mu\text{s}$  which should correspond to the half of the splitting of  $\Delta \approx 2000 \text{Hz}$  (see Fig. 3b). We note that the above estimated period of oscillation is affected in the measurements by the transverse relaxation during the excitation/reconversion periods which shifts the times at which the maxima in oscillations occur (see below).

The large amplitude of these oscillations for the orientation angle  $\theta = 0^\circ$  is due to the larger value of the residual dipolar coupling. This fact is a direct consequence of the anisotropy of the dipolar tensor orientation. It was shown previously by simulation that for a

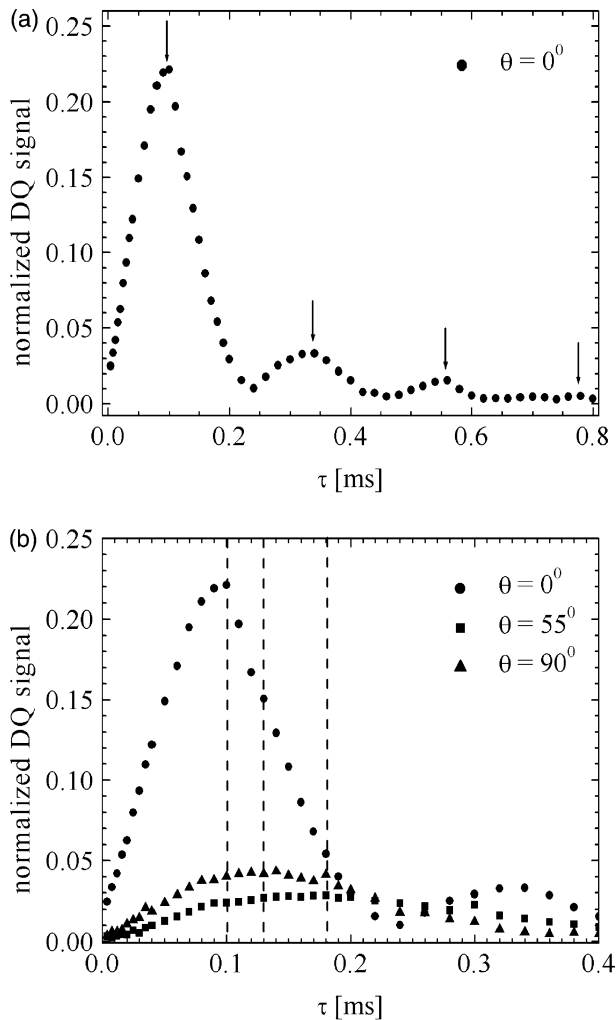


Fig. 5. (a) Normalized integrals of the  $^1\text{H}$  DQ-filtered NMR spectra (see Eq. (16)) versus the excitation/reconversion time  $\tau$  for sheep tendon at the orientation angle  $\theta = 0^\circ$ . Four attenuated oscillations (marked by arrows) are detected at this orientation where the residual dipolar coupling has the largest strength. (b) DQ build-up curves for orientation angles  $\theta = 0^\circ$ ,  $55^\circ$ , and  $90^\circ$  between the  $z$ -axis of the tendon plug and the direction of the static magnetic field. The maxima of the DQ build-up curves are marked by broken lines. The measurements were performed at room temperature  $20 \pm 1^\circ\text{C}$ .

spin-1/2 pair attached to an isolated polymer chain fixed between two joint points, the DQ build-up curve is given by a step function which exhibits a small oscillation for longer values of  $\tau$  [21]. If this spin-system response is multiplied by the transverse relaxation decay function  $\exp\{-(k + 2/T_2^*)\tau\}$  the oscillations are attenuated. This is exactly the case of the tendon having collagen fibers oriented in a small range of angles along the direction of the tendon plug.

Three trends are noticeable in the results shown in Fig. 5. Upon changing the sample orientation from  $0^\circ$  to  $90^\circ$  to  $54.7^\circ$  the rise and the fall times get longer and the maxima are shifted towards longer times. The normalized integrated intensities decrease. The shift of maxima

and the lengthening of the rise time are expected as the residual dipolar interaction is declining. However, the lengthening of the decay along with the decrease of the intensity requires that the effect of chemical exchange of water molecules between free and bound sites as well as proton exchange among water molecules should be considered. As can be seen from previous publication [4] as well as from Eq. (9), the effect of the above two processes on the DQ signal is two folds: (i) scaling down [4,5] of the residual dipolar interaction and (ii) relaxation that may depend on the orientation.

The value of the  $^1\text{H}$  residual dipolar interaction was measured by fitting the normalized DQ build-up curves in the initial regime, up to  $50\ \mu\text{s}$  by a polynomial in  $\tau^2$ . The coefficient of the term linear in  $\tau^2$  is given by  $(3/2)\langle\varpi_d^2\rangle$  (cf. Eq. (7)). The normalization factor is given by the integral intensity obtained after deconvolution of the SQ spectrum shown in Fig. 3a. The quantity  $\langle\varpi_d^2(\theta)\rangle^{1/2}$  is shown in Fig. 6a as a function of the orientation angle  $\theta$ . If the angular dependence of this quantity were described by the function  $P_2(\cos\theta)$ , the minimum value of  $\langle\varpi_d^2(\theta)\rangle^{1/2}$  would be zero and the angular contrast would be  $|\langle\varpi_d^2(0)\rangle^{1/2}/\langle\varpi_d^2(90^\circ)\rangle^{1/2}| = 2$ . Nevertheless, the experimental data of Fig. 6a show that the minimum value of  $\langle\varpi_d^2(\theta)\rangle^{1/2}$  represents about 13% of the value at  $\theta = 0^\circ$ , and the angular contrast in residual dipolar couplings is  $|\langle\varpi_d^2(0)\rangle^{1/2}/\langle\varpi_d^2(90^\circ)\rangle^{1/2}| \approx 2.7$ . Both these facts can be explained by a distribution of the tendon fibril orientation described by Eqs. (16) and (17).

In the approximation of a Gaussian angular distribution function given by Eq. (15), the normalized square of the residual dipolar couplings  $\langle\varpi_d^2(\theta)\rangle/\langle\varpi_d^2(0)\rangle$  shown in Fig. 6b was fitted with Eqs. (16) and (17). The best fit leads to the Gaussian distribution function characterized by a standard deviation of  $\sigma_{\bar{\beta}} = 12^\circ \pm 1^\circ$  and a center of distribution of  $\bar{\beta}_0 = 4^\circ \pm 1^\circ$ . The value of the standard deviation of the Gaussian angular distribution obtained in this study is in good agreement with the value  $\sigma_{\bar{\beta}} = 13^\circ$  reported previously on bovine Achilles Tendon by Berendsen [5] by the measurements of the anisotropy of the second van Vleck moments.

#### 4.3. Anisotropy of the $^1\text{H}$ residual dipolar couplings by DQ-filtered spectra

The  $^1\text{H}$  DQ-filtered spectra were recoded for  $\tau = 50\ \mu\text{s}$  with the pulse sequence of Fig. 2 and half of the splitting  $\Delta$  is shown in Fig. 7a as a function of the orientation angle  $\theta$ . From the definition of  $\langle\varpi_d^2\rangle^{1/2}$  given by Eqs. (3) and (7) and the frequency splitting [20] we get the following relation:  $(\langle\varpi_d^2\rangle^{1/2}/2\pi) = \sqrt{2/3}\Delta$ . Using this equation for splitting at  $\theta = 0^\circ$  of  $\Delta \cong 2200\ \text{Hz}$  (see Fig. 7a) we get  $\langle\varpi_d^2\rangle^{1/2}/(2\pi) \cong 1800\ \text{Hz}$ . The discrepancy between this value and the value shown in Fig. 6a is probably due to the inaccuracy in the evaluation of the integral intensity of the SQ spectrum used for normali-

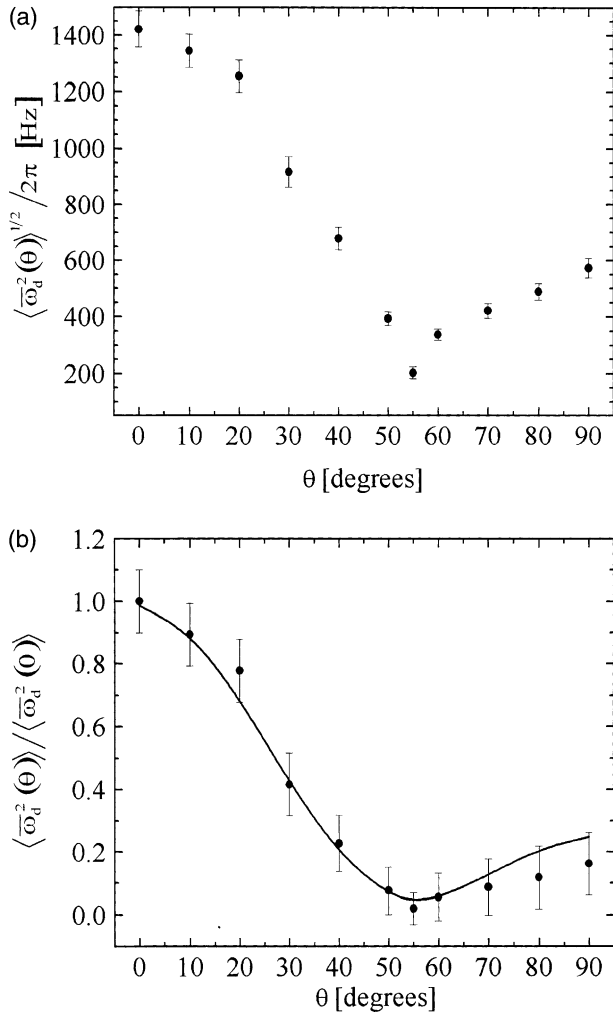


Fig. 6. (a) Values of the  $^1\text{H}$  residual dipolar couplings determined from the DQ build-up curves of Fig. 5 in the initial regime of excitation/reconversion times up to  $\tau = 50\mu\text{s}$ , for different orientations of the sheep-tendon plug. (b) The normalized values of the square of the  $^1\text{H}$  residual dipolar couplings versus the orientation angle  $\theta$ . The solid line represents the least-square fit of the data with the angular function given by Eqs. (16) and (17). The best fit is obtained for a Gaussian angular distribution function characterized by the standard deviation  $\sigma_{\tilde{\beta}} = 12^\circ \pm 1^\circ$  and the center of the distribution at  $\tilde{\beta}_0 = 4^\circ \pm 1^\circ$ . The measurements were performed at room temperature  $20 \pm 1^\circ\text{C}$ .

zation of the DQ-filtered signals. Nevertheless, we note that the normalized orientation dependent residual dipolar couplings shown in Fig. 6b are not affected by the normalization procedure.

The normalized values of the square of the splitting of the DQ-filtered spectra are shown in Fig. 7b versus the angle  $\theta$ . The best fit using Eqs. (16) and (17) (solid line in Fig. 7b) leads to the Gaussian distribution function characterized by a standard deviation of  $\sigma_{\tilde{\beta}} = 19^\circ \pm 1^\circ$  and a center of distribution of  $\tilde{\beta}_0 = 2^\circ \pm 1^\circ$ . In Fig. 7b the angular distribution function obtained from the residual dipolar couplings measured from the slopes of DQ build-up curves are also shown. The angular distribution

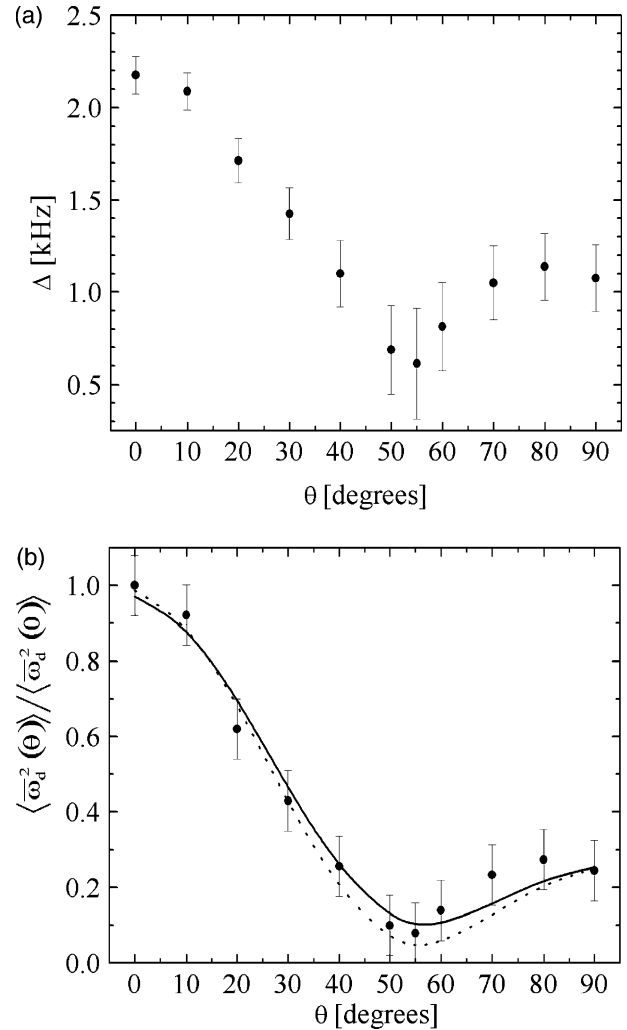


Fig. 7. (a) Values of the  $^1\text{H}$  residual dipolar couplings determined from half of the doublet splitting  $\Delta$  of the DQ-filtered spectra (see Fig. 4a) measured in the initial regime of excitation/reconversion times, i.e., for  $\tau = 50\mu\text{s}$  at different orientations  $\theta$  of the sheep-tendon plug. (b) The normalized values of the square of the  $^1\text{H}$  residual dipolar couplings measured from the spectral splitting versus the orientation angle  $\theta$ . The solid line represents the least-square fit of the data with the angular function given by Eqs. (16) and (17). The best fit (solid line) is obtained for a Gaussian angular distribution function characterized by the standard deviation  $\sigma_{\tilde{\beta}} = 19^\circ \pm 1^\circ$  and the center of the distribution at  $\tilde{\beta}_0 = 2^\circ \pm 1^\circ$ . The angular distribution function obtained from the  $^1\text{H}$  DQ build-up curves is also shown with a broken line. The measurements were performed at room temperature  $20 \pm 1^\circ\text{C}$ .

function differs more in the region of angles around the magic angle where the errors in the measurements of residual dipolar couplings are larger.

A dipolar interaction between/among the bound water molecule protons and those of the collagen may contribute to the measured residual dipolar coupling and may affect the estimates we obtain for the distribution of the fibril orientation. However, we believe that this is not the case due to the following reason: the intermolecular dipolar interaction between protein and



water is typically much smaller (distances of more than 0.2 nm) than the water molecule intramolecular dipolar interaction. Moreover, the existence of well-defined dipolar spectrum splitting and especially the behavior at the magic angle cannot be explained if the space distributed interactions at different angles with the protons of the collagen are taken into account. Furthermore, in addition to the fast reorientation motion of the water molecules on the protein surface along with the exchange between bound and free states that reduces both intra and intermolecular interactions, the latter ones may be further reduced by diffusion. Thus, making the effects of the intermolecular dipolar interactions on the measurements negligible.

## 5. Conclusions

The  $^1\text{H}$  DQ-filtered NMR signals of bound water were used to quantitatively determine the anisotropy of the residual dipolar couplings in tendon. The values of the residual dipolar couplings were obtained from the DQ build-up curves as well as the DQ-filtered spectra measured in the initial regime of the excitation/reconversion periods. At the magic angle the value of the residual dipolar coupling is different from zero which is a manifestation of a distribution of the collagen fibril directors around the macroscopic symmetry axis of the tendon. In the first approximation this distribution can be described by a Gaussian angular distribution function with a center of the distribution slightly shifted from the tendon axis.

These  $^1\text{H}$  residual dipolar couplings which carry information about the orientation of the collagen fibrils in tendon are determined model free from the DQ build-up curves and doublet splitting. To a good approximation these values measured from the slopes of the DQ build-up curves are not affected by the anisotropy of the transverse relaxation rates and the inhomogeneities of the magnetic field. The sensitivity to the anisotropy of the ordered tissue can be increased as well as the signal-to-noise ratio if the maxima of the DQ build up curves are detected. However, the quantitative analysis of the data becomes more complex. Furthermore, the use of higher-order MQ coherences can further increase the sensitivity to the collagen fibril orientations because the initial slope of the MQ build-up curves increases with the order of the MQ coherence. The measurements of the  $^1\text{H}$  residual dipolar couplings at lower temperatures than the room or body temperatures will decrease the adverse effect of the proton exchange on the strength of DQ coherences. Moreover, our results show that the measurements at room temperature can be used in the initial regime of the excitation/reconversion periods to obtain information about the anisotropy of the residual dipolar couplings.

The anisotropy of the proton residual dipolar coupling measured by the splitting of the DQ-filtered spectra was used to validate the measurements made from the slopes of the DQ build-up curves. The parameters of the Gaussian distribution function obtained with both methods are in reasonable agreements. The method using the doublet splitting is, at least in principle, more accurate than the method based on the slopes of the DQ build-up curves. The advantage of the last method is that it can be used independent of the existence of a well-defined splitting. For instance,  $^1\text{H}$  DQ-filtered spectra of bovine Achilles tendon recorded at temperatures of 24 and 41 °C do not show any splitting [4]. Moreover, it is not affected by the normalization procedure. Nevertheless, more experimental data are needed in order to define a DQ build-up curve.

The method based on the DQ build-up curves described in this work can be used for a quantitative investigation of the anisotropy in different ordered tissues and to establish a correlation between the degree of orientation of collagen fibrils and the biological functionality. Moreover, degeneration processes and injuries can be characterized by the changes in the angular distribution of the fibrils. This method can be combined with MRI procedures [23] to measure in vivo the heterogeneities of the degree of orientation of various ordered tissues.

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