Solid-State NMR Studies of Liquid Crystals 
and of the HET-s(218-289) Prion

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Abbreviations

1D one-dimensional
2D two-dimensional
3D three-dimensional
CP cross-polarisation
CS chemical shift
CSA chemical shift anisotropy
CsPFO cesium perfluorooctanoate
FID free induction decay
FSR frequency-selective REDOR
FWHM full width at half maximum
INEPT intensive nuclei enhanced by polarization transfer
LC liquid crystal
MA magic angle
MAS magic angle spinning
MW molecular weight
NMR nuclear magnetic resonance
OD optical density
PBLG poly-γ-benzyl-L-glutamate
PMSF phenylmethanesulfonyl fluoride
REDOR rotational-echo double-resonance
RF radio frequency
SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNR signal to noise ratio
w/w weight over weight
Prions are infectious proteins with the ability to spread through self-replication of an altered conformation. Prions are responsible of a number of diseases including scrapie in sheep, bovine spongiform encephalopathy in cattle, and Creutzfeld-Jakob in humans. The diseased form of prions is characterized by a $\beta$-sheet-rich molecular aggregate called amyloid fibrils. The presence of amyloid fibrils has also been observed in the fungus *Podospora anserina*, where the HET-s prion triggers a programmed cell death. The HET-s molecule is composed of an N-terminus globular domain and a C-terminus prion forming domain, represented by residues 218 to 289 [HET-s(218-289)]. HET-s is a model of choice to study amyloid fibrils because of its suggested similarities with mammalian prions, as well as its well-defined structure at physiological pH, which does not show polymorphism, and the ease to express, purify and fibrilize the HET-s(218-289) monomers. The structure of HET-s(218-289) fibrils has been measured with solid-state nuclear magnetic resonance (NMR). Solid-state NMR does not require crystalline nor soluble samples, which are a prerequisite for X-ray crystallography and solution-state NMR, respectively. Solid-state NMR is therefore an appropriate technique to study amyloid fibrils at atomic resolution. In solid-state NMR flexible residues of proteins are measured with the INEPT transfer, whereas the CP sequence is used to obtain signal from the more rigid residues. The HET-s(218-289) fibrils show resonances in both CP- and INEPT-based spectra.

In this thesis the Rotational-Echo D0uble-Resonance (REDOR) sequence was tested on liquid crystals (LCs) displaying very small residual dipolar couplings (RDCs) in order to determine whether REDOR could be used the measure RDCs in very flexible residues of proteins. With our sample, RDCs around 60 Hz could be measured with REDOR with an accuracy of $\pm 5$ Hz. HET-s(218-289) fibrils were then studied with solid-state NMR using INEPT-based experiments. It was suggested that the signals observed in INEPT-based spectra originate either from flexible residues framing the $\beta$-sheets or from HET-s(218-289) monomers. A series of experiments was performed to test these hypotheses. REDOR experiments and spectra measured at an angle different than the "magic angle" showed that the RDCs of the residues displaying resonances in INEPT spectra have a value close to 0 Hz. Furthermore a NMR diffusion experiment indicated that these residues are part of very big units. These resonances were assigned using HNCA and HNCO experiments and showed striking spectral similarities with a sample containing HET-s(218-289) monomers in solution. The resonances observed in INEPT-based spec-
tra of HET-s(218-289) fibrils therefore probably belong to HET-s(218-289) monomers attached to the fibrils, which would explain the very slow diffusion coefficient. A limited proteolysis with the enzyme Proteinase K was then carried out in order to remove the HET-s(218-289) monomers, while keeping the fibrils intact. After optimization of the enzymesubstrate ratio, as well as of the incubation time, it was impossible to eliminate all the signals from the INEPT spectra without cleaving off the fibrils. The intensity of CP spectra was used as reference to evaluate the amount of fibrils in the sample. The resonances remaining after limited proteolysis likely originate from parts of HET-s(218-289) monomers less accessible to the enzyme for cleavage. The possibility that these signals come from flexible residues of HET-s(218-289) fibrils was therefore excluded.

In the second part of this work, a dynamic analysis of the HET-s(218-289) fibrils was performed by recording CP-based experiments. The timescales and amplitudes of motion of the $^1$H-$^{15}$N and $^1$Hα-$^{13}$Cα bonds were characterized by measuring $R_1$, $R_1ρ$, and REDOR experiments. The presence of two timescales of motion was observed: a faster timescale (ps-ns range) and a slower timescale ($\mu$s range). The faster timescale describes a very localized motion, which makes the interpretation of the results difficult. The slower timescale of motion suggests that the entire molecule sees a single motion.
Résumé

Les prions sont des protéines contagieuses qui se propagent en réplicant une conformation anormale. Les prions sont responsables d’un nombre de maladies comprenant la tremblante du mouton, l’encéphalopathie spongiforme bovine, aussi appelée “maladie de la vache folle”, ainsi que la maladie de Creutzfeldt-Jakob. La forme infectieuse du prion est caractérisée par un agrégat de protéines adoptant la structure typique du feuillet β, aussi appelé fibres amyloïdes. La présence de fibres amyloïdes a également été observée chez le champignon Podospora anserina, où le prion HET-s déclenche une mort cellulaire programmée. La molécule HET-s est composée d’une extrémité N-terminale formant un domaine globulaire et d’une extrémité C-terminale où est située la séquence codant pour le prion, représentée par les résidus 218 à 289 [HET-s(218-289)]. HET-s est un modèle de choix pour l’étude des fibres amyloïdes grâce à ses similitudes suggérées avec les prions mammifères, ainsi qu’à sa structure bien définie à pH physiologique, exempt de polymorphisme, et à la facilité à exprimer, purifier et fibriliser les monomères de HET-s. La structure des fibres de HET-s(218-289) a été mesurée par résonance magnétique nucléaire (RMN) du solide. La RMN du solide n’exige pas d’avoir des échantillons cristallins ou solubles, qui sont un prérequis pour la cristallographie à rayons X et la RMN en solution, respectivement. La RMN du solide est par conséquent une technique de choix pour l’étude des fibres amyloïdes. En RMN du solide, le transfert de magnétisation INEPT est habituellement utilisé pour mesurer les résidus flexibles alors que le transfert CP est employé pour obtenir le signal des résidus plus rigides. La présence de résonances est visible sur les spectres des fibres de HET-s(218-289) utilisant le transfert CP ainsi que le transfert INEPT.

La séquence REDOR a été testée sur des cristaux liquides présentant de petits couplages dipolaires résiduels afin de déterminer si REDOR pouvait être utilisé pour mesurer les couplages résiduels de résidus très flexibles de protéines. Avec notre échantillon, des couplages résiduels d’environ 60 Hz ont pu être mesurés avec REDOR avec une précision de ±5 Hz. Les fibres de HET-s(218-289) ont ensuite été mesurées avec la RMN du solide en utilisant des expériences employant le transfert INEPT. L’hypothèse a été émise que les signaux observés sur les spectres INEPT proviennent soit des résidus entourant les feuillet β, soit de monomères de HET-s(218-289). Une série d’expériences a été réalisée dans le but de tester ces hypothèses. Les expériences REDOR et les spectres mesurés à un angle de rotor différent de l’“angle magique” ont montré que les couplages résiduels
des résidus montrant des signaux dans les spectres INEPT sont très petits. De plus, les expériences mesurant le coefficient de diffusion de ces résonances ont indiqué que ces résidus font partie d’unité de grande taille. Ces résonances ont été attribuées en utilisant les séquences HNCA et HNCO basées sur le transfert INEPT et ont montré des similarités saisissantes avec un échantillon contenant uniquement des monomères de HET-s(218-289) en solution. Les résonances observées dans les spectres INEPT appartiennent donc très probablement aux monomères de HET-s(218-289) attachés aux fibres, ce qui expliquerait la très lente diffusion des monomères. Une protéolyse limitée avec l’enzyme Proteinase K a ensuite été effectuée afin de digérer les monomères de HET-s(218-289) tout en gardant les fibres intactes. Après optimisation du ratio enzyme:substrat ainsi que du temps d’incubation, il était impossible d’éliminer tous les signaux des spectres INEPT sans détruire les fibres de HET-s(218-289). L’intensité des spectres utilisant un transfert CP a été utilisée pour l’évaluation de la quantité de fibres dans l’échantillon. Les résonances restantes après protéolyse limitée appartiennent probablement aux parties des monomères de HET-s(218-289) moins accessibles au clivage par l’enzyme. La possibilité que ces signaux proviennent des résidus flexibles des fibres de HET-s(218-289) a donc été exclue.

Dans la deuxième partie de ce travail, une analyse de la dynamique des fibres de HET-s(218-289) a été effectuée par l’enregistrement d’expériences utilisant un transfert CP. Les échelles de temps ainsi que les amplitudes de mouvement des liaisons $^1\text{H}-^{15}\text{N}$ et $^1\text{H}-^{13}\text{C}$ ont été caractérisées en mesurant des expériences $R_1$, $R_\rho$, et REDOR. La présence de deux échelles de temps de mouvement a été observée: une plutôt rapide (échelle de la ps-ns) et une plutôt lente (échelle de la µs). La première échelle de temps décrit un déplacement très localisé, ce qui rend difficile l’interprétation des résultats dans leur ensemble. La deuxième suggère que la molécule entière est soumise à un seul mouvement.
Chapter 1

Introduction to solid-state NMR

Solid-state NMR is a powerful technique that allows the study of both the average structure and dynamics of molecules using the combination of magic-angle spinning and radio-frequency (RF) pulse sequences. In NMR, the Hamiltonians describe two kinds of interactions: the interaction between a spin and a magnetic field and the interaction between two spins. Three Hamiltonians characterize the interaction between a spin and a magnetic field: the Zeeman Hamiltonian $\hat{H}_Z$, the chemical shift Hamiltonian $\hat{H}_{CS}$ and the RF field Hamiltonian $\hat{H}_{RF}$. The interaction between two spins is given by the dipolar-coupling Hamiltonian $\hat{H}_D$, the quadrupolar-coupling Hamiltonian $\hat{H}_Q$ and the J-coupling Hamiltonian $\hat{H}_J$, also known as “scalar coupling”. For a spin-$\frac{1}{2}$ nuclei, the total NMR Hamiltonian is a sum of all the Hamiltonians described previously:

$$\hat{H} = \hat{H}_Z + \hat{H}_{CS} + \hat{H}_{RF} + \hat{H}_D + \hat{H}_J.$$  

(1.1)

Some of these interactions are defined in Sections 1.3, 1.4, 1.5, and 1.6.

In solid-state NMR, in contrast to solution-state NMR, the anisotropic part of the chemical shift tensor, as well as the dipolar coupling tensor, are not averaged out to zero because of the absence of molecular tumbling, and lead to broad spectral lines. To circumvent this issue, the NMR sample is spun at an angle $\theta \approx 54.7^\circ$ against the static magnetic field $\vec{B}_0$, the so-called “magic-angle”, at a spinning frequency higher than most of the anisotropic interactions. The remaining interactions, for example the strongly dipolar-coupled nuclei or the J-coupled interactions, can be decoupled using an RF field, whose strength depends on the interactions to decouple. For J couplings, an RF field of 2-3 kHz can be enough, while the decoupling of highly coupled protons sometimes requires more than 100 kHz RF field. Specific decoupling pulse sequences are used depending on the experimental conditions: strength of the coupling, spinning rate, for example. Well-known pulse sequences are, for example, WALTZ [1], SPINAL [2], TPPM [3] or XIX [4].

\footnote{The Equations of this Chapter have been derived from Lecture Notes, Physical Chemistry IV: Magnetic Resonance, HS 2015, written by Prof. Matthias Ernst, Prof. Beat H. Meier, and Prof. Gunnar Jeschke, as well as from Advanced Magnetic Resonance, HS 2013, Relaxation Theory in Liquid-State and Solid-State NMR Spectroscopy, written by Prof. Matthias Ernst.}
1.1 Orientation of the molecules

The orientation of a molecule relative to the laboratory frame is defined by three Euler angles $\alpha$, $\beta$ and $\gamma$. These angles describe a rotation from the principal-axis system (PAS) to the laboratory frame. The PAS is a particular molecular fixed coordinate system, where the chemical-shift tensor $\sigma$ is diagonal

$$
\sigma = \begin{pmatrix}
\sigma_{11} & 0 & 0 \\
0 & \sigma_{22} & 0 \\
0 & 0 & \sigma_{33}
\end{pmatrix}
$$

(1.2)

The Euler angles $\alpha$, $\beta$ and $\gamma$ describe three successive rotations: first by $\alpha$ around the original $z$-axis, then by $\beta$ around the new $y'$-axis and last by $\gamma$ around the new $z''$-axis.

A powder sample contains molecules with many different orientations having each the same probability of appearance. The sample is called isotropic. If some molecular orientations are preferred, the sample is anisotropic. Figure 1.2 shows a spectrum of an isotropic sample, characterized by its powder pattern, as well as spectra of anisotropic samples with molecules aligned with $\beta=0^\circ$ and $\beta=90^\circ$.

---

**Figure 1.1:** The Euler angles $\alpha$, $\beta$, and $\gamma$ describe three rotations of the PAS coordinate frame: first by $\alpha$ around the original $z$-axis, then by $\beta$ around the new $y'$-axis and last by $\gamma$ around the new $z''$-axis.
1.2 The rotating-frame Hamiltonian

In solid-state NMR experiments the sample is rotated about an axis inclined by an angle \( \theta_r \) with respect to the static magnetic field \( B_0 \), which is usually equal to \( \theta_r = \theta_m \approx 54.7^\circ \). The determination of the chemical-shift and dipolar-coupling tensor is performed in two steps. The PAS is first transformed into a rotor-fixed coordinate system. The rotor-fixed coordinate system is in a second step rotated into the laboratory frame. The spherical-tensor components in the rotor-fixed frame are described by

\[
A_{lm''}^{(lab)} = \sum_{m'=-l}^{l} D_{m'r,m''r}^l(\omega_r t, -\theta_r, 0) \sum_{m=-l}^{l} D_{m,m'}^l(\alpha, \beta, \gamma) \rho_{lm}^{(PAS)},
\]

(1.3)

where \( D \) is the Wigner rotation matrix, \( \rho \) the density operator, \( l \) the rank of the tensor, \( \alpha, \beta \) and \( \gamma \) are the Euler angles. The index \( m'' \) denotes the laboratory frame, \( m' \) the rotor-fixed frame and \( m \) the PAS frame. In the high-field approximation only the \( A_{l0}^{(lab)} \) are considered. Equation (1.3) simplifies therefore to

\[
A_{l0}^{(lab)} = \sum_{m'=-l}^{l} D_{m',0}^l(\omega_r t, -\theta_r, 0) \sum_{m=-l}^{l} D_{m,m'}^l(\alpha, \beta, \gamma) \rho_{lm}^{(PAS)}.\]

(1.4)
The Wigner rotation matrix for the angles \(-\omega_r, -\theta_r, 0\) is defined as
\[
\mathcal{D}^l_{m,m'}(-\omega_r, -\theta_r, 0) = e^{i m' \omega_r} d^l_{m'm}(-\theta_r)
\]
and for the Euler angles \(\alpha, \beta\) and \(\gamma\).
\[
\mathcal{D}^l_{m',0}(\alpha, \beta, \gamma) = e^{-i \alpha_0} d^l_{0m'}(\beta) e^{-i m' \gamma}.
\]
Equation 1.4 can therefore be written as
\[
A^{(lab)}(l_0) = \sum_{m'=-l}^{l} e^{i m' \omega_r} d^l_{m'm}(-\theta_r) A^{(rot)}_{lm'},
\]
where
\[
A^{(rot)}_{lm'} = \sum_{m=-l}^{l} \mathcal{D}^l_{m,m'}(\alpha, \beta, \gamma) \rho^{(PAS)}_{lm}.
\]

The time-depending high-field truncated Hamiltonian of rank \(l\) is therefore
\[
\hat{\mathcal{H}}(t) = \sum_{m'=-l}^{l} e^{i m' \omega_r t} d^l_{m'm}(-\theta_r) A^{(rot)}_{lm'} \hat{T}^0_l.
\]
\(\hat{T}^0_l\) is the spin part of the Hamiltonian. The parameter \(d^l\) denotes the reduced Wigner elements of rank \(l\). The values of the reduced Wigner elements of rank 2 are shown in Appendix E.

### 1.3 The dipolar-coupling Hamiltonian

The rotating-frame dipolar-coupling Hamiltonian for an heteronuclear pair of spins 1 and 2 is given by
\[
\hat{\mathcal{H}}^D = -\frac{\mu_0 \gamma_1 \gamma_2 \hbar}{4 \pi r_{12}^3} \left( \frac{3 \cos^2 \theta_{12} - 1}{2} \right) \hat{I}_{1z} \hat{I}_{2z},
\]
where \(\gamma_1\) and \(\gamma_2\) indicate the gyromagnetic ratios of spins 1 and 2, \(r_{12}\) the effective bond length between the spins, \(\mu_0\) the magnetic permeability of free space, and \(\hbar\) the Planck’s constant. The parameter \(\theta_{12}\) is the angle between the vector joining the spins 1 and 2 and the external magnetic field \(B_0\). The brackets indicate the average over all of the orientations, weighted by their probabilities. \(\hat{I}_z\) is an operator that represents the z-component of the nuclear spin angular momentum.

The constant
\[
\delta_D = -\frac{2 \mu_0 \gamma_1 \gamma_2 \hbar}{4 \pi r_{12}^3} \left( \frac{3 \cos^2 \theta_{12} - 1}{2} \right)
\]
is the anisotropy of the dipolar-coupling Hamiltonian. For isotropic samples Equation 1.11 simplifies to
\[
\delta_D = -\frac{2 \mu_0 \gamma_1 \gamma_2 \hbar}{4 \pi r_{12}^3}
\]
In the following Sections the Hamiltonians of interactions considered in the rest of this work are described.

1.4 The chemical-shift Hamiltonian

The chemical shift (CS) of a nucleus depends on its local magnetic field, which is influenced by the local electronic environment of the nucleus. The magnetic field at the position of the nucleus $k$ is given by

$$\vec{B}_k = \vec{B}_0 + \vec{B}_S,$$  \hfill (1.13)

where $\vec{B}_S$ is the correction field and is defined as

$$\begin{pmatrix} B_{S,x} \\ B_{S,y} \\ B_{S,z} \end{pmatrix} = - \begin{pmatrix} \sigma_{xx}^{(k)} & \sigma_{xy}^{(k)} & \sigma_{xz}^{(k)} \\ \sigma_{yx}^{(k)} & \sigma_{yy}^{(k)} & \sigma_{yz}^{(k)} \\ \sigma_{zx}^{(k)} & \sigma_{zy}^{(k)} & \sigma_{zz}^{(k)} \end{pmatrix} \begin{pmatrix} 0 \\ 0 \\ B_0 \end{pmatrix},$$  \hfill (1.14)

The CS Hamiltonian that describes the interaction with the correction field is therefore given by

$$\hat{\mathcal{H}}_{CS} = \sum_k \gamma_k (\hat{I}_{k,x}, \hat{I}_{k,y}, \hat{I}_{k,z}) \begin{pmatrix} \sigma_{xx}^{(k)} & \sigma_{xy}^{(k)} & \sigma_{xz}^{(k)} \\ \sigma_{yx}^{(k)} & \sigma_{yy}^{(k)} & \sigma_{yz}^{(k)} \\ \sigma_{zx}^{(k)} & \sigma_{zy}^{(k)} & \sigma_{zz}^{(k)} \end{pmatrix} \begin{pmatrix} 0 \\ 0 \\ B_0 \end{pmatrix},$$  \hfill (1.15)

where $\gamma_k$ is the gyromagnetic ratio of the nucleus $k$, $\hat{I}_k$ represents the nuclear spin angular momentum of the nucleus $k$ and the quantity $\sigma$ is the chemical-shift tensor. The anisotropy $\delta$ of the tensor is defined by

$$\delta = \sigma_{zz}^{(k)} - \bar{\sigma},$$  \hfill (1.16)

where $\bar{\sigma}$ is the trace of the tensor. The asymmetry $\eta$ is given by

$$\eta = \frac{\sigma_{yy}^{(k)} - \sigma_{xx}^{(k)}}{\delta}.$$  \hfill (1.17)

1.5 The J-coupling Hamiltonian

The J coupling is an interaction between spins mediated by the binding electrons. In the rotating frame, the Hamiltonian for the heteronuclear J coupling in the high-field approximation is given by

$$\hat{\mathcal{H}}_J^{(k,n)} = 2\pi J^{(k,n)} \hat{I}_{k,z} \hat{S}_{n,z}.$$  \hfill (1.18)
1.6 The quadrupolar-coupling Hamiltonian

The quadrupolar coupling is present for nuclei with a spin quantum number $I > 1/2$. For these nuclei the uneven distribution of charges within the nucleus generates an electric field gradient, which gives rise to the quadrupolar interaction. The Cartesian formulation of the quadrupolar Hamiltonian is defined by

$$\hat{\mathcal{H}}_Q = \hat{T}_k Q^{(k)} \hat{T}_k = \left( \hat{I}_{kx} \hat{I}_{ky} \hat{I}_{kz} \right) \left( \begin{array}{ccc} Q^{(k)}_{xx} & Q^{(k)}_{xy} & Q^{(k)}_{xz} \\ Q^{(k)}_{yx} & Q^{(k)}_{yy} & Q^{(k)}_{yz} \\ Q^{(k)}_{zx} & Q^{(k)}_{zy} & Q^{(k)}_{zz} \end{array} \right) \left( \begin{array}{c} \hat{I}_{kx} \\ \hat{I}_{ky} \\ \hat{I}_{kz} \end{array} \right),$$

where

$$Q^{(k)} = \frac{eQ}{2I(2I-1)\hbar} V^{(k)},$$

with the nuclear quadrupolar moment $eQ$ and the electric field gradient-tensor $V$.

1.7 Transfer of polarization

In solid-state NMR, the method of choice to transfer polarization from a high-$\gamma$ nuclei to a low-$\gamma$ nuclei is the Hartmann-Hahn cross-polarization (CP) [5–7], where the transfer of polarization is mediated by dipolar couplings using specific RF irradiation, as illustrated in Figure 1.3A. In solution NMR the preferred method to transfer polarization is the INEPT scheme [8, 9] shown in Figure 1.3B. INEPT is also employed in solid-state NMR to measure flexible residues of a protein, for instance. The rigid parts of proteins have a short $T_2$ and the efficiency of INEPT is therefore very low.

![Figure 1.3](image)

**Figure 1.3:** The CP scheme in **A** and the INEPT scheme in **B** to transfer the magnetization from $^1H$ to X. The delay $\Delta$ is equal to $1/4J$. The second 90° pulse on $^1H$ is of phase $y$.

1.8 Dynamics studies

Understanding the dynamics of a protein is a *sine qua non* condition to characterize its biological function [10, 11]. Proteins can be modeled as small machines and are responsible
for a vast variety of tasks including inner cell organisation and transmission of signals from outside to inside the cell. Each of these steps concerns transitions between different protein subconformations. These transitions are characterized by different timescales and amplitudes of motion. Slow motions involve for instance ligand binding or signal transduction, and indicate large-scale rearrangement of a protein. Faster motions are more local, small-amplitude motions. Solid-state NMR is a method of choice to study a broad range of timescales of motion (from $\sim$1 second to $\sim$1 picosecond).

The amplitude of motion, characterized by the order parameter $S^2$, depends on the anisotropy of the dipolar coupling $\delta_D$ between two nuclei 1 and 2, and can be measured with solid-state NMR. The order parameter $S^2$ is calculated using the experimentally measured dipolar coupling $\delta_{D}^{\text{expt}}$.

$$\langle S^2 \rangle = \left( \frac{\delta_{D}^{\text{expt}}}{\delta_{D}^{\text{rigid}}} \right)^2,$$

where the rigid-limit value of the dipolar coupling $\delta_{D}^{\text{rigid}}$ is calculated with Equation 1.11 using the distance between nuclei 1 and 2. The order parameter satisfies the inequalities $0 \leq S \leq 1$. If the motion is isotropic, $S^2 = 0$; if the motion is fully restricted, $S^2 = 1$.

If multiple motions are present, $S^2$ is the product of all motions acting on the dipolar coupling

$$S^2 = S_1^2 \times S_2^2 \times ...$$

### 1.8.1 Amplitude of the motion: REDOR

Dipolar couplings can be recoupled with a variety of NMR pulse sequences. According to Schanda et al. [12], REDOR [13] appears to be more robust against RF inhomogeneities and incorrect setting of the RF field strength than T-MREV [14] or symmetry-based sequences [15].

REDOR was developed in 1989 by Gullion and coworkers [13] to recouple the $^{13}\text{C}-^{15}\text{N}$ dipolar couplings in $^{13}\text{C}$- and $^{15}\text{N}$-labeled alanine. After a $^1\text{H}-^{13}\text{C}$ cross-polarization, the averaging of the $^{13}\text{C}-^{15}\text{N}$ dipolar coupling by MAS is prevented by inserting two $^{15}\text{N} \pi$ pulses per rotor period, as shown in Figure 1.4. One of the $\pi$ pulses is synchronized with the full rotor period and the other with a time smaller or equal to half the rotor period. The $^{13}\text{C}-^{15}\text{N}$ coupling can be measured by recording the difference between a $^{13}\text{C}$ NMR spectrum obtained under these conditions ($S$), and one measured without $^{15}\text{N} \pi$ pulses ($S_0$). REDOR can be more generally used to recouple heteronuclear dipolar coupled spin pairs.
Figure 1.4: REDOR pulse sequence for the measurement of $^{13}$C-$^{15}$N RDCs. The magnetization is transferred from $^1$H to $^{13}$C by CP (1). During the REDOR period, rotor synchronized 180° pulses are applied on $^{15}$N to reintroduce $^{13}$C-$^{15}$N dipolar couplings (2). A 180° pulse on $^{13}$C in the middle of the REDOR periods refocuses the $^{13}$C chemical shift (3). The magnetization evolves under $^1$H decoupling. The REDOR period is highlighted with the red box.

Figure 1.5: Simulated REDOR curves for a powder sample with a number of $\gamma$ angles set to 40. The dipolar coupling $D$ of the red curve is twice smaller as the one of the blue curve.

The difference between $S_0$ and $S$ is plotted against the dephasing time of the REDOR period to obtain REDOR curves. We call “dephasing” of the REDOR curve the part of the curve between 0 and its maximum $(S-S_0)/S_0$. The dephasing of the REDOR curve gives access to the strength of the dipolar coupling and depends only on the anisotropy of the dipolar coupling. As illustrated in Figure 1.5, the dephasing of the REDOR curve becomes faster with increasing dipolar coupling. Since REDOR data are recorded in a normalized manner using a reference experiment, REDOR has the advantage that fitting is very robust and handy: one can fit the data using the dipolar coupling of interest as single parameter.
Variations of REDOR

2- and 4-pulse REDOR. A molecule can adopt different orientations within a sample. Depending on the orientation of the molecule, the number of $\pi$ pulses per rotor period of the REDOR sequence varies in order to recouple the residual dipolar coupling between two atoms of the molecule. We employed two variations of REDOR: REDOR containing two and four $\pi$ pulses per rotor period, called 2- and 4-pulse REDOR respectively, illustrated in Figure 1.6. In the presence of a powder sample, both the 2- and 4-pulse REDOR yield to a curve reaching 1 and displaying oscillations, as illustrated in Figure 1.7A, the 2-pulse REDOR dephasing however slightly faster than the 4-pulse REDOR. If the sample solely contains molecules with an angle $\beta=90^\circ$, only the component of the rotating frame dipolar coupling Hamiltonian of the form of Equation 1.9 that oscillates at $\pm 2$ times the rotor frequency is non-equal to zero. This term is recoupled using the 4-pulse REDOR, as depicted in Figure 1.7B. However, if the molecules orient with $\beta=0^\circ$, the rotating frame dipolar coupling Hamiltonian of the form of Equation 1.9 is equal to 0 and cannot be recoupled with 2- nor 4-pulse REDOR as shown in Figure 1.7C.

**Figure 1.6:** REDOR period employing two or four $180^\circ$ pulses on Y per rotor period. The $180^\circ$ pulse on X between the two rotor periods refocuses the X chemical shift. The magnetization is on X during the whole sequence.

**Figure 1.7:** Simulated 2- and 4-pulse REDOR curves, in blue and black, respectively, obtained with a powder sample in A, with $\beta=90^\circ$ in B, and with $\beta=0^\circ$ in C. The number of $\gamma$ angles was set to 40 for all simulations.
**Shifted REDOR** If the standard REDOR sequence recouples dipolar couplings too strongly, which means that the dephasing of the curve does not contain enough experimental data points, the measurement of these dipolar couplings cannot be done accurately. The dephasing of the REDOR curve can be reduced by employing a shifted version of REDOR [16], where the delay \( \tau \) can be decreased as depicted in Figure 1.8. The delay \( \tau \) is equal to half a rotor period in the non-shifted 2-pulse REDOR.

![Figure 1.8](image)

**Figure 1.8:** A Pulse sequence employed to record a shifted version of REDOR for an isotropic sample. Reducing the delay \( \tau \) leads to a slower dephasing of the REDOR curve, as depicted in panel B for simulated REDOR curves with three different \( \tau \) values and an arbitrary dipolar coupling and spinning frequency.

### 1.8.2 Correlation time of the motion: \( R_1 \) and \( R_{1,\rho} \)

The correlation time of the motion can be characterized by the measurements of several longitudinal relaxation rates (\( R_1 \)) and transverse relaxation rates (typically \( R_{1,\rho} \)). In order to calculate a relaxation-rate constant the spectral density functions \( J(\omega) \) have to be known. Spectral density functions are Fourier transforms of correlation functions, which are functions characterizing the motion and are often described by a sum of exponentially decaying functions. Single relaxation rate does not provide enough information for the determination of the spectral density functions. A more exact form of the correlation function is obtained with multiple relaxation measurements.
CHAPTER 1. INTRODUCTION TO SOLID-STATE NMR

In theory the motion is described by several correlation times. Solid-state NMR studies of the dynamics however generally characterize two correlation times of motion. The information of the faster motion (typically $\tau_c<10$ ns) is obtained with $R_1$, while information on slower motion (typically $\tau_c>1$ ns) is provided by $R_{1\rho}$, although the information collected with $R_1$ and $R_{1\rho}$ often overlap. The dynamics is therefore usually characterized by fitting the measured parameters to a dynamic model, in general the Lipari-Szabo model-free analysis [17]. Lipari and Szabo suggested that the fast internal motions can be described by only two model-independent quantities: an effective correlation time $\tau_e$ and a generalized order parameter $S^2$. Using this model, the spectral density function is given by

$$J(\omega) = \frac{2}{5}(1-S^2) \frac{\tau_e}{1 + (\omega \tau_e)^2}. \quad (1.23)$$

If a slow and a fast motion are present, the spectral density function is defined using the extended model-free approach [18]

$$J(\omega) = \frac{2}{5}(1-S_{fast}^2) \frac{\tau_{c,fast}}{1 + (\omega \tau_{c,fast})^2} + \frac{2}{5}(1-S_{slow}^2) S_{fast}^2 \frac{\tau_{c,slow}}{1 + (\omega \tau_{c,slow})^2}. \quad (1.24)$$

The $R_1$ relaxation of spin I generated by fluctuations of a dipole coupling with spin S ($\delta^{IS}$) is given by

$$R^{IS}_1 = \left(\frac{\delta^{IS}}{4}\right)^2 (J(\omega_I - \omega_S) + 3J(\omega_I) + 6J(\omega_I + \omega_S)), \quad (1.25)$$

and the $R_1$ relaxation of spin I induced by a CSA ($\omega_I \sigma_{zz}$) is given by

$$R^{CSA}_1 = \frac{3}{4}(\omega_I \sigma_{zz})^2 J(\omega_I). \quad (1.26)$$

The total relaxation is therefore expressed as

$$R_1 = R^{IS}_1 + R^{CSA}_1. \quad (1.27)$$

Kurbanov et al. [19] derived the equations to calculate $R_{1\rho}$ using the model-free calculation and taking into account the effects of both MAS and an applied RF field. The $R_{1\rho}$ relaxation of spin I by the dipole coupling with spin S is given by

$$R^{IS}_{1\rho} = \frac{1}{2} R^{IS}_1 + \left(\frac{\delta^{IS}}{4}\right)^2 \left(\frac{1}{3} J(\omega_S) + \frac{2}{3} J(\omega_1 - 2\omega_r) + \frac{2}{3} J(\omega_1 - \omega_r) + \frac{2}{3} J(\omega_1 + \omega_r) + \frac{1}{3} J(\omega_1 + 2\omega_r)\right), \quad (1.28)$$

where $\omega_r$ and $\omega_1$ are the rotor frequency and the RF field strength applied to the spin, respectively. For the $R_{1\rho}$ relaxation of spin I resulting from the CSA, one obtains

$$R^{CSA}_{1\rho} = \frac{1}{2} R^{CSA}_1 + \frac{1}{6}(\omega_I \sigma_{zz})^2 \left(\frac{1}{2} J(\omega_1 - 2\omega_r) + J(\omega_1 - \omega_r) + J(\omega_1 + \omega_r) + \frac{1}{2} J(\omega_1 + 2\omega_r)\right). \quad (1.29)$$
The total $R_{1\rho}$ relaxation is therefore given by

$$R_{1\rho} = R_{1\rho}^{IS} + R_{1\rho}^{CSA}.$$  

(1.30)
Part I

Liquid Crystals
Chapter 1

Introduction

Liquid crystals (LCs) have been observed for the first time in the end of the XIXth century by F. Reinitzer [20] and O. Lehmann [21]. LCs are in an intermediate state of aggregation between amorphous liquid and crystalline solid. They are highly anisotropic and yet show some fluidity, and occur for molecules that are geometrically anisotropic in shape, like a disk or most often like a rod. The fluidity of the LCs comes from the ease with which the molecules slide past one another while still retaining their parallelism [22]. LCs go through one or more phases before being transformed into the isotropic liquid. These phase transitions may take place when heat is brought to the system but also depend on the concentration of solvent. In 1922, M. G. Friedel, classified the LCs into three types: nematic, cholesteric and smectic [23]. In 1970, A. de Vries used X-ray diffraction to define the structure of a nematic LC [24]. He claimed that, in the nematic phase, the only restriction on the arrangement is that the molecules have a more or less parallel orientation with respect to their neighbors. In a static magnetic field $\mathbf{B}_0$, LCs with positive magnetic anisotropy ($\Delta \chi_m > 0$) tend to align with the director parallel to $\mathbf{B}_0$ and those with negative magnetic anisotropy ($\Delta \chi_m < 0$) perpendicular to $\mathbf{B}_0$. The director results from averaging all possible orientations weighted by their probabilities.

LCs are recognized since the 1960s as alignment media [25, 26]. However, the development of partial alignment leading to small tunable RDCs started about forty years later [27, 28]. When a molecule is dissolved in a LC, the orientation of this molecule is affected by the alignment of the LC molecules: it acquires a partial orientation by interactions with the LC molecules, as sketched in Figure [1.1]. In this thesis nematic LCs were used to study RDCs of a $^1$H-$^{13}$C isolated 2-spin system. Cesium perfluorooctanoate in heavy water (CsPFO/D$_2$O) was first investigated. Phe-$2^{-13}$C was dissolved in the LCs in order to study the $^1$H$\alpha$-$^{13}$C$\alpha$ RDCs of Phe-$2^{-13}$C. Poly-$\gamma$-benzyl-L-glutamate in chloroform (PBLG/$^{13}$CHCl$_3$) was then examined. The RDCs $^1$H-$^{13}$C spin pair of $^{13}$CHCl$_3$ were considered.
Part I. Liquid Crystals

Figure 1.1: Sketch of LCs with positive magnetic anisotropy and solvent molecules that tend to be oriented with their long axis parallel to the director.

The RDC, represented by the letter $D$, can be calculated by measuring the total spin-spin constant $T_{\text{static}}$, which is the total spectral $^{13}\text{C}$ splitting. In the case of a static sample with positive magnetic anisotropy, $T_{\text{static}}$ can be calculated by using

$$T_{\text{static}} = J_{\text{iso}} + \frac{2D}{2\pi},$$

where $D$ is given by

$$D = \delta_{D}/2,$$

with $\delta_{D}$ defined in Equation 1.1.

In this thesis, only LCs with positive magnetic anisotropy were considered. When LCs with positive magnetic anisotropy are spun in a rotor around an axis tilted by an angle $\theta_r$ with respect to the magnetic field smaller than the MA, the director of the LCs is aligned parallel to the rotor axis, in order to minimize the magnetic energy [29, 30]. The dipolar interactions are scaled by the second Legendre polynomial of the cosine of the angle $\beta$ [$P_2(\cos\beta) = (3\cos^2\beta - 1)/2$. $P_2(\cos\beta)$ is equal to 1, if the LCs are aligned parallel to the rotor axis ($\beta = 0^\circ$). By changing the angle $\theta_r$ the dipolar interactions are scaled by a factor proportional to the second Legendre polynomial of the cosine of the angle $\theta_r$ [$P_2(\cos\theta_r) = (3\cos^2\theta_r - 1)/2$].

The total spin-spin constant of the spinning sample $T_{sp}$ can therefore be obtained from Equation 1.1

$$T_{sp} = J_{\text{iso}} + \frac{3\cos^2\theta_r - 1}{2} \left( \frac{3\cos^2\beta - 1}{2} \right) \left( \frac{2D}{2\pi} + J_{\text{aniso}} \right),$$
where $T_{sp}$ is the total spin-spin coupling constant, $\theta_r$ the angle to the magnetic field, $J_{iso}$ the isotropic scalar coupling constant (also simply called $J$), $D$ the residual dipolar coupling and $J_{aniso}$ the anisotropic scalar coupling, which is usually neglected. The same types Equations as Equation 1.1 and Equation 1.3 are valid for the quadrupolar coupling $Q$ as well

$$T_{\text{static, } Q} = 2Q,$$  \hspace{1cm} (1.4)

$$T_{sp, Q} = \frac{3\cos^2\theta_r - 1}{2}(\frac{3\cos^2\beta - 1}{2})2Q.$$  \hspace{1cm} (1.5)
Chapter 2
Cesium perfluorooctanoate/water

This work was done in collaboration with Albert A. Smith and Giorgia Zandomeneghi. Albert A. Smith provided support in performing and evaluating the experiments, and in implementing the simulations; Giorgia Zandomeneghi gave very helpful input for the progression of the project.

2.1 Introduction

About thirty years ago, Boden et al. [31] determined the high-resolution phase diagram of CsPFO/D$_2$O using $^2$H NMR spectroscopy combined with a variety of other techniques. They showed the occurrence of a nematic phase over a broad range of temperatures and concentrations. The nematic CsPFO/D$_2$O LCs have a director aligned parallel to the magnetic field $B_0$ due to the high positive magnetic anisotropy of the fluorinated alkyl chains [32]. The amphiphilic molecules shown in Figure 2.1 aggregate to form very long cylinders. The CsPFO/D$_2$O samples were prepared by dissolving 1 % weight fraction of Phe-2-$^{13}$C in the LCs. The $^1$H-$^{13}$C RDCs of Phe-2-$^{13}$C were studied by measuring $^{13}$C NMR spectra.

![Figure 2.1: A CsPFO molecule in solution.](image)

2.2 Results and Discussion

CsPFO was dissolved in D$_2$O to a weight fraction of 0.4, as described in 2.3.1. According to Boden et al. [31], the nematic phase is found around 300 K at this concentration. To
determine with accuracy the temperature range of the nematic phase, a series of $^2H$ NMR spectra of CsPFO/D$_2$O was measured between 290 and 302 K. Figure 2.2 shows the $^2H$ splitting as a function of the temperature. The nematic phase is found approximately between 295 and 302 K. The nematic phase is visible by a steeper slope of the curve than between 290 and 294 K where the lamellar phase is found. At 303 K, the sample enters the isotropic phase, characterized by the absence of RDCs, and only a single peak is visible in the $^2H$ NMR spectrum.

![Figure 2.2](image)

**Figure 2.2:** The $^2H$ NMR doublet measured between 290 and 302 K shows the presence of two phases: a nematic phase and a lamellar phase. The nematic phase is characterized by a steeper slope than the lamellar phase. The straight lines are a guide to the eye but do not represent a fit.

The measurements were performed at a temperature of 299 K, which roughly corresponds to the middle of the temperature range of the nematic phase in Figure 2.2. A $^{13}C$ spectrum shown in Figure 2.3 was recorded without sample spinning to determine the total spin-spin constant $T_{static}$ between $^1H\alpha$ and $^{13}C\alpha$ of Phe-2-$^{13}C$. The MAS spectrum in Figure 2.3 displays a splitting corresponding to the $^1H\alpha$-$^{13}C\alpha$ J coupling of Phe-2-$^{13}C$. From these spectra, we measured $T_{static}=\pm1840$ Hz and $J=145$ Hz. Since the sign of $T_{static}$ can be positive or negative, the residual dipolar coupling $D$, which is calculated using Equation 1.1, can have two values, namely $D_1=-993$ Hz or $D_2=848$ Hz.
The sample was then spun at a spinning frequency $\nu_r=450$ Hz and at a rotor angle $\theta_r=52.4^\circ$. The splitting measured was 247 Hz, as shown in Figure 2.4A, which corresponds to the value of $T_{sp}$ calculated using Equation 1.3 with $D_2=848$ Hz, $\theta_r=52.4^\circ$ and $\beta=0^\circ$. We therefore discarded $D_1$ and concluded that $D=D_2=848$ Hz. The spinning frequency was gradually increased to 5000 Hz. A loss of orientation of the LCs was observed with increasing spinning frequency as illustrated in Figures 2.4B-H. At a spinning frequency of 450 Hz, the LCs orient with $\beta=0^\circ$, as expected from theory [29]. However, at spinning frequencies equal to 850 Hz or higher, more than two peaks are visible in the spectra. Two of these peaks can be assigned to isotropic $^{13}$C$\alpha$ of Phe-2-$^{13}$C, highlighted with dashed black lines, and characterized by a $^{13}$C splitting of 145 Hz, which represent the J coupling between $^1$H$\alpha$ and $^{13}$C$\alpha$. The other part of the spectrum could be fitted using a powder pattern with a dipolar constant of 1500 Hz, as depicted in Figure 2.5 for a spinning frequency of 5000 Hz. The isotropic:anisotropic ratio was $\sim1/3$ (estimated by integrating the different peaks). The CSA of $^1$H and $^{13}$C were assumed to be equal to 0. The dipolar coupling of 1500 Hz is however larger than $D=848$ Hz measured in the static spectrum and in the spectrum recorded at a rotor angle of 52.4° and at a spinning frequency of 450 Hz. A possible explanation for this larger dipolar coupling is the loss of water in a part of the sample at higher MAS frequencies.

Figure 2.3: $^{13}$C spectra of the CsPF/O/D$_2$O system measured without sample spinning in blue and with a MAS of 5000 Hz in red.
Figure 2.4: $^{13}$C spectra of the CsPFO/D$_2$O system measured at spinning frequencies between 450 and 5000 Hz and at a rotor angle of 52.4°. At slow spinning frequencies, most of the LCs are aligned parallel to the spinning axis, as highlighted with the red dashed lines. With increasing spinning frequency, the orientation of the LCs tends to be lost.
Figure 2.5: $^{13}$C spectrum measured at a rotor angle of 52.4° and a spinning frequency of 5000 Hz, in blue. Simulated powder pattern using a RDC of 1500 Hz and a line broadening of 10 Hz, in black. The SIMPSON script used for the simulations is shown in Appendix C.

Figure 2.6: REDOR pulse sequence used to recouple RDCs between $^1$H-$^{13}$C in the LCs samples. The pulse sequence starts with a 90° pulse on $^{13}$C and is then followed by the REDOR periods containing two or four 180° pulses on $^1$H per rotor period. The 180° pulse on $^{13}$C in the middle of the two REDOR periods refocuses the $^{13}$C chemical shift. WALTZ16 is used on $^1$H during acquisition to decouple the $^1$H-$^{13}$C J coupling.

The 2- and 4-pulse REDOR sequences shown in Figure 2.6 were employed at a spinning frequency of 5000 Hz to recouple the $^1$H-$^{13}$C RDCs of Phe-2-$^{13}$C dissolved in the LCs. The experimental REDOR data as well as simulations are displayed in Figure 2.7.
amplitude of the 4-pulse REDOR curve reaches a maximum of about 0.3. A probable explanation for this reduced amplitude is the presence of isotropic LCs within the sample. We multiplied the experimental data by a factor 3.3 in order to obtain a 4-pulse REDOR curve dephasing to 1. The factor 3.3 is however more than twice higher than expected by estimating the ratio of anisotropic to isotropic LCs from Figure 2.5. The experimental 4-pulse REDOR curve obtained after multiplication dephases slightly faster than the simulated curve. The 2-pulse REDOR curve, even after multiplication by the factor 3.3, does not show oscillations and displays an amplitude much lower than observed in the simulated 2-pulse REDOR curve. This discrepancy between the experimental and the simulated data is probably a result of the difficulty to estimated the distribution of angles $\beta$ in the sample at a spinning frequency of 5000 Hz. In fact, Figure 2.5 shows that the simulated powder pattern does not fit perfectly the experimental data. The simulated powder pattern seems to lack LCs with $\beta$ close or equal to 0° in order to form a perfect powder pattern.
Figure 2.7: 2- and 4-pulse REDOR at a spinning frequency $\nu_r = 5000$ Hz and at a rotor angle $\theta_r = 52.4^\circ$. The continuous lines show SIMPSON simulations performed with a dipolar coupling $D = 1500$ Hz and a powder averaging using 40 $\gamma$ angles. The $(S_0- S)/S_0$ values of experimental 4-pulse REDOR curve has been multiplied by a factor 3.3 so that it dephases to 1. The gray stars are the multiplication by 3.3 of the black stars. The experimental 2-pulse REDOR curve has been multiplied by the same factor (blue and light blue triangles represent the data before and after multiplication, respectively).

The much more preserved orientation of the LCs at slower spinning frequencies observed in Figure 2.4 indicate that REDOR should be measured close to a spinning frequency of 450 Hz in order to avoid an amplitude reduction of the REDOR curve. However, at a rotor angle of 52.4°, the LCs align with $\beta = 0^\circ$, and the RDCs cannot be recoupled with REDOR, as we have considered in Section 1.8.1. However, with a perpendicular orientation ($\beta = 90^\circ$), the 4-pulse REDOR sequence theoretically gives to a REDOR curve dephasing to 1 and displaying oscillations. This could be achieved by performing experiments at a rotor angle larger than the MA, where LCs theoretically align preferentially with $\beta = 90^\circ$ [29]. However, at a spinning frequency of 450 Hz, where the rotor period is $\sim 2.2$ ms, the signal intensity is significantly reduced due to $T_2$ relaxation, as shown by the $S_0$ data points in Figure 2.8.
Figure 2.8: $S_0$ data points recorded at a spinning frequency of 450 Hz.

2.3 Material and Methods

2.3.1 Sample preparation

Perfluorooctanoic acid (PFOA) and cesium hydroxide monohydrate (CsOH) were obtained from Sigma Aldrich and used without further purification. CsPFO was prepared by dissolving PFOA in water and titrating with a solution of CsOH to a pH of 7.0, as described in [31]. The salt solution was evaporated to dryness in a lyophilizer. The crystals were dissolved to a weight fraction of 0.4 in D$_2$O containing 0.01 weight fraction of Phe-2-$^{13}$C. Homogeneous solutions were obtained by heating the sample into the isotropic phase ($>303$ K) and shaking.

2.3.2 Solid-state NMR

All NMR spectra of CsPFO/D$_2$O were recorded on a Bruker Avance spectrometer operating at a static magnetic field of 9.4 T (400 MHz $^1$H resonance frequency) using a Bruker 4 mm double resonance probe. The NMR spectra were measured either without sample spinning or with spinning frequencies between 450 and 5000 Hz. The temperature was set to 299 K. A decoupling with an RF amplitude of 6 kHz on $^1$H using WALTZ16 was applied during the evolution period. The total coupling constants ($T_{static}$, $T_{sp}$) and scalar coupling constants ($J$) were recorded using a direct 90° pulse on $^{13}$C with an RF
amplitude of 37 kHz. The REDOR sequence was applied using pulses with an RF amplitude of 36 Hz on $^1\text{H}$ and of 55 kHz on $^{13}\text{C}$. The recycle delay was set to 3 s. The $^1\text{H}$ and $^{13}\text{C}$ pulses were optimized using INEPT. The rotor angle was calibrated using trimethyl phosphine sulfide (TMPS). The NMR spectra were processed without window apodization.

### 2.3.3 Calibration of the rotor angle $\theta_r$

The rotor angle $\theta_r$ was calibrated using trimethyl phosphine sulfide (TMPS). Since the crystal structure of TMPS is very simple, the $^{31}\text{P}$ NMR spectrum shows only a single peak. Moreover, the three-fold rotation axes of the phosphorus atoms ensures an uniaxial chemical shift anisotropy (CSA) tensor ($\eta=0$). A direct pulse on $^{31}\text{P}$ with an RF amplitude of 84 kHz was used. The methyl protons were decoupled during the $t_2$ evolution period using SPINAL64 with an RF amplitude of 50 kHz. The spinning frequency of the sample was 5 kHz and the temperature was set to 296 K. The spectra were simulated with SIMPSON [33] using the CSA parameters $\delta_{\text{aniso}} = 78.8$ ppm and $\eta = 0$ [34]. The spectra are shown in Figure A in the Appendix.

### 2.3.4 Simulations

All simulations have been performed using SIMPSON [33] and are shown in Appendix B.1 and C.
Chapter 3

Poly-γ-benzyl-L-glutamate/chloroform

This work was done in collaboration with Christina Thiele, Albert A. Smith and Giorgia Zandomeneghi. Christina Thiele prepared the PBLG samples; Christina Thiele and Albert Andrew Smith provided support in recording and evaluating the experiments; Giorgia Zandomeneghi gave very helpful input for the progression of the project.

3.1 Introduction

Poly-γ-benzyl-L-glutamate (PBLG) was the first synthetic polymer to show a LC phase [35]. PBLG is soluble in many organic solvents, where it adopts an α-helical structure. It has been shown that there is a close relationship between the order parameter of PBLG in LC phase and the volume fraction of PBLG [36,37]. It is also known that the concentration range for stability of the LC phase for rod-like molecules like PBLG increases with increasing the axial ratio $L/d$ (where $L$ is the length of the rod and $d$ its diameter) and therefore with increasing MW, since $d$ stays constant [37–39]. It is however true only when $L$ is smaller than the persistence length $P$ of the polymer [40]. Unfortunately, commercial PBLG orient most compounds too strongly, which makes the interpretation of the spectra challenging. In 2009, Marx et al. [41] performed a systematic investigation into the LC phases of PBLG. Isopinocampheol was used as solute to analyse its orientational properties. It was shown that the critical concentrations for the phase transition LC to biphasic LC/isotropic as a function of the MW decreases steeply in the low-MW region. It was also demonstrated that the spectral quality was improved significantly with high MW samples. With very high MW PBLG, RDCs values of about 10 Hz could be obtained.

3.2 Results and Discussion

The LCs were prepared by mixing PBLG with $^{13}$CHCl$_3$ and CDCl$_3$. RDCs between $^1$H and $^{13}$C of $^{13}$CHCl$_3$ were investigated. The sample was spun at a frequency of 800 Hz
at four different rotor angles (52.4°, 54.7°, 56.3°, 58.9°) and, since the quadrupolar interaction was in this case stronger than the dipolar interaction, $^2$H spectra were measured in order to determine the orientation of the LCs. Figure 3.1 shows the $^2$H splitting of the static sample, as well as of the sample spinning at a rotor angle of 52.4°, 56.3°, and 58.9°. We measured $T_{^2H, static}=709$ Hz and $T_{sp}$ at the various rotor angles. The angle $\beta$ was determined from $T_{sp}$ and $\theta_r$. The values of $\theta_r$, $T_{sp}$ and $\beta$ are displayed in Table 3.1.

![Figure 3.1](image)

**Figure 3.1:** $^2$H spectra of PBLG/3CHCl$_3$ without sample spinning, in blue, and spinning at an angle $\theta_r$ of 52.4°, 56.3°, and 58.9°, in black, red, and green, respectively. The orientation of the LCs was determined from the $^2$H splitting: LCs adopt a parallel orientation ($\beta=0^\circ$) below the MA (52.4°) and a perpendicular alignment ($\beta=90^\circ$) above the MA (56.3°, 58.9°).

<table>
<thead>
<tr>
<th>$\theta_r$ [°]</th>
<th>$T_{sp}$ [Hz]</th>
<th>$\beta$ [°]</th>
</tr>
</thead>
<tbody>
<tr>
<td>52.4</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>56.3</td>
<td>12</td>
<td>90</td>
</tr>
<tr>
<td>58.9</td>
<td>35</td>
<td>90</td>
</tr>
</tbody>
</table>

**Table 3.1:** Table showing the measured spectral splittings $T_{sp}$ at various rotor angles $\theta_r$ for a spinning frequency of 800 Hz. The $\beta$ values are determined from $\theta_r$ and $T_{sp}$. 
From the $^{13}$C static spectrum in Figure 3.2 one observes $T_{\text{static}}=\pm 339(\pm 10)$ Hz. According to Equation 1.1 since $J=209$ Hz, $D$ can have the two following values: $D_1=65$ Hz and $D_2=-274$ Hz. The measurement of $T_{\text{sp}}$ at four different rotor angles $\theta_r=52.4^\circ$, $54.7^\circ$, $56.3^\circ$, and $58.9^\circ$ allowed us to conclude that the value of $D$ obtained from the static $^{13}$C spectrum was equal to $65(\pm 10)$ Hz. The $D$ values extracted from $T_{\text{sp}}$ at the various rotor angles are shown in Table 3.2. The $D$ values have an uncertainty between 9 and 25 Hz depending on the FWHM of the spectrum. The value of 65 Hz is always within uncertainty.

$$\begin{array}{|c|c|}
\hline 
\theta_r[^\circ] & D[\text{Hz}] \\
\hline
\text{static} & 65(\pm 10) \\
52.4 & 60(\pm 9) \\
56.3 & 50(\pm 25) \\
58.9 & 60(\pm 10) \\
\hline
\end{array}$$

Table 3.2: Table showing the $^1H$-$^{13}$C dipolar coupling values without sample spinning and with a sample spun at different rotor angles $\theta_r$ obtained from the spectral splitting $T_{\text{static}}$ and $T_{\text{sp}}$.

REDOR experiments with two and four 180° pulses per rotor period were recorded with a sample spinning at a frequency $\nu_r=800$ Hz at the four rotor angles $\theta_r=52.4^\circ$, $54.7^\circ$, $56.3^\circ$, and $58.9^\circ$. Figures 3.3C and D show that, above the MA, the dephasing of the
4-pulse REDOR fits the simulations quite well, although the oscillations are damped. The 2-pulse REDOR exhibits a dephasing as well, whereas the difference between $S$ and $S_0$ should be zero. At the MA, as displayed in Figure 3.3B, both the experimental 2-pulse and 4-pulse REDOR dephase, the 2-pulse REDOR fits however better the simulations than the 4-pulse REDOR: it dephases to 1 and displays oscillations. Below the MA, none of the versions of REDOR should dephase, since the LCs are aligned parallel to the rotor axis. However, one observes a dephasing of both the experimental 2-pulse and 4-pulse REDOR as shown by Figure 3.3A. In Figures 3.3C and D for the 4-pulse REDOR and in Figure 3.3B for the 2-pulse REDOR, one sees that the dephasing of the experimental curve fits reasonably well the simulated curve but that the frequency of the oscillations is slightly lower in the experimental data compared to the simulations. We therefore performed simulations of REDOR curves using various values of dipolar coupling to determine whether one could obtain a better fit of the experimental REDOR curves showing oscillations. Simulations were done with a dipolar coupling $D=55$, $65$, and $75$ Hz, and were compared to the experimental data, as presented in Figure 3.4. One observes that the best fit of the oscillations of the curves is around $D=60$ Hz, for the 2-pulse at the MA displayed in Figure 3.4A, and for the 4-pulse REDOR above the MA shown in Figures 3.4B and 3.4C. The uncertainty on $D$ is around $\pm 5$ Hz. Dipolar coupling values displayed in Table 3.3 obtained with REDOR are very similar to the dipolar coupling values measured with the spectral splitting $T_{sp}$.

<table>
<thead>
<tr>
<th>$\theta_r$ [$^\circ$]</th>
<th>$D$ [Hz]</th>
<th>REDOR</th>
</tr>
</thead>
<tbody>
<tr>
<td>static</td>
<td>$65(\pm 10)$</td>
<td></td>
</tr>
<tr>
<td>52.4</td>
<td>$60(\pm 9)$</td>
<td>$60(\pm 5)$</td>
</tr>
<tr>
<td>56.3</td>
<td>$50(\pm 25)$</td>
<td>$60(\pm 5)$</td>
</tr>
<tr>
<td>58.9</td>
<td>$60(\pm 10)$</td>
<td>$60(\pm 5)$</td>
</tr>
</tbody>
</table>

Table 3.3: Table showing the $^1\text{H}$-$^{13}\text{C}$ dipolar coupling values obtained from the $^{13}\text{C}$ spectral splitting $T_{\text{static}}$ and $T_{sp}$ ($D$) and the ones resulting from the fit of the REDOR curves, without sample spinning and with a sample spun at different rotor angles $\theta_r$.

The damping of the oscillations, as well as the dephasing of the 2-pulse REDOR above the MA and of both the 2- and 4-pulse REDOR below the MA, are probably a result of a change of orientation of the LCs within the time course of the experiment. Further work could be invested in finding a dynamic model that explains the behaviour of all the REDOR curves in Figure 3.3.
Figure 3.3: 2- and 4-pulse REDOR at a spinning frequency $\nu_r=800$ Hz and at a rotor angle $\theta_r=52.4^\circ$, 54.7°, 56.3°, and 58.9°, in A, B, C, and D respectively. The black dots indicate experimental 4-pulse REDOR curves, the blue triangles show experimental 2-pulse REDOR curves. The continuous lines depict SIMPSON simulations performed with $D=65$ Hz. The Euler angle $\beta$ was set to 90° for the experiments performed above the MA, to 0° below the MA, and a powder averaging was used at the MA.
Figure 3.4: 2-pulse REDOR curve recorded at a rotor angle $\theta_r=54.7^\circ$, in A, and 4-pulse REDOR at $\theta_r=56.2^\circ$, and $58.9^\circ$, in B and C, respectively. The blue triangles and the black dots indicate the experimental data, while the straight lines represent the result of simulations using a $D=55$, 65, and 75 Hz in blue, green and red, respectively.
3.3 Material and Methods

3.3.1 Sample preparation

The PBLG polymers were kindly provided by Prof. Dr. Christina Thiele from Technische Universität Darmstadt. They were prepared using the Deming protocol [42–44]. This protocol employs the living polymerization, which favours the synthesis of block copolypeptides by the elimination of side reactions. The results is a rather constant chain growth and a very similar length between the chains. The final product is shown in Figure 3.5. The Deming protocol has the advantage to make use of a class of initiators based on organonickel compounds which are able to eliminate significant competing termination and transfer steps from NCA polymerization. The monomers are synthetized by adapted procedures from Albert et al. [45] and Goodman et al. [46]. As described in [41], in the first step, L-glutamic acid is reacted with benzyl alcohol in the presence of two equivalents of tetrafluoroboric acid to give the γ-ester 2 shown in Figure 3.6. This γ-ester is reacted with phosphogene in dry tetrahydrofuran to give the α-amino acid-N-carboxyanhydride 3.

Figure 3.5: The final PBLG product after polymerization.

Figure 3.6: Synthesis of monomer, as described in [41] and adopted from Albert et al. [45] and Goodman et al. [46].
The number average molecular mass of the polymer, measured by gel permeation chromatography, was $\overline{M}_n=2.0\times10^5$ gmol$^{-1}$ with a polydispersity index (PDI) of 3.0. The polymers were mixed in a tube with 4.1 weight fraction of CDCl$_3$ and 2.0 weight fraction of $^{13}$CHCl$_3$ and centrifuged. CDCl$_3$ was used for deuterium imaging in order to check the homogeneity of the sample. The 4 mm Bruker rotors were filled at room temperature using a pipet.

3.3.2 Solid-state NMR

All NMR spectra of PBLG were recorded on a Bruker Avance spectrometer operating at a static magnetic field of 9.4 T (400 MHz $^1$H resonance frequency) using a Bruker 4 mm double resonance probe. The NMR spectra were measured either without sample spinning or with a spinning frequency varying between 780 and 820 Hz ($\pm$1 Hz). The temperature was set to 296 K. The decoupling on $^1$H was achieved during the $t_2$ evolution period using WALTZ16 with an RF amplitude of 3.5 kHz. The total coupling constants ($T$) and scalar coupling constants ($J$) were recorded using a direct 90° pulse on $^{13}$C with an RF amplitude of 36 kHz. The REDOR sequence was applied using an RF amplitude of 36 kHz on $^1$H and of 83 kHz on $^{13}$C. The recycle delay was set to 5 s. The rotor angle was calibrated using trimethyl phosphine sulfide (TMPS) as described in 2.3.3. The NMR spectra were processed without window apodization.

3.3.3 Simulations

All simulations have been performed using SIMPSON [33] and are shown in Appendix B.1 and B.2.
Chapter 4

General Discussion and Conclusion

Although LCs appear to be an appropriate alignment medium without sample spinning [27, 47], the situation becomes more complicated upon spinning, since the orientation of the LCs is no longer conserved. The CsPFO/D₂O LCs are mostly aligned parallel to the axis of rotation at a spinning frequency of 450 Hz. However, the system loses its ordering with increasing spinning frequency and appears to be inhomogeneous: the emergence of an isotropic part between 850 and 5000 Hz spinning frequency is observed, while the anisotropic part is composed of LCs displaying many different orientations. The presence of isotropic LCs within the sample leads to an amplitude reduction of the REDOR curve. At a spinning frequency of 450 Hz, where the sample is homogeneous, a significant part of the signal intensity is lost during the long REDOR delays because of $T_2$ relaxation, which makes the data analysis difficult.

The PBLG/¹³CHCl₃ LCs offer more advantages than CsPFO/D₂O LCs. The sample remains homogeneous at least up to spinning frequencies equal to 800 Hz. The molecules are aligned either with $\beta=90^\circ$ (above the MA) or with $\beta=0^\circ$ (below the MA). The orientation at the MA is not known. We assume that no particular orientation is energetically favorable at this rotor angle. Furthermore, the $¹³C$ signal is very good due to the abundance of the $¹³CHCl₃$ molecules in the sample. And last, the PBLG/¹³CHCl₃ LCs equilibrate very quickly (5-10 minutes) after a change of condition (temperature, spinning frequency), while CsPFO/D₂O sometimes takes hours until it reaches equilibrium.

Even though the alignment of the PBLG/¹³CHCl₃ molecules seems to be clearly defined, parts of the REDOR data remain unexplained. However, if one only considers the REDOR curves displaying oscillations, one can determine the RDC of the LCs with an accuracy of $\pm5$ Hz by fitting the curve with simple SIMPSON simulations, where the dipolar coupling is the only parameter that varies. REDOR can therefore be used here to measure RDCs of $\sim60$ Hz with an accuracy of $\pm5$ Hz. The RDCs values obtained from the spectra splitting are very similar to the result of the REDOR fits. The unexplained behaviour of the 2-pulse REDOR above the MA, of the 4-pulse REDOR at the MA, and of both the 2- and 4-pulse REDOR below the MA could be caused by slow motions of the LCs. This hypothesis would have to be confirmed by a dynamic model.
Further work could be invested in using other samples displaying small RDCs but that are more stable under spinning. Strain-induced alignment in a gel has been extensively used for measurements in glass NMR tubes [48, 49]. A setup is required to make a sample of gel that is held stretched inside the tube. As described in [50], this operation is usually performed with a plugged silicone tube filled with gel. After stretching, a thumbscrew holds the silicone tube in the stretched state. One would have to develop a procedure to efficiently stretch the gel in a NMR rotor. This can be difficult to achieve since adding extra apparatus around the rotor could impair its spinning stability.

An alternative is to use molecules bound to swollen resins [51–54]. Studies have been carried out on the tetrapeptide Ala-Ile-Gly-Met bound to a Wang resin swollen in DMF-d$_7$ and it has been demonstrated that the mobility of the peptide is reduced when bound to the resin compared the free state [55]. RFDR experiments have been also used to retrieve the weak proton dipolar interaction present in this sample [56]. One could synthesized the tetrapeptide containing an amino acid with an isolated $^1$H-$^{13}$C pair, $^1$H$_\alpha$-$^{13}$C$_\alpha$ for instance. The REDOR sequence could be used to recouple the RDCs of this spin pair.
Part II

The HET-s(218-289) prion domain
Prions are proteins with the ability to spread through self-replication of an altered conformation. These proteins are therefore contagious and responsible for a class of mammalian diseases such as scrapie in sheep [57], bovine spongiform encephalopathy in cattle [58], and Creutzfeld-Jakob in humans [59]. The presence of prions has also been observed in yeast and in the fungus *Podospora anserina* [60], [61]. These fungal prions are valuable models to study prion propagation because of their similarities in the structure with mammalian prions: both form amyloids *in vitro* [62–65]. Amyloids are characterized by a β-sheet-rich molecular aggregate and increased resistance to Proteinase K digestion.

In *Podospora anserina*, the HET-s prion triggers a programmed cell death, which may prevent parasitism [66]. *Podospora anserina* has two allelic incompatibility genes $s$ and $S$, both encoding a 30 kDa protein differing in only 14 amino acids between the two genes. The fusion of a strain bearing the protein $s$ with a strain bearing the protein $S$ leads to the destruction of the heterokaryotic cell [67]. The HET-s prion is composed of an N-terminus globular domain and of a proteinase-K resistant C-terminus prion forming domain (residues 218–289). It has been shown that an increase of the β-sheet content takes place in the prion domain during the transition from the soluble to the aggregated state [65]. The well-defined structure of HET-s(218–289) fibrils at physiological pH, which is exempt of polymorphism caused by different molecular structure having the same stability, as well as its similarities with mammalian prions [62–65] and the ease to express, purify and fibrilize the HET-s(218–289) monomers makes of HET-s a model of choice to study the structure and dynamics of prions.

Solid-state NMR is the preferred method for the study of the structure and dynamics of amyloid fibrils at atomic resolution. Solid-state NMR presents the advantage to X-ray diffraction or solution NMR methods that it does not require crystalline nor soluble samples. For HET-s(218–289), the solid-state NMR lines are comparable to the ones of typical microcrystalline samples because of their high structural order. Typically, the $^{13}$C line width is in the order of 0.25-0.5 ppm [68]. In 2008, it has been shown, based on 134 distance-restraints, that HET-s(218-289) forms a left-handed β-solenoid, with each molecule forming two helical windings [69]. The model also explains the presence of a
loop connecting $\beta_2b$ and $\beta_3a$. In 2010, the HET-s(218-289) fibrils structure was refined using a collection of HET-s(218-289) samples including diluted, homogeneous and mixed samples [70].
Chapter 2

INEPT-based experiments

This work was done in collaboration with Albert A. Smith, Riccardo Cadalbert and Marielle Wälti. Albert A. Smith provided support in recording and evaluating the spectra; Riccardo Cadalbert helped in preparing the samples; Marielle Wälti performed the solution-state NMR experiments.

2.1 Introduction

In NMR spectroscopy only rigid parts of the sample contribute to the spectrum of dipolar-coupling mediated experiments. The more flexible parts of the sample, where the dipolar couplings are scaled and the spin-spin relaxation $T_2$ longer, are more sensibly detected by INEPT-based experiments. The HET-s prion in its fibrillar form contains residues that give sharp signals in CP-based experiments, as well as residues showing narrow lines in INEPT-based experiments. The structure of the rigid core of HET-s(218-289) proposed in 2008 by Wasmer et al. [69] was calculated from CP-based experiments. The HET-s(218-289) rigid core was found to form a left-handed $\beta$ solenoid. However, only 58 of the 71 residues of the HET-s(218-289) sequence were observable in these experiments [70]. Furthermore, resonances were visible in INEPT-based spectra [71, 72]. These resonances were so far only tentatively assigned and were believed to belong to the HET-s(218-289) segments framing the $\beta$-sheets [71], the segments that are invisible in CP-based experiments.

In this Chapter we aim to characterize the resonances observed in the INEPT-based spectra using a series of NMR experiments. We formulated a second hypothesis that these signals originate instead from HET-s(218-289) monomers present in the fibril sample. We performed a REDOR experiment as well as an experiment measured at a rotor angle different than the MA (off-MA) to obtain information about the RDCs present in these residues. We believe that the residues framing the $\beta$-sheets in HET-s(218-289) fibrils are less flexible than the HET-s(218-289) monomers because they are in close contact with rigid parts of the protein. We then carried out a diffusion experiment to examine the size of the molecules at the origin of the INEPT signals. We eventually assigned almost all
the resonances using a 3D HNCA and HNCO experiments.

2.2 Results

2.2.1 CP vs INEPT intensity

We measured a $^{1}$H-$^{15}$N CP as well as a $^{1}$H-$^{15}$N INEPT to perform a quantitative comparison between the signals coming from the rigid parts and the ones from the flexible parts of HET-s(218-289) fibrils. These spectra were acquired on $^{15}$N using a recycle delay of 6.5 s to get near thermal polarization, and with 83 kHz RF amplitude to decouple $^{1}$H during the time evolution of the direct dimension $t_{2}$. We also recorded 1D spectra of $^{1}$H and $^{15}$N to determine the spin-spin relaxation time $T_{2}$ in order to correct for the loss of intensity occurring during the INEPT delays. The relative amount of CP to INEPT signal is roughly 8:1. The HET-s(218-289) sequence contains 71 residues, while 6 additional residues come from the His$_{6}$-tag. The number of residues observable in CP-based experiments is 58. Therefore, about 25% of the sequence is not visible in CP-based experiments. If we expected that this 25% is observable in the INEPT-based spectra, the CP to INEPT ratio would be 4:1. The INEPT signal is however twice lower than expected. Furthermore, one sees that nearly half (45%) of the INEPT signal originates from the tentatively assigned His$_{6}$-tag, whereas only 40% is coming from the remaining backbone amide resonances (not including resonances above 125 ppm). These results show either that the INEPT signals originate from parts of the HET-s(218-289) fibrils that are in an intermediate motional regime where the CP transfer gives almost no signal, whereas the INEPT transfer works but is not fully efficient, or that these signals originate from HET-s(218-289) monomers representing a minority of the sample.
CHAPTER 2. INEPT-BASED EXPERIMENTS

Material and Methods

HET-s(218-289) expression, purification and fibrilization

The HET-s(218-289) protein expression, purification and fibrilization was always performed according to the following protocol, unless stated otherwise. Uniformly $^2$H, $^{13}$C, $^{15}$N-labeled histidine-tagged HET-s(218-289) was recombinantly expressed in Escherichia coli BL21 in M9 minimal medium, containing $^2$H, $^{13}$C glucose as the sole carbon source and $^{15}$N$^2$H$_4$. All buffers were prepared using D$_2$O. The purification steps were performed in H$_2$O-based buffer solutions. The standard expression steps are described in [73]. After expression, the cells were harvested by centrifugation and frozen at -80°C. The cells were lysed in 150 mM NaCl and 1 M Tris-HCl, pH 8 and disrupted using a microfluidizer (Microfluidics). The lysate was centrifuged for 90 min at 8,000 × g. The pellet was re-suspended in denaturing buffer (6 M guanidinium HCl, 150 mM NaCl and 1 M Tris-HCl, pH 8) and incubated overnight at 60°C. The supernatant was cleared by centrifugation for 3 h at 186,000 × g and filtered over 0.2 μm pore-size filters. The sample was applied to a HiTrap desalting column (GE Healthcare) and concentrated to approximately 0.5-1 mM. The buffer was exchanged with 150 mM acetic acid pH 2.5 using a HiPrep 26/10 desalting column (GE Healthcare). The pH was then immediately adjusted to 7.4 by
addition of 3 M Tris at 25 °C, which triggers fibrilization. The sample was placed on a rotator during the fibrils formation. The aggregation of HET-s(218-289) into fibrils was visible after about 10 min. After 3 d, the fibrils were washed 3 × with H$_2$O and 1 × with 20 mM citrate buffer at pH 5.0.

**Solid-state NMR**

The spectra have been measured on a Bruker Avance spectrometer operating at a static magnetic field of 14.1 T, which corresponds to a $^1$H resonance frequency of 600 MHz, using a 3.2 mm Bruker probehead. The spinning frequency was automatically stabilized at 13.2 kHz and the temperature set to 298 K. The $^1$H-$^{15}$N CP and INEPT were recorded with an RF amplitude of 83 kHz on $^1$H and of 31 kHz on $^{15}$N. $^1$H decoupling was applied during the time evolution $t_2$ at an RF amplitude of 83 kHz for CP using SPINAL64 and of 6 kHz for INEPT using WALTZ16. The recycle delay was set to 6.5 s. The $^1$H spin-spin relaxation time $T_2$ was determined by using the $^1$H-$^{15}$N INEPT sequence and adding a variable delay with a 180° pulse on $^1$H after the first 90° pulse. The $^{15}$N $T_2$ was measured in the same way by adding a variable delay at the end of the $^1$H-$^{15}$N INEPT sequence.

### 2.2.2 REDOR

![Pulse sequence](image)

**Figure 2.2:** Pulse sequence derived from Schanda and coworkers [16] used for the measurement of $^1$H-$^{15}$N dipolar couplings in uniformly [2H,13C,$^{15}$N]-labeled back-exchanged HET-s(218-289) fibrils. Filled and open rectangles indicate 90 and 180° pulses, respectively. The delay $\Delta$ was set to 2.6 ms. WALTZ16 decoupling was applied on $^1$H at an RF-field amplitude of 2 kHz during the acquisition time. The solvent saturation pulse was applied for 200 ms at an RF-field amplitude of 1 kHz. The pulse and receiver phases were set to $\phi_1 = y, y, -y, -y; \phi_2 = x, -x, x, -x; \phi_3 = -y, -y; \phi_4 = -y, -y; \phi_5 = y, y,$ and $\phi_{rec} = x, -x, -x, x.$ The phases of the 180° $^1$H pulses during the REDOR pulse train were incremented according to the XY-16 scheme [74]. The phases of all other pulses were set to x.
REDOR was measured on HET-s(218-289) fibrils using the pulse sequence shown in Figure 2.2 derived from Schanda and coworkers [16]. The pulse sequence starts with an INEPT transfer from $^1$H to $^{15}$N and is followed by a REDOR period containing two or four 90° pulses per rotor period. The signal is acquired on $^1$H after an INEPT transfer from $^{15}$N to $^1$H. A series of 1D spectra shown in Figure 2.3 has been acquired. As we have previously observed, most of the signal comes from the His$_6$-tag. The peak at $\sim$7.4 ppm was tentatively assigned to AsnHδ. The remaining resonances overlap with the His$_6$-tag peaks. The REDOR curves have been measured based on the integral of the signal between the two dashed lines and are plotted in Figure 2.4. They show a damped dephasing and a reduced amplitude lacking oscillations. Some of the REDOR curves measured on the LCs in Section 1.11 display the same features. At this stage of the project, the origin of this behaviour is not yet understood. However, we believe that the $^1$H-$^{15}$N RDCs present in the HET-s(218-289) sample are on the order of 200 Hz.
Figure 2.3: $^1$H spectrum of uniformly [2H, $^{13}$C, $^{15}$N]-labeled back-exchanged HET-s(218-289) fibrils. The area between the two vertical dashed lines is integrated and used to calculate the REDOR curves.
2.2.3 Off-magic angle

In this Section we performed NMR experiments spinning the HET-s(218-289) fibril sample at a rotor angle $\theta_r=48^\circ$. These experiments give an indication of the range of RDCs present in the sample. The rotor angle was calibrated using the C$\beta$ tensor of L-Alanine-2,3,3,3-d$_4$.

The $^1$H-$^{15}$N RDCs present in U-[$^{13}$C,$^{15}$N]-labeled HET-s(218-289) fibrils were measured using the pulse sequence in Figure 2.5 at a rotor angle $\theta_r=48^\circ$ and at the MA. The pulse sequence is composed of a double INEPT to transfer the magnetization from $^1$H to $^{15}$N and back to $^1$H and provides a 2D spectrum displaying splittings in the indirect dimension as no $^1$H decoupling is applied during the $t_1$-time evolution period. The spectral splittings measured at the MA in the indirect dimension only shows the $^1$H-$^{15}$N J couplings, whereas the RDCs are reintroduced in the spectrum recorded at $\theta_r=48^\circ$. 

Figure 2.4: REDOR curves obtained with the 2- and 4-pulse REDOR pulse sequence, in blue and red respectively, used to measure the $^1$H-$^{15}$N RDCs in HET-s(218-289) fibrils. The filled circles indicate experimental data, while the continuous lines show the SIMPSON simulations performed with a dipolar coupling of 200 Hz and a powder averaging.
Figure 2.5: Pulse sequence used to measure $^1$H-$^{15}$N RDCs during the evolution time $t_1$, where no decoupling on $^1$H is applied. Filled and open rectangles indicate 90 and 180° pulses, respectively. The water signal is suppressed by means of pulse field gradients $G_z$. The pulse and receiver phases were set to $\phi_1 = x, -x$; $\phi_2 = x, x, -x, -x$; $\phi_3 = x, x, -x, -x$; $\phi_{rec} = -x, x, x, -x$. All other phases were set to $x$ unless indicated otherwise.

Figure 2.6 displays the spectrum measured at the MA. The traces of both spectra are displayed in Figure 2.7. The size of splittings are comparable, which means that the $^1$H-$^{15}$N RDCs for these resonances are very small. The size of the $^1$H-$^{15}$N RDCs was estimated by assuming that the scaled $^1$H-$^{15}$N RDCs at $\theta_r=48^\circ$ are smaller than 10 Hz. The full $^1$H-$^{15}$N RDC obtained by multiplying 10 Hz with the second order Legendre polynomial $P_2=(3\cos^2\theta_r - 1)/2$, was smaller than 100 Hz, which is in the same order of magnitude as the $^1$H-$^{15}$N RDCs measured with REDOR in the previous Section. The order parameter calculated using Equation 1.21 using $\delta_D^{\text{expt}} < 100$ Hz and $\delta_D^{\text{rigid}} = 11888$ Hz (with 1.008 Å for the distance between $^{15}$N and the amide protons [75]) is $S<0.01$. From this experiment we concluded that the signals observed belong to very flexible residues.
Figure 2.6: $^1$H-$^{15}$N correlations of uniformly $[^2$H,$^{13}$C,$^{15}$N]-labeled back-exchanged HET-s(218-289) fibrils measured using the pulse sequence depicted in Figure 2.5 at the MA. The vertical dashed grey lines indicate the traces shown in Figure 2.7.
**Figure 2.7:** In blue, traces of the MA spectrum shown in Figure 2.6 and, in green, of the spectrum measured at a rotor angle of 48°.

**Material and Methods**

**Solid-state NMR** All NMR spectra of L-Alanine-2,3,3,3-d₄ and of HET-s(218-289) fibrils were measured on a Bruker Avance spectrometer operating at a static magnetic field of 14.1 T (600 MHz ¹H resonance frequency) using a Bruker HR-MAS probehead. The NMR spectra were recorded at an MAS frequency of 6 kHz. The spectra of L-
Alanine-2,3,3,3-d$_4$ were measured using a direct pulse on $^{13}$C with an RF amplitude of 19 kHz. No decoupling was applied on $^1$H during the $t_2$ evolution period. The recycle delay was set to 7 s. The HET-s(218-289) fibril spectra were acquired using an RF amplitude of 42 kHz on $^1$H and of 14 kHz on $^{15}$N. $^1$H decoupling during the $t_2$ evolution period was performed with WALTZ16 with an RF amplitude of 2 kHz. The sample temperature was set to 298 K.

**Rotor angle calibration** The C$\beta$ tensor of L-Alanine-2,3,3,3-d$_4$ was used to calibrate the rotor angle. No high $^1$H decoupling was needed, thanks to the high level of deuteration of the sample. A $^{13}$C direct pulsed MAS spectrum of L-Alanine-2,3,3,3-d$_4$ was recorded and fitted the spectrum to obtain the FWHM. The rotor angle was then changed (off-MA) and another $^{13}$C direct pulsed spectrum was measured. The off MA spectrum was then fitted using the CSA parameters of Alanine from [76] and the FWHM of the MAS spectrum.
Figure 2.8: Direct pulse $^{13}$C spectra of L-Alanine-2,3,3,3-d$_4$ A at the MA B at 48°. The experimental data and the simulations are shown in green and blue respectively. The Cβ CSA tensor of L-Alanine-2,3,3,3-d$_4$ at $\theta_r=48^\circ$ was simulated using the parameters from [76] and the FWHM of the MAS spectrum.

2.2.4 Diffusion

In this Section the diffusion coefficient of the HET-s(218-289) fibril signals present in the INEPT-based spectra was measured. The diffusion of particles depends, among other factors, on the size of the particle. For a spherical particle the Einstein relation links the diffusion constant $D$ to the radius $r$ of the particle:

$$D = \frac{k_B T}{6\pi \eta r},$$

(2.1)

where $k_B$ is the Boltzmann’s constant, $T$ the temperature, and $\eta$ the viscosity. Diffusion-ordered NMR spectroscopy (DOSY) can be performed to determine the translational
dynamics of a particle. The pulse sequence makes use of the dependence of signal intensity on the diffusion coefficients and gradient strengths. It employs a modified version of the longitudinal eddy current delay (LED), where each gradient pulse has been replaced by a pair of gradient pulses with different polarities and separated by a 180° pulse in order to reduce the eddy current. The pulse sequence is illustrated in Figure 2.9. The pulse sequence starts with an INEPT step to transfer the magnetization from $^1$H to $^{15}$N and is followed by the diffusion dimension. The diffusion was obtained by assuming a single diffusion coefficient and using the following equation to find the amplitude of the fast induction decay (FID) [77]

$$I(q) = I(0)\exp[-Dq^2(\Delta - \delta/3 - \tau/2)],$$  \hspace{1cm} (2.2)

where $q = \gamma \delta g$ is the area of the gradient pulse in cm$^{-1}$, $\gamma$ the gyromagnetic ratio, $g$ and $\delta$ the amplitude and duration of the gradient pulses, respectively, $\tau$ the delay for gradient switching and $D$ the diffusion coefficient. After diffusion, the water signal is saturated and the magnetization transferred back from $^{15}$N to $^1$H for acquisition.

![Figure 2.9: Pulse sequence used for the measurement of the diffusion coefficient of U-[D$_2$, $^{13}$C,$^{15}$N] labeled HET-s(218-289) fibril samples. The pulse sequence starts with an INEPT to transfer the magnetization from $^1$H to $^{15}$N. The diffusion occurs in the second part of the sequence, where the total gradient pulse length $\delta = 3$ ms, the diffusion time $\Delta' = 807$ ms, and the delay for gradient switching $\tau = 600$ $\mu$s. The gradient strength was incremented in 100 steps between $g = 1 \times 10^{-4}$ and $64 \times 10^{-4}$ T/m. The pulse and receiver phases were set to $y$ unless indicated otherwise.]

Diffusion coefficients were measured using the pulse sequence in Figure 2.9 with the total gradient pulse length $\delta$=3 ms, the diffusion time $\Delta'$=807 ms, and the delay for gradient switching $\tau=600$ $\mu$s. The gradient strength was incremented in 100 steps between $g=1 \times 10^{-4}$ and $64 \times 10^{-4}$ T/m. The diffusion constant obtained by fitting the intensity decay curve with Equation 2.2 is $D=5.0 \times 10^{-11}$ m$^2$/s. This value was obtained by assuming a single diffusion coefficient and by integrating the total signal from the series of
one-dimensional experiments. The experimental data and the fit is shown in Figure 2.10. The different parts of the spectra were then integrated separately. The results are given in Figure 2.11. The diffusion coefficient is similar for the different regions of the spectra and is equal to the value obtained by Siemer et al. in 2006 [71], who estimated the size of the particle to be in the order of 16 MDa. The signals observed in our INEPT-based experiments are therefore part of very large units, most probably HET-s(218-289) fibrils.

**Figure 2.10:** Experimental data measured using the LED pulse sequence with bipolar gradients, represented with the blue dots. The diffusion coefficient obtained with the best fit is $D=5.0 \times 10^{-11} \text{ m}^2/\text{s}$. 
Material and Methods

Solid-state NMR The diffusion spectra of HET-s(218-289) fibrils were recorded on a Bruker Avance spectrometer operating at a static magnetic field of 14.1 T (600 MHz $^1$H resonance frequency) with an HR-MAS probehead from Bruker. The temperature was set to 298 K and the spinning frequency to 6 kHz. The RF amplitude applied on $^1$H and $^{15}$N was 42 kHz and 14 kHz, respectively. The RF amplitude of the $^1$H saturation pulses was 12 kHz during $4 \times 50$ ms. The INEPT delay $\Delta$ was set to 2.4 ms. The acquisition time was 40 ms and the spectral width 21 ppm. The spectra were processed using an exponential function with and a line broadening of 10 Hz.
**Z axis gradient strength calibration** To calibrate the gradient strength, a 4 mm Bruker rotor with a 12 µl insert was used. The rotor was filled with H₂O. The filled length of the rotor was 0.26 cm. A spin-echo sequence with two gradient pulses shown in Figure 2.12 was employed. The first gradient was applied after the 90° pulse. The second gradient, having the same strength but twice the duration of the first, was applied during the acquisition period where the spin-echo refocuses. The excitation profile, measured after phase correction, was 7135.3 Hz, as shown in Figure 2.13. The gradient strength, calculated with

\[ G_z = \frac{2\pi \Delta \nu}{\gamma H \Delta z}, \]  

(2.3)

where \( \Delta \nu \) is the width of the excitation profile in Hz and \( \Delta z \) is the length of the filled space in the rotor, was 6.3 Gcm\(^{-1}\)A\(^{-1}\).

**Figure 2.12:** Pulse sequence used to calibrate the z axis gradient strength of the HR-MAS probe. The pulse sequence starts with a 90° pulse on \(^1\)H, which is followed by a gradient pulse. A second gradient pulse is applied after the 180° pulse. This second gradient pulse has the same strength but twice the duration of the first gradient pulse.
Figure 2.13: The excitation profile of a 4 mm Bruker rotor with a 12 µl insert filled with H₂O. The width of the profile is 7135 Hz, corresponding to a gradient strength of 0.63 Gcm⁻¹, which is 10% of the maximum gradient strength.

### 2.2.5 Assignment

The resonances present in INEPT-based spectra of U-[²H,¹³C,¹⁵N] labeled HET-s(218-289) fibrils were assigned with an HNCA and an HNCO experiment [78, 79] using INEPT transfers. The HNCA sequence is shown in Figure 2.14. The pulse sequence starts with an INEPT transfer from ¹H to ¹⁵N (A). After the INEPT transfer the operator is I_z S_z. The ¹H signal coming from the water is knocked-out by mean of a selective pulse (B). The first gradient pulse dephases the water signal, which is rephased by the two other gradients pulses applied before the enhanced INEPT [80] and prior the t₂-evolution period. An antiphase three-spin coherence H_xN_yC_α is created during the first delay T (C). The first ¹³C selective 90°-pulse converts this term to H_xN_yC_α (D), which evolves during the t₁-evolution period (E). It is then rotated back to the H_xN_yC_α antiphase. The rephasing of the three-spin coherence to an ¹⁵N coherence takes place during the following delay T (F). The t₂-evolution time happens simultaneously, which has the intent to minimize loss of signal due to relaxation of transverse relaxation. The enhanced INEPT converts the
coherence back to amide protons for observation (G). Since the J coupling between $^{15}$N and $^{13}$C$_{\alpha}$ of the same residue (i) and of $^{13}$C$_{\alpha}$ of the preceding residue (i-1) in the protein primary sequence are of similar size ($J_{N_{i}C_{\alpha_{i}}} = 11$ Hz and $J_{N_{i-1}C_{\alpha_{i-1}}} = 7$ Hz), as illustrated in Figure 2.15A, both correlations are visible in the HNCA spectrum. The HNCO sequence transfers the magnetization between $^1$H$_N$, $^{15}$N and $^{13}$CO of the preceding residue, as shown in Figure 2.15B. The J coupling between $^{15}$N and $^{13}$C of the same residue is too small and the correlation is therefore not observable in the spectrum.

![Figure 2.14](image)

**Figure 2.14:** HNCA pulse sequence based on INEPT transfers. $\Delta$ is set to 2.4 ms, $T$ to 12 ms and $\gamma$ to 5.5 ms. The delay $\epsilon$ corresponding to the length of the gradient and the recovery is 1.2 ms. $^1$H and $^{15}$N using WALTZ16 with an RF field of 3 kHz. The application of a selective 180° pulse during the $t_2$ evolution period decouples the $^{13}$CO. The shaped pulses used were E-BURP on $^1$H [81] and Q5 and Q3 [82] on $^{13}$C. The pulse and receiver phase were set to $\phi_1 = x, -x$; $\phi_2 = x, x, -x, -x$; $\phi_3 = y, y, -y, -y$; $\phi_{rec} = x, -x, -x, x$. The phases of all other pulses were set to x unless indicated otherwise.

![Figure 2.15](image)

**Figure 2.15:** Sketch of the HNCA magnetization transfer in A and of the HNCO magnetization transfer in B.
The 3D HNCA experiment displayed in Figure 2.14 was employed to connect the residues of the protein backbone. All the peaks of the HNCA were assigned with the exception of a pair of $C^\alpha_i$ and $C^\alpha_{i-1}$ and four single peaks. The parts of the HET-s(218-289) fibrils that have been employed for assignment are indicated in Figure 2.16. We observe that a large portion of the HET-s(218-289) residues are visible in the HNCA spectrum. A detail of the assignment is shown in Figure D in the Appendix.

Figure 2.16: HET-s(218-289) sequence showing the assigned residues of HET-s(218-289) fibrils, in green. The residues assigned by Lange et al. [72] using CP-based experiments are highlighted in gray.
**Figure 2.17:** $^1$H-$^{15}$N correlation spectrum using INEPT transfers of U-$[^2]$H-$[^3]$C-$[^15]$N]-labeled HET-s(218-289) fibrils with the assignment from the 3D HNCA.

We believe that the resonances visible in the INEPT-based experiments are not the flexible parts of the HET-s(218-289) fibrils because there seems to be no correlation between the residues observed in the CP-based experiments by Lange *et al.* [72] and
Those observed in the INEPT-based experiments, as illustrated in Figure 2.16. These results strongly support the hypothesis that the INEPT signals come from HET-s(218-289) monomers. We think that these monomers are tethered to the surface of the HET-s(218-289) fibrils, which would explain their very slow diffusion measured in Section 2.2.4. The presence of monomers tethered to the surface of fibrils has already been observed in the Aβ peptide [83, 84], which is believed to be involved in Alzheimer’s disease [85, 86].

To confirm our assumption a sample of HET-s(218-289) monomers was prepared in the same buffer used to wash the HET-s(218-289) fibrils. The solution was transferred into an NMR tube and the signal intensity of the $^1$H spectra was checked at time $t=0$ and $t=+2$ days, as shown in Figure 2.18 to make sure that fibrilization does not occur. A 2D HN as well as a 3D HNCA of the U-[2H,$^3$C,$^1$5N]-labeled HET-s(218-289) monomers were then recorded in order to assign the resonances. The same pulse sequence as for the fibril (solid) sample shown in Figure 2.14 was employed. Figure 2.19 displays the parts of the HET-s(218-289) sequence that have been assigned in the 3D HNCA of the solid as well as of the solution sample. All of the peaks present in the solid spectrum are also visible in the solution spectrum, with one exception. The solution spectrum contains few more peaks. Furthermore, the peaks of both spectra share comparable chemical shifts and comparable linewidth, as illustrated in Figure 2.20.

Figure 2.18: $^1$H spectra of U-[2H,$^3$C,$^1$5N]-labeled HET-s(218-289) solution sample. The blue spectrum was recorded at time $t=0$, while the red spectrum was measured 2 days later. The signal intensity is constant over time, which means that fibrilization does not occur. The broad peak at a chemical shift around 5 ppm is attributed to the rest of the water signal.
**Figure 2.19:** HET-s(218-289) sequence showing the assigned residues for the solid and solution in green.
Figure 2.20: $^1$H-$^{15}$N correlation spectra using INEPT transfers of U-[2H, $^{13}$C, $^{15}$N]-labeled HET-s(218-289) solid sample in grey and solution sample in purple. The peaks of both spectra have comparable linewidth and chemical shift.
PART II. THE HET-S(218-289) PRION DOMAIN

Lower NMR intensity of the C-terminus

Figure 2.21 depicts the intensities of the assigned and unassigned peaks in the solid (B) and in the solution (C) samples. The unassigned peaks have on average a lower intensity than the assigned peaks. As shown in Figure 2.21A, most of the unassigned peaks are located at the C-terminus of the HET-s(218-289) sequence, both for the solid and solution sample. The likely explanation for the lower intensity of the C-terminus of HET-s(218-289) is the presence of two aromatic surface-exposed amino acids located at the C-terminus of the HET-s(218-289) sequence, namely Phe286 and Trp287, which interact with Phe286 and Trp287 of other HET-s(218-289) monomers. It has been suggested that \( \pi-\pi \) interactions between aromatic residues may play a major role in molecular recognition and self-assembly, for example by providing stability and directionality in the formation of amyloid fibrils [87]. These interactions are believed to include van der Waals, hydrophobic and electrostatic forces [88]. The replacement of aromatic residues with nonaromatic substitutes has shown reduced tendency for aggregation for a variety of peptides [89, 90]. These ring moieties are therefore a good target for the development of novel directions of inhibition of the mechanism of nucleation and elongation of fibril. For instance, several inhibitors are believed to interact with Trp residues and prevent fibril formation [91, 92]. The residues Phe286 and Trp287 may therefore trigger fibril formation of HET-s(218-289). The lower intensity of the peaks corresponding to residues located at the C-terminus of the solution sample may be due to aromatic interactions between different HET-s(218-289) molecules. The C-terminus is therefore less flexible than the rest of the molecule and appears with lower intensity in the NMR spectrum. However, probably because of the low concentration of molecules in solution, the HET-s(218-289) monomers do not form fibrils but remain flexible enough to be detected using INEPT-based experiments. The situation is a bit different concerning the solid sample. The fibrils have been harvested by centrifugation before filling the rotor and the supernatant has been discarded. We therefore expect that the sample contains only high MW aggregates. Furthermore, we have shown using NMR diffusion experiments that the HET-s(218-289) monomers are part of very large aggregates of 16 MDa. We therefore believe that the HET-s(218-289) monomers are tethered to the the surface of HET-s(218-289) fibrils from their C-terminus.

Intense His\(_6\)-tag peaks in the solid sample

The His\(_6\)-tag peaks are much more intense than the peaks assigned to residues of HET-s(218-289) monomers. The His\(_6\)-tag is most probably part of the HET-s(218-289) fibrils but is flexible enough to be seen by INEPT-based experiments, while the rest of the fibrils is invisible.
Influence of the pH of the buffer on the HET-s(218-289) monomers

The HET-s(218-289) fibrils were washed with a 20 mM citrate buffer at pH 5 in order to gain sensitivity in NMR experiments involving magnetization transfer from the amide protons, thanks to the much slower $^1$H exchanged compared to the same protein in a pH 7 buffer. However, the effect of a pH 5 buffer on the structure of the HET-s(218-289) fibrils was so far not known. It has been shown that the HET-s(218-289) fibrils formed at pH 2 are morphologically distinguishable from the ones resulting from fibrillation at neutral pH [93, 94]. The pH 2 fibrils are less infectious than pH 7 fibrils and form an alternative structure. We measured HET-s(218-289) fibrils prepared following the same protocol as for the pH 5 sample, and washed with water at neutral pH instead of citrate buffer at pH 5. 2D $^1$H-$^{15}$N INEPT spectra of pH 5 and pH 7 samples shown in Figure 2.22 were then recorded. The much faster exchange of protons at the amide sites at pH 7 compared to pH 5 leads to very low SNR in the $^1$H-$^{15}$N INEPT of the pH 7 fibrils. The total acquisition time was $\sim$1 hour for pH 5 and $\sim$30 hours for pH 7. The SNR in the pH 7 spectrum does not allow the measurement of a 3D HNCA in a reasonable amount of time in order to assign the peaks. However, the most intense peaks of the pH 5 spectrum are also visible in the pH 7 spectrum, as illustrated in Figure 2.22. The presence of 230Lys, 231Ile, 233Thr, and 237Ala, for instance, confirms that the signals observed do not come from
the residues framing the β-sheets of the HET-s(218-289) fibrils but rather from HET-s(218-289) monomers. We therefore concluded that the presence of the HET-s(218-289) monomers in fibrils samples are not due to the low pH.

Figure 2.22: 1H-15N correlation spectra using INEPT transfers of U-[3H,13C,15N]-labeled HET-s(218-289) fibrils, the “usual” sample (pH 5) is shown in grey and the pH 7 sample in red.
Material and Methods

Solid-state NMR sample preparation The pH 5 sample was prepared as described in 2.2.1. The pH 7 sample was made using the same protocol as for pH 5 sample with the exception of the last washing steps, which were made with H$_2$O at pH 7.

Liquid-state NMR sample preparation HET-s(218-289) monomers were dissolved in a solution containing 20 mM citric acid in H$_2$O and 5 % D$_2$O. The solution was then filtered over 0.2 µm pore-size filters. The final concentration of monomers measured by OD$_{280}$ was 360 µM. The pH was adjusted to 5.0 using HCl. A NMR tube (Shigemi) was filled with 500 µL of the solution. Proton spectra were recorded at time t=0 and t=+2 days. The absence of significant fibril formation was shown by the constant $^1$H NMR signal intensity illustrated in Figure 2.18.

Spectra Assignment The spectra have been analysed with ccpNMR analysis 2.3 [95].

Solid-state NMR All the solid-state NMR spectra were measured at a static magnetic field of 14.1 T (600 MHz $^1$H resonance frequency) on a Bruker Avance spectrometer using a Bruker HR-MAS probehead. The temperature was set to 298 K and the spinning frequency to 6 kHz.

Liquid-state NMR All the liquid-state NMR spectra were recorded on a Bruker Avance II spectrometer operating at a static magnetic field of 14.1 T, which corresponds to a $^1$H resonance frequency of 600 MHz using a Bruker triple resonance cryoprobehead. The temperature was set to 280 K. The water signal in $^1$H spectra was suppressed using the watergate W5 [96] pulse sequence with gradients. The HNCA pulse sequence employed is shown in Figure 2.14.

2.3 Discussion

2.3.1 Limited Proteolysis

We made the hypothesis that the signals seen in the INEPT-based experiments come from HET-s(218-289) monomers tethered to the surface of HET-s(218-289) fibrils. In this Section we checked whether the NMR signals from the HET-s(218-289) monomers overlap possible signals coming from the flexible residues framing the $\beta$-sheets. We therefore biochemically removed the HET-s(218-289) monomers present in the fibrils sample using limited proteolysis. Proteolysis is the cleavage of the protein by an enzyme called “protease”. To bind on the protein and be active the protease typically needs up to 10 residues in an accessible and flexible conformation [97]. It is therefore expected that
cleavage occurs at mobile sites of a protein, such as loops, whereas the protein core remains quite rigid and thus resistant to proteolysis. In order to extract information about the higher order structure of a protein the proteolytic reaction has to be limited. Limitation can be done, for instance, by restricting the enzyme:substrate ratio, and is typically accomplished by ensuring that the substrate conserves its native state.

Additionally to their role in probing the structure of a protein, proteases are a powerful tool in many other areas of biochemistry including sequencing and complete enzyme degradation. Trypsine, for instance, is a well-known enzyme that cleaves proteins mainly at the carboxyl side of the amino acids lysine or arginine and produces therefore a “proteolysis map”, which can be used for protein identification. However, since the reaction is performed in denaturing conditions, no information about the high order structure of the protein can be obtained from this reaction. Other well-known proteases are chymotrypsin, subtilisin or lactoferrin, for example.

**Proteinase K**

Proteinase K is a protease that cleaves preferentially peptide bonds adjacent to the carboxyl group of aliphatic and aromatic amino acids [98]. Proteinase K originates from the fungus *Tritirachium album* and has an active-site composed by the catalytic triad Asp39-His69-Ser224. Proteinase K has the ability to digest even keratin and led therefore to the letter K [99]. Proteinase K is commonly used in biochemistry to prevent degradation of DNA or RNA preparations by nucleases, thanks to its ability to digested these enzymes [100, 101]. The use of Proteinase K to perform limited proteolysis is widely used in biochemistry fields since about 50 years. An example of this application was carried out in 1977 by Williamson *et al.* [102] to show the heterogeneity of rabbit muscle creatine kinase. The mixture of the two peptides composing the protein can be digested by a low concentration of Proteinase K, which cleaves about 38 amino acids from each chain. Limited proteolysis using Proteinase K has also been applied to amyloid fibrils. Limited proteolysis can identify regions of amyloids that are directly involved in the β-sheet structure [103]. Because of their high degree of hydrogen bonds, the β-sheet parts of amyloid, which compose the core of the fibrils, are not affected by limited proteolysis. In 1990, Borchelt and coworkers [104] showed that the cellular and scrapie isoforms of the prion protein (PrP), PrP<sup>C</sup> and PrP<sup>sc</sup>, both copies of the same gene, can be distinguished under conditions where PrP<sup>C</sup> is hydrolyzed, while PrP<sup>sc</sup> is resistant. They demonstrated that the resistance of PrP<sup>sc</sup> to proteolytic cleavage results from a posttranslational event. The prion protein HET-s has also been submitted to limited proteolysis with Proteinase K. In 2002, Dos Reis and coworkers demonstrated that a HET-s fragment of ∼7 kDa remains after limited proteolysis of full-length HET-s with Proteinase K [65]. Furthermore, it has been shown that the remaining fragment of HET-s(218-289) is fibrillar and infectious [105]. About a year later, an analysis by N-terminal Edman microsequencing revealed a KIDAIIV sequence at its N-terminus, which corresponds to residues 218-223.
of the HET-s sequence [73]. Moreover, the resistant peptide binds Ni-NTA in a western blot experiment, which means that it still bears the histidine tag, in other words that the resistant fragment includes the C-terminus of HET-s. The resistant fragment is therefore HET-s(218-289).

### 2.3.2 Limited proteolysis of the HET-s(218-289) monomers

Assuming that the monomers are attached on the outside of the fibril bundle, they are accessible for cleavage by Proteinase K. The integrity of the HET-s(218-289) fibrillar core after limited proteolysis with Proteinase K has been tested by measuring an NCA spectrum using CP transfers shown in Figure 2.23. The enzyme:substrate ratio was 1:10 and the incubation time 30 minutes at 37 °C. The position of the expected chemical shifts from Lange et al. [72] is indicated by the red crosses. The peaks of the digested sample match the expected chemical shifts with a maximal error of ±0.2 ppm for \(^{13}\)C and ±0.4 ppm for \(^{15}\)N, which is within expected tolerance. The absence of some peaks, for instance 223Val, 251His, and 261Thr, can be explained by their position in the HET-s(218-289) fibril. In fact, these residues are located at the limit between a \(\beta\)-sheet and a loop. The SNR is probably too low to observe these resonances in our spectrum.
**Figure 2.23:** NCA correlation spectrum using CP transfers of U-$^{13}$C, $^{15}$N-labeled HET-s(218-289) fibrils after limited proteolysis with Proteinase K at an enzyme:protein ratio of 1:10 during an incubation of 30 minutes at a temperature of 37°C. The red crosses show the chemical shifts measured by Lange et al. [72].

**Optimization of the reaction**

The cleavage of molecules must be restricted to the most flexible parts of the fibrils in order to remove the HET-s(218-289) monomers without digesting the fibrils. The enzymesubstrate ratio, as well as the incubation time, were optimized. In a first step, two different enzyme:substrate ratios were tested, namely 1:25 (w/w) and 1:10 (w/w), during 30 minutes incubation time at 37°C. Spectra with magnetization transfer from $^1$H to $^{15}$N and back to $^1$H using CP and INEPT were recorded for each sample. The CP spectra were used as a reference to quantify the amount of HET-s(218-289) fibrillar core in the sample. The CP signal is expected to be constant before and after proteolysis if no digestion of the fibrillar core takes place. On the other hand, if the monomers are completely removed, no INEPT signal remains after digestion. The INEPT spectra are therefore a good indication of which portion of the monomers are digested by Proteinase K. Figure 2.24 shows the comparison between the HET-s(218-289) fibrils submitted to limited proteolysis and the undigested HET-s(218-289) fibrils. An increase of the enzyme concentration leads to a decrease in INEPT signal intensity. The CP signal remains
however constant after proteolysis. In a second step, the incubation time of Proteinase K with the HET-s(218-289) fibrils was increased to 1 hour. The result is visible in Figure 2.23. The CP signal is reduced by approximately one half after 1 hour incubation time compared to 30 minutes, which means that 1 hour incubation time is too long, since digestion of the fibrillar core occurs. The resolution of the 1D spectra is too low to determine with certainty whether the monomers are completely cleaved off but we observe that the decrease in the INEPT signal is also about one half for the asparagine side chain. However, the peak assigned to the His<sub>6</sub>-tag is only 20 % less intense after 1 hour.

2D INEPT-based spectra

We then performed a 2D analysis to gain insight into the remaining resonances after proteolysis. Since the amount of protein was limited, 1.3 mm rotors (Bruker) were used and 2D <sup>1</sup>H-<sup>15</sup>N INEPT experiments were recorded. Each spectrum showed in Figure 2.26 have been recorded during 3 days. One notices the disappearance of some of the peaks after limited proteolysis. Most of the peaks are located at the N-terminus of HET-s(218-289) sequence, for example Gly224, Ser227, Lys229. This observation corroborates our hypothesis that the HET-s(218-289) monomers are attached to the fibril from their C-terminus, although it is very difficult to draw any conclusion from such low-resolution spectra. One can also observe a slight chemical shift difference between the two spectra, which can be explained by a change of environment in vicinity of the corresponding amino acids.
Figure 2.24: $^1$H spectra of U-$^{13}$C, $^{15}$N]-labeled HET-s(218-289) fibrils without limited proteolysis in blue and after limited proteolysis with Proteinase K at an enzyme:substrate ratio of 1:10 in red, and at a ratio of 1:25 in black, both with 30 min incubation time. A $^1$H-$^{15}$N-$^1$H CP. B $^1$H-$^{15}$N-$^1$H INEPT showing the lowest signal intensity for the 1:10 ratio.
Figure 2.25: $^1$H spectra of U-[13C, $^{15}$N]-labeled HET-s(218-289) brils without limited proteolysis in blue, and after limited proteolysis with Proteinase K with an incubation time of 30 min in red and 60 min in black, both at an enzyme:substrate ratio of 1:10. A $^1$H-$^{15}$N-$^1$H CP. B $^1$H-$^{15}$N-$^1$H INEPT. The decrease in CP intensity of roughly a factor two shows that the fibrillar core is cleaved off with an incubation time of 60 min.
Figure 2.26: $^1$H-$^{15}$N correlation spectra using INEPT transfers of U-[13C, $^{15}$N]-labeled HET-s(218-289) fibrils without limited proteolysis (black contours), and after 30 min digestion at 37°C with Proteinase K (red contours). The black assignments show the resonances that disappear in the digested sample, while the red assignments indicate the remaining peaks after digestion.

Proteinase K inhibition

The fibrils were first incubated with Proteinase K and then harvested by centrifugation. The supernatant was discarded in order to remove the enzyme. However, this purification step did not lead to the complete removal of Proteinase K from the fibrils. If
Proteinase K is not inhibited, it continues to cleave the fibrils. Figures 2.27A and B show the effect over time on the sample if the enzyme is not inhibited. While the CP intensity remains roughly constant, the INEPT intensity increases dramatically after 2 days for an enzyme-substrate ratio of 1:10. The same signal raise is obtained after 3 weeks for a ratio of 1:25. This change in intensity is most probably due to the cleavage of the fibrillar core of HET-s(218-289) by Proteinase K. It is likely that the CP is not sensitive enough to show the decrease of the amount of fibrils within the sample.

Phenylmethanesulfonyl fluoride (PMSF) irreversibly inhibits Proteinase K. PMSF reacts by sulfonation with the serine residue of the catalytic triad (Ser-His-Asp) in the active site of the enzyme [106]. The half-life of PMSF in aqueous solution at pH 8 is 35 minutes [107]. PMSF is therefore usually added to a final concentration of 5 mM at point-of-use from a 100 mM dry stock solution [108]. We prepared a stock solution of PMSF 100 mM in methanol. The final concentration of PMSF to inhibit the enzyme was 5 mM. The mixture was incubated 15 minutes at RT. Figure 2.27D illustrates the effect of the enzyme inhibition with PMSF.
Figure 2.27: $^1$H spectra of U-[^{13}C, $^{15}$N]-labeled HET-s(218-289) fibrils after limited proteolysis without inhibition with PMSF in A (CP) and B (INEPT) and with an enzyme:substrate ratio of 1:10 in red and 1:25 in black. The straight lines represent the signal at time 0 and the dashed line the signal after 2 days for the 1:10 ratio in red, and 3 weeks for the 1:25 in black. The significant raise of the INEPT signal over time is an indication of the proteolysis of the fibrillar core. C and D show the CP and INEPT, respectively, of the sample after limited proteolysis with inhibition with PMSF, at time 0, 2 days and 4 days, in red, blue, and black, respectively.
2.3.3 Conclusions

HET-s(218-289) monomers can be partially removed by limited proteolysis with Proteinase K. The remaining signals most likely originate from parts of monomers that are not accessible to the enzyme. A fraction of the His$_6$-tag also subsists after limited proteolysis. This result could be explained by a model of the fibril bundles where parts of the His$_6$-tag point outside of the bundle, whereas the rest of the His$_6$-tag is confined inside the bundle and can therefore not be reached by the enzyme. The probability of the presence of signals coming from flexible parts of HET-s(218-289) fibrils is quite unlikely since we observe a significant loss of intensity for most of the peaks after digestion.

The fact that these monomers are bound to the fibrils from their C-terminus would explain why they cannot be completely digested by limited proteolysis, the access to the C-terminus of the monomers being less accessible to the enzyme. Additionally to the C-terminus, other regions of the monomers might be in contact with the fibrils, particularly the regions that are absent from the INEPT-based spectra.

Material and Methods

Solid-state NMR

The NCA spectrum was measured on a Bruker Avance II+ wide-bore NMR spectrometer operating at a static magnetic field of 20.0 T (850 MHz $^1$H resonance frequency) using a Bruker 1.3 mm triple-resonance probe. The MAS frequency was actively stabilized at 60 kHz and the sample temperature was set to 4 °C. Proton decoupling was applied during the time evolutions $t_1$ and $t_2$. All the other spectra were acquired on a Bruker Avance spectrometer at a static magnetic field of 11.7 T (500 MHz $^1$H resonance frequency). The MAS frequency was manually stabilized at 10 kHz and the sample temperature was set to 296 K. Decoupling was applied on $^{15}$N during the time evolution $t_2$ using SPINAL64 at an RF amplitude of 30 kHz for $^1$H-$^{15}$N-$^1$H CP and WALTZ16 at an RF amplitude of 3 kHz for $^1$H-$^{15}$N-$^1$H INEPT. For both sequences the RF amplitude was 100 kHz on $^1$H and 50 kHz on $^{15}$N and the recycle delay was set to 1 s. The water signal was suppressed using MISSISSIPPI. The 2D spectra were acquired with a spectral width of 250 ppm in $F_1$ and 100 ppm in $F_2$. The acquisition times were 25 ms in $F_1$ and 50 ms in $F_2$ and the number of scans was set to 400.

Sample preparation

An amount of 0.5 mg of Proteinase K was dissolved in 1 mL Proteinase K buffer containing 50 mM Tris and 150 mM NaCl at pH 8. After fibrilization, the fibrils were washed 3 times with H$_2$O. The concentration of the stock solution of HET-s(218-289) monomers was calculated by measuring the OD$_{280}$. An amount of 1.5 mg of HET-s(218-289) fibrils were transferred to a 1.5 mL tube. The fibrils were harvested 10 min at 10,000 × g. The
supernatant was discarded and replaced by Proteinase K buffer. A volume of 0.15 mL of Proteinase K solution was added to the fibrils solution to obtain a final enzymesubstrate ratio of 1:10. The mixture was placed 30 minutes at 37 °C on a rotor at slow rotation speed. After incubation, the fibrils were washed twice with the buffer containing 50 mM citric acid at pH 5.
Chapter 3

CP-based experiments: dynamics studies

This work was done in collaboration with Albert A. Smith and Riccardo Cadalbert. Albert A. Smith provided significant support in recording and evaluating the spectra, and in performing the simulations, Albert A. Smith also implemented the automated spectrum fitting routine; Riccardo Cadalbert helped in preparing the samples.

3.1 Introduction

We have previously shown that the HET-s(218-289) signals observed in INEPT-based experiments belong to HET-s(218-289) monomers attached to the surface of the fibrils. Furthermore, CP-based experiments indicate the presence of resonances that can be assigned to the core of the HET-s(218-289) fibrils [72]. In this Chapter, the dynamics of HET-s(218-289) fibrils was measured with CP-based experiments. Dynamics of protein implies a change of its conformation, which leads to a modification of its local environments. A spin therefore experiences variations of its CS, as well as of its bond vector orientations. This will affect its dipolar coupling interactions and CSA, which are, in solid-state NMR, not averaged out by molecular tumbling as it is the case in solution-state NMR. Furthermore, in solid-state NMR, conformational exchange might interfere with other time-dependant processes such as sample rotation or RF irradiation. For example, the HORROR condition ($\omega_1=\omega_r/2$, where $\omega_r$ is the sample rotation frequency and $\omega_1$ is the nutation frequency around an applied resonant radio frequency) [109] should generally be avoided.

We analyzed the site-specific dynamics of the $^1$H$\alpha$-$^{13}$C$\alpha$, as well as of the $^1$H-$^{15}$N bonds of HET-s(218-289) fibrils, by measuring $R_1$, $R_{1\rho}$, and REDOR experiments to collect information on the faster ($\tau_c<10$ ns) and slower ($\tau_c>1$ ns) timescales of motion, and on the amplitude of the motion, respectively. We prepared two U-$[^{13}$C, $^{15}$N] labeled HET-s(218-289) fibril samples with partial deuteration. The sample used for $^1$H$\alpha$-$^{13}$C$\alpha$ measurements
was prepared as described in 3.3.3 to obtain protonation at the $^{1}$H$\alpha$ positions and limited protonation at other positions [110]. For $^{1}$H-$^{15}$N data we employed fully deuterated HET-s(218-289) fibril sample with back-exchanged amide sites; the protocol for sample preparation is described in 2.2.1. Sample deuteration minimizes coherent interference (dipolar dephasing) in the measurements. Furthermore, we employed fast MAS (60 kHz) to further limit dipolar dephasing. Fast MAS has also the advantage to reduce linewidths and, used in combination with $^{1}$H detection, significantly enhances the sensitivity of the measurements.

3.1.1 Pulse sequences

Both $R_{1}$ and $R_{1\rho}$ pulse sequences illustrated in Figures 3.1A and B start with a transfer of magnetization from $^{1}$H to the nucleus of interest. The magnetization is then allowed to decay as longitudinal magnetization and as spin-locked magnetization, respectively. The $R_{1\rho}$ measurements are corrected based on the $^{1}$H flip rate, which may influence the rate of magnetization decay. The rate of $^{1}$H flips depends on the MAS frequency, $^{1}$H concentration, and on the spin-lock strength on the heteronucleus. The $^{1}$H flip rate is measured with the sequence shown in Figure 3.1C. Magnetization is first transferred to the heteronucleus, the $t_{1}$-evolution time is performed, magnetization is transferred back to $^{1}$H, and the $^{1}$H flip rate is measured. Magnetization is then again transferred to the heteronucleus and back to suppress the signal from the solvent. The $^{1}$H magnetization is frequency encoded before the decay period to allow for spin diffusion to other spins during the delay $\tau$. A simple inversion at the beginning of the sequence would not show the loss, since the initial net magnetization is roughly equal at all sites, quenching any net spin-diffusion. We used REDOR to measure the residual $^{1}$H-$^{15}$N and $^{1}$H-$^{13}$C$\alpha$ couplings of the U-$^{13}$C, $^{15}$N] labeled HET-s(218-289) fibril sample. Since the standard REDOR sequence recouples the one-bond dipole couplings too strongly, we employed the shifted version of REDOR [16] shown in Figure 3.1D. Measuring $^{1}$H-$^{13}$C$\alpha$ dipolar couplings is more challenging than measuring $^{1}$H-$^{15}$N dipolar couplings, since the sample contains proteins with both $^{1}$H$\alpha$ and $^{1}$H$\beta$ protonated, while the $^{1}$H-$^{1}$N pair is isolated. The frequency-selective REDOR (FSR) [111] is a solution of choice to recouple the $^{1}$H-$^{13}$C$\alpha$ dipolar coupling. FSR replaces the first pulse of the $\pi$ pulses train of the second half of the sequence with a frequency selective pulse in the center of the sequence in order to only recouple the nuclei that have been inverted by the selective pulse. However, using a shifted version of the FSR leads to a loss of its selectivity: the nuclei that are not inverted by the selective pulse are still recoupled, albeit at a different rate. We have therefore developed what we refer to as finite-pulse REDOR, shown in Figure 3.1E. Finite-pulse REDOR uses two $\pi$ pulses placed immediately next to each other in the center of the rotor period, which reduces significantly the effective dipole coupling during that period. If the $^{1}$H is not inverted in the middle of the sequence, the dephasing in the first half of the sequence
is refocused in the second half. The result is a selective REDOR that can be used to recouple $^1\text{H}\alpha-^{13}\text{C}\alpha$ couplings without recoupling the $^1\text{H}\beta-^{13}\text{C}\alpha$ couplings.

**Figure 3.1:** Pulse sequences to measure dynamics data. A and B are $R_1$ and $R_{1\rho}$ measurement sequences, respectively, where the incrementation of $\tau$ gives relaxation curves. C is used to measure the $^1\text{H}$ flip rate, under the same conditions as $R_{1\rho}$ experiments. D is the shifted REDOR sequence employed to measure the $^1\text{H}-^{15}\text{N}$ RDCs and E the finite-pulse REDOR, which measures $^1\text{H}\alpha-^{13}\text{C}\alpha$ couplings by frequency-selecting the $^1\text{H}\alpha$ resonances. The parameter $n$ is incremented to obtain REDOR curves.
3.2 Results and Discussion

For the dynamic characterization of both $^1$H-$^1$C$\alpha$ and $^1$H-$^15$N bonds, $R_1$ was measured at three different magnetic fields (9.4, 11.8 and 20.0 T), $R_{1\rho}$s were acquired at five spin-locking field strengths, and $S^2$ was measured. The parameters are summarized in Table 3.1. For all calculations we assumed a $^1$H-$^1$C$\alpha$ bond distance of 1.09 Å and a $^1$H-$^15$N bond distance of 1.02 Å, and a $^13$C$\alpha$ CSA of 20 ppm and a $^15$N CSA of 100 ppm. Figures 3.2 and 3.3 display reference spectra of $^1$H-$^1$C$\alpha$ and $^1$H-$^15$N correlations, respectively, using CP-based experiments.

<table>
<thead>
<tr>
<th>Nucleus</th>
<th>Type</th>
<th>#Exp</th>
<th>Critical parameters</th>
</tr>
</thead>
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<tr>
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<td>REDOR</td>
<td>1</td>
<td>$\omega_2/2\pi=60$ kHz</td>
</tr>
<tr>
<td>$^13$C</td>
<td>$R_{1\rho}$ (+1H flip rate)</td>
<td>5(+5)</td>
<td>$\omega_2/2\pi=60$ kHz</td>
</tr>
<tr>
<td>$^13$C</td>
<td>$R_1$</td>
<td>3</td>
<td>$\omega_0/2\pi(1H)=400$ MHz</td>
</tr>
<tr>
<td>$^15$N</td>
<td>REDOR</td>
<td>1</td>
<td>$\omega_2/2\pi=60$ kHz</td>
</tr>
<tr>
<td>$^15$N</td>
<td>$R_{1\rho}$</td>
<td>5</td>
<td>$\omega_2/2\pi=60$ kHz</td>
</tr>
<tr>
<td>$^15$N</td>
<td>$R_1$</td>
<td>3</td>
<td>$\omega_0/2\pi(1H)=400$ MHz</td>
</tr>
</tbody>
</table>

Table 3.1: Dynamics experiments and parameters.
Figure 3.2: $^1$Hα-$^{13}$Cα correlations using a CP-based experiment of U-$[^{13}$C,${}^{15}$N]-labeled HET-s(218-289) fibrils expressed using 75% D$_2$O and 25% H$_2$O. The red crosses indicate unassigned peaks.
Figure 3.3: $^1$H-$^{15}$N correlations using a CP-based experiment of U-[^13]C,$^{15}$N-labeled HET-s(218-289) fibrils expressed in D$_2$O, fibrillized in H$_2$O and washed in H$_2$O pH 5.0.
Figure 3.4: Experimental parameters for $^1$H-$^13$C dynamics fitted using the method in 3.3.1. A–E give the $R_{1\rho}$ values measured at various spin-lock strengths and MAS frequencies. F–H give $R_1$ measurements for different magnetic fields. I gives $S^2$ values measured with finite-pulse REDOR. All measurements also include error bars, indicating one standard deviation on the measurement data, determined via bootstrapping.
Figure 3.5: Experimental parameters for $^1$H-$^{15}$N dynamics fitted using the method in 3.3.1. A-E give the $R_{1\rho}$ values measured at various spin-lock strengths. F-H give $R_1$ measurements for different magnetic fields. I gives $S^2$ values measured with shifted REDOR. All measurements also include error bars, indicating one standard deviation on the measurement data, determined via bootstrapping.
Reference spectra of $^1$H-$^{13}$Cα and $^1$H-$^{15}$N correlations are plotted in Figures 3.2 and 3.3 respectively. The values of $R_{1\rho}$, $R_1$ and $S^2$ for $^1$H-$^{13}$Cα and $^1$H-$^{15}$N obtained from fitting the experimental data using the method described in 3.3.1 are plotted in Figure 3.4 and in Figure 3.5 respectively. One observes variations of $R_{1\rho}$ values measured at different spin-lock fields for $^1$H-$^{13}$Cα as well as for $^1$H-$^{15}$N data, as shown in Figures 3.4A-E and 3.5A-E, which indicates a slow motion on the order of the rotor period ($\sim$1-100 $\mu$s). It has been shown that timescales in the microsecond range indicate concerted motions of at least several atoms or more [112]. In fact, the changes of $R_{1\rho}$ with the magnetic field are roughly constant over the whole HET-s(218-289) molecule, which implies a narrow distribution of timescales and suggests that the entire molecule may see a single motion. Variations are also visible in the $^{13}$C and $^{15}$N $R_1$ values recorded at different magnetic fields, as depicted in Figures 3.4F-H and 3.5F-H. The changes of $R_1$ values across the magnetic field $B_0$ are larger for $^{13}$C than for $^{15}$N. Using the Kurbannov Equations shown in 1.8.2, the timescale of motion is estimated to be in the picosecond range for $^{13}$C and in the nanosecond range for $^{15}$N. These fast timescales are, like the slower timescales, very similar over the whole sequence. They represent very localized motions, it is therefore not straightforward to interpret the results as a whole.

Additionally, $R_1$ and $R_{1\rho}$ indicate that residues at the turns between $\beta$-sheets are more mobile than those in the core of the fibril. Moreover, residues framing the $\beta$-sheets show greater motion, which is consistent with the expected motions of the HET-s(218-289) prion that have been predicted using molecular dynamics simulations by Lange et al. [72]. The order parameter $S^2$ of the $^1$H-$^{13}$Cα in Figure 3.4I has a value between $\sim$0.5 and $\sim$0.7 for the whole sequence, which is smaller than the one of $^1$H-$^{15}$N ($\sim$0.8-1) in Figure 3.5I. This behaviour can be explained by a motional restriction of the $^1$H-$^{15}$N motion due to hydrogen binding of the amide $^1$H between layers of the fibrils. Furthermore, since $^1$H-$^{13}$Cα directly sees the sidechain motion and since its motion is not restricted by hydrogen bonding, the errors on the $^1$H-$^{13}$Cα data are considerably smaller. Additionally, 2D $^1$H-$^{13}$Cα have significantly more resolution than $^1$H-$^{15}$N spectra, as shown in Figures 3.2 and 3.3 making the extraction of the data much easier.

It is necessary to fit the experimental data ($R_1$, $R_{1\rho}$, and $S^2$, for each $^1$H-$^{13}$Cα and $^1$H-$^{15}$N spin pair) to a dynamic model in order to obtain more accurate values of amplitude ($S^2_\nu$) and timescales of motion ($\tau_c$). The dynamic model is characterized by $S^2_\nu$ and $\tau_c$. The experimental parameters are, in a second step, compared to those calculated from the dynamic model using a $\chi^2$-statistics

$$
\chi^2_\nu = \frac{(S_{exp} - S_{calc})^2}{\sigma(S)^2} + \sum_{k=1}^{N_{R_1}} \frac{(R_{1\rho,k}^{exp} - R_{1\rho,k}^{calc})^2}{\sigma(R_{1\rho,k})^2} + \sum_{k=1}^{N_{R_1}} \frac{(R_{1,k}^{exp} - R_{1,k}^{calc})^2}{\sigma(R_{1,k})^2}.
$$

Equation 3.1 takes into account the calculated values for all experiments, the experimental values, and the experimental standard deviation. The degrees of freedom $\nu$ of the $\chi^2$ distribution is given by the number of experimental parameters ($N_{R_1}+R_{1\rho}+1$) minus
PART II. THE HET-S(218-289) PRION DOMAIN

the number of fit parameters. The value of $\chi^2$ is minimized by varying the dynamic parameters, so that the more accurately measured parameters are favored. The Lipari-Szabo model-free analysis [17] is the dynamic model usually employed. As the correlation times become longer, the model starts to lose its accuracy to fit the experimental data and the analysis requires the use of a different model. The fit to a dynamic model will be performed in a later work. However, the direct data analysis, without the use of a dynamic model, already provides useful information on the amplitude and timescales of motion of HET-s(218-289) fibrils.

3.2.1 Flexible residues of the HET-s(218-289) fibrils

The HET-s(218-289) protein contains 71 residues, among which 58 are assigned using CP-based experiments [70, 71]. The remaining 13 residues are either very weak or invisible in the CP spectra and can only be tentatively assigned. The spectrum in Figure 3.2 shows $^1$H-$^{13}$Cα peaks having significantly lower intensity than the rest of the $^1$H-$^{13}$Cα peaks. These low intensity peaks are marked with a red cross and are not assigned in [70, 71]. One can identify the presence of unassigned Gly residues with their very distinct $^{13}$C chemical shift of $\sim$45 ppm, as well as two other unassigned peaks with higher $^{13}$C chemical shifts. We believe that these peaks belong to the more flexible part of the HET-s(218-289) fibrils, namely the C- and N-termini as well as the loop. Unfortunately, the low sensitivity of these peaks makes a site-specific dynamic analysis difficult to achieve.

3.3 Material and Methods

3.3.1 Data analysis

$R_1$, $R_{1\rho}$ and REDOR experiments were recorded as a series of 2D experiments. For $R_1$ and $R_{1\rho}$ the value of $\tau$ was incremented and for REDOR the value of $n$. Since the spectra do not show well-separated resonances, we used a spectrum-fitting program developed in our group by A. A. Smith et al.. This program employs an automated spectrum fitting routine, using MATLAB [113] and calculates amplitudes for each resonance, which are then fitted to determine relaxation rates and RDCs. For all relaxation rates except $^{15}$N $R_{1\rho}$, the intensity data was fitted to

$$I_k = A \exp(-R\tau_k),$$

where $R=R_1$ or $R_{1\rho}$, $A$ represents the intensity variations for different resonances, and $k$ indexes the different experiments in the time series. For $^{15}$N $R_{1\rho}$, because both $^1$H-$^{15}$N dipole coupling and $^{15}$N CSA contribute significantly to the relaxation, we used a biexponential function to fit the data
The contributions from $^1$H-$^{15}$N dipole coupling and $^{15}$N CSA are constructive or destructive, depending on the state of the $^1$H, and are partially averaged out with each other due to periodic $^1$H flips. In the limit of fast flipping $R_a$ and $R_b$ are equal. One can however not predict the ratio of $R_a$ to $R_b$, since it is a function of the $^1$H flip rate, but the average of these two rates is independent of the flip rate. Therefore, we report $R_{1\rho} = (R_a + R_b)/2$. REDOR data are composed of $S$ and $S_0$ curves. Since the length of the dephasing period is relatively short (1 ms for $^1$H-$^{13}$C, 1.33 ms for $^1$H-$^{15}$N), we assumed the decay of the $S_0$ curve to be linear in this range. We therefore recorded several $S_0$ spectra, linearly fitted them using a slope $m$ and an offset $b$ and calculated the value of $S_0$ for a particular residue using

$$S_{0,k} = m\tau_k + b. \quad (3.4)$$

The intensity $I_k$ of the curves are given by

$$I_k = (S_{0,k} - S_k)/S_{0,k}. \quad (3.5)$$

The order parameter $S^2$ was obtained by fitting the REDOR curves with SIMPSON simulations using a two-spin system. An additional amplitude scaling parameter was used to fit the $^1$H-$^{13}$C REDOR data as imperfect inversion of the $^1$H with the selective pulse leads to a decrease in the dephasing amplitude.

The error on the measurements were obtained by bootstrapping the data, since the sample size was insufficient to allow straightforward error calculation. The first step was to take the best fit of the data and assuming that each experimental data deviates from the model by the error $\epsilon_k$

$$I_{exp,k} = I_{model,k} + \epsilon_k \quad (3.6)$$

A bootstrapped data set is then generated using

$$I_{bootstrap,k} = I_{model,k} + \epsilon_m, \quad (3.7)$$

where $\epsilon_m$ is a randomly selected error from the $\epsilon_k$ of the original fit. The bootstrapped intensities are then refit using $\epsilon_m$ to calculate a new value of the parameter of interest ($R_1$, $R_{1\rho}$, $S^2$). This process is repeated 100 times to obtain the standard deviation from the bootstrapped fits, which is used as standard deviation of the measurements.

### 3.3.2 Solid-state NMR

All experiments were carried out with a Bruker 1.3 mm triple resonance ($^1$H, $^{13}$C, $^{15}$N) probe. The sample temperature was set to 297.5 K. The pulse sequences used to measure
dynamics data are shown in Figure 3.1. All sequences employ a CP to transfer polarization from $^1\text{H}$ to $^{15}\text{N}$ or $^{13}\text{C}$. A modified MISSISSIPPI sequence [114], where $\pi/2$ pulses are inserted after each saturation period, destroys all the remaining $^1\text{H}$ magnetization. Before detection, the $^{15}\text{N}$ or $^{13}\text{C}$ magnetization is transferred back to $^1\text{H}$. The $^1\text{H}$ resonances were assigned based on the existing $^{15}\text{N}$, $^{13}\text{C}_\alpha$, $^{13}\text{C}_\beta$, and $^{13}\text{CO}$ assignment [70, 71]. $^1\text{H}_\alpha$ assignment was obtained from a 3D HACAN spectrum using CP transfers, and a 3D HACACB spectrum using CP and INEPT transfers, based on [115]. $^1\text{H}$ amide assignment was achieved using a 3D HNCA based on CP transfers.

### 3.3.3 Sample preparation

The U-[$^{13}\text{C},^{15}\text{N}$] HET-s(218-289) monomers for measurements of $^1\text{H}$-$^{15}\text{N}$ were expressed in D$_2$O, fibrilized in H$_2$O and washed with H$_2$O pH 5.0, according to 2.2.1. For $^1\text{H}_\alpha$-$^{13}\text{C}_\alpha$ measurements, the protein was expressed and purified as described in 2.2.1 using a M9 minimum medium containing 75% D$_2$O and 25% H$_2$O and $^{15}\text{NH}_4\text{Cl}$ and [${^{13}\text{C}_6,^{2}\text{H}_7}$]-glucose as the sole nitrogen and carbon sources. It has been shown that this medium produces proteins with $\alpha$ positions having on average a higher level of protonation than all other positions [110, 116]. A proton concentration between 15 and 25% in the M9 medium gives the best balance between sensitivity and resolution [117].
Conclusions and Outlook
In this thesis solid-state NMR was employed to study LCs, as well as the HET-s(218-289) prion. LCs were used as model system to test the REDOR pulse sequence on samples containing small RDCs. We showed that REDOR could be used to recouple $^{1}$H-$^{13}$C RDCs around 60 Hz with an accuracy of $\pm 5$ Hz. These results open new avenues for the measurements of RDCs in flexible parts of proteins. Further work could be invested understanding the behaviour of the LCs to explain the shape of the REDOR curves that do not display oscillations and show a damped dephasing.

The experiments on the HET-s(218-289) prion have shown the signals observed in INEPT-based experiments originate from HET-s(218-289) monomers attached to the surface of HET-s(218-289) fibrils. It is believed that, during fibril formation, monomers accumulate along the surface of the protofilaments and then convert into a new protofilament [118]. According to this hypothesis, the HET-s(218-289) monomers present in fibril samples might play an important role in fibril formation. However, this assumption still has to be verified experimentally.

In the last part of this work, we employed CP-based experiments to look into the dynamics of the more rigid parts of the HET-s(218-289) fibrils. We measured a fast timescale of motion (picosecond range for $^{13}$C and nanosecond range for $^{15}$N) as well as a slower timescale of motion in the order of 1-100 microseconds, which is a concerted motion of several atoms and seems to be rather constant over the whole HET-s(218-289) protein. We therefore suppose that the whole fibrils sees a single slow motion. Using a dynamic model to fit the experimental data would measure with more precision the different amplitudes and timescales of motion present in the HET-s(218-289) fibrils. Getting insight into the structure and dynamics of amyloid fibrils could be of paramount importance for the development of therapies.
Appendix

A  Rotor angle calibration

Figure 6: Centerband of the $^1$H decoupled $^{31}$P spectra of TMPS recorded at a spinning frequency of 5 kHz. The experimental data are shown in blue. The simulations have been performed with SIMPSON.
B SIMPSON scripts for REDOR simulations

B.1 2-pulse REDOR

```plaintext
spinsys {
  channels 13C 1H
  nuclei 13C 1H
  shift 1 6p 0 0 0 0
  shift 2 6p 0 0 0 0
  dipole 1 2 -75 0 0 0
  jcoupling 1 2 200 0 0 0 0
}

par {
  spin_rate 810
  rotor_angle 54.7
  proton_frequency 40006
  crystal_file rep144
  gamma_angles 40
  start_operator I1x
  detect_operator I1x
  np 120
  variable pwr1 50000
  variable pwr2 50000
  variable d21 1e6/spin_rate
  variable p1 0.25e6/pwr1
  variable p12 0.5e6/pwr2
  variable d25 d31-p12*.5
  variable d26 d31*0.5-p12
  variable d27 d31-p12*.5
}

proc block1 () {
  global par
  maxdt 1
  delay $par(d25)
}

proc block2 () {
  global par
  maxdt 1
  reset [expr $par(d25)/2]
  delay $par(d25)
}

proc pulseq () {
  global par
  maxdt 1
  reset
  block1
  store 1
  reset
  block2
  store 2
  reset
  block3
  store 3
  reset
  block4
  store 4
  reset
  block5
  store 5

  for {set i 0} {$i < $par(np)} {incr i 1} {
    reset
    prop 1
    prop 2 $i
    prop 3
    prop 4 $i
    prop 5
    acq
  }
}

proc main () {
  global par

  set par(verbose)
  set par(pulse sequence) pulseq
  set f [fsimpson]
  fsave $f $par(name).xy
}
```


B.2 4-pulse REDOR

```plaintext
spinsys {
  channels 13C 1H
  nuclei 13C 1H
  shift 1 2p 0p 0 0 0 0
  shift 2 2p 0p 0 0 0 0
  dipole 1 2 -75 0 0 0
  jcoupling 1 2 209 0 0 0 0
}

par {
  spin_rate 810
  rotor_angle 54.7
  proton_frequency 400e6
  crystal_file alpha0beta90
  gamma_angles 200
  start_operator 11x
  detect_operator 11x
  np 128
  variable pwr1 50000
  variable pwr2 50000
  variable d31 1e6/spin_rate
  variable p1 0.25e6/pwr1
  variable p12 0.5e6/pwr2
  variable d25 d31-p12*.5
  variable d26 d31-p12*.5
  variable d27 d31-p12*.5
}

proc block1 () {
  global par
  maxdt 1
  delay $par(d25)
}

proc block2 () {
  global par
  maxdt 1
  reset [expr $par(p12)/2]
  delay $par(d25)
}

proc pulse () {
  global par
  maxdt 1
  reset
  block1
  store 1
  reset
  block2
  store 2
  reset
  block3
  store 3
  reset
  block4
  store 4
  reset
  block5
  store 5
  for (set i 0) {si < $par(np)} {incr i 1} {
    reset
    prop 1
    prop 2 $i
    prop 3
    prop 4 $i
    prop 5
    acq
  }
}

proc main () {
  global par

  set par(verb Soc) 1
  set par(pulse_sequence) pulseq
  set f [fsimpson]
  fsave $f $par(name),xy
}
```
C SIMPSON script for powder pattern $^{13}$C simulations

```plaintext
spinsys {
   channels 1H 13C
   nuclei 1H 13C
   shift 1 0 0 0 0 0 0
   shift 2 0 0 0 0 0 0
   dipole 1 2 1500 0 0 0 0
   jcoupling 1 2 145 0 0 0 0
}

par {
   crystal_file rep144
   sw 5000
   np 4096
   proton_frequency 400e6
   start_operator I1x
   detect_operator I1p
   method direct
   gamma_angles 5
   spin_rate 5000
   rotor_angle 52.33
   variable dw 1e6/sw
   dipole_check false
}

proc pulseq () {
   global par

   acq
   for {set i 1} {$i < $par(np)} {incr i} {
      delay $par(dw)
      acq
   }
}

proc main () {
   global par

   set f [fsimpson]
   faddlb $f 10 0
   fsave $f $par(name).xy
}
```
D  HET-s(218-289) assignment

Figure 7: Strip plots from the HNCA spectrum using INEPT transfers of HET-s(218-289) solid (blue) and solution sample (green).
E Reduced Wigner Rotation Matrix Elements of Rank 2

The reduced Wigner rotation matrix elements are given by \( d_{m',m}^2(\beta) \).

<table>
<thead>
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<th>( m' )</th>
<th>+2</th>
<th>+1</th>
<th>0</th>
<th>-1</th>
<th>-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>+2</td>
<td>((1+\cos^2\beta)) &amp; (-\frac{1+\cos^2\beta}{2}\sin\beta)</td>
<td>(\frac{3}{8}\sin^2\beta)</td>
<td>(-\frac{1-\cos^2\beta}{2}\sin\beta)</td>
<td>(-\frac{1-\cos^2\beta}{2}\sin\beta)</td>
<td></td>
</tr>
<tr>
<td>+1</td>
<td>(\frac{1+\cos^2\beta}{2}\sin\beta) &amp; (\cos^2\beta - \frac{1-\cos^2\beta}{2})</td>
<td>(-\sqrt{\frac{3}{8}}\sin(2\beta))</td>
<td>(-\cos^2\beta + \frac{1-\cos^2\beta}{2})</td>
<td>(-\frac{1-\cos^2\beta}{2}\sin\beta)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>(\sqrt{\frac{3}{8}}\sin^2\beta) &amp; (\frac{3}{8}\sin(2\beta))</td>
<td>(\frac{3\cos^2\beta - 1}{2})</td>
<td>(-\sqrt{\frac{3}{8}}\sin(2\beta))</td>
<td>(\frac{3}{8}\sin^2\beta)</td>
<td></td>
</tr>
<tr>
<td>-1</td>
<td>(\frac{1-\cos^2\beta}{2}\sin\beta) &amp; (-\cos^2\beta + \frac{1-\cos^2\beta}{2})</td>
<td>(\frac{3}{8}\sin(2\beta))</td>
<td>(\cos^2\beta - \frac{1-\cos^2\beta}{2})</td>
<td>(-\frac{1+\cos^2\beta}{2}\sin\beta)</td>
<td></td>
</tr>
<tr>
<td>-2</td>
<td>(\frac{1-\cos^2\beta}{2}\sin\beta) &amp; (\frac{1-\cos^2\beta}{2}\sin\beta)</td>
<td>(\sqrt{\frac{3}{8}}\sin^2\beta)</td>
<td>(\frac{1+\cos^2\beta}{2}\sin\beta)</td>
<td>(\frac{1+\cos^2\beta}{2}\sin^2\beta)</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Reduced Wigner rotation elements of rank 2.


Bibliography


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Publications
