<http://www.diva-portal.org>

Preprint

This is the submitted version of a paper published in *Journal of magnetic resonance*.

Citation for the original published paper (version of record):

Kaderavek, P., Zapletal, V., Fiala, R., Srb, P., Padrta, P. et al. (2016)
Spectral density mapping at multiple magnetic fields suitable for C-13 NMR relaxation
studies
Journal of magnetic resonance, 266: 23-40
<https://doi.org/10.1016/j.jmr.2016.02.016>

Access to the published version may require subscription.

N.B. When citing this work, cite the original published paper.

Permanent link to this version:

<http://urn.kb.se/resolve?urn=urn:nbn:se:su:diva-130855>

Spectral Density Mapping at Multiple Magnetic Fields Suitable for ^{13}C NMR Relaxation Studies

Pavel Kadeřávek^{a,b,c}, Vojtěch Zapletal^a, Radovan Fiala^b, Pavel Srb^b, Petr Padrta^b, Mária Šoltésová^d, Jozef Kowalewski^e, Göran Widmalm^f, Josef Chmelík^g, Vladimír Sklenář^{a,b}, Lukáš Žídek^{*a,b}

^a National Centre for Biomolecular Research, Faculty of Science, Masaryk University, Kamenice 5, CZ-625 00 Brno, Czech Republic

^b Central European Institute of Technology, Masaryk University, Kamenice 5, CZ-625 00 Brno, Czech Republic

^c Institute of Biophysics of Academy of Sciences of the Czech Republic, Královopolská 135, CZ-612 65 Brno, Czech Republic

^d Faculty of Mathematics and Physics, Charles University, V Holešovičkách 2, CZ-180 00 Prague, Czech Republic

^e Department of Materials and Environmental Chemistry, Arrhenius Laboratory, Stockholm University, S-106 91 Stockholm, Sweden

^f Department of Organic Chemistry, Arrhenius Laboratory, Stockholm University, S-106 91 Stockholm, Sweden

^g Institute of Microbiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, CZ-142 00 Prague 4 - Krč, Czech Republic

Abstract

Standard spectral density mapping protocols, well suited for the analysis of ^{15}N relaxation rates, introduce significant systematic errors when applied to ^{13}C relaxation data, especially if the dynamics is dominated by motions with short correlation times (small molecules, dynamic residues of macromolecules). A possibility to improve the accuracy by employing cross-correlated relaxation rates and on measurements taken at several magnetic fields has been examined. A suite of protocols for analyzing such data has

*Corresponding author, phone +420-549 498 393

Email addresses: kada@chemi.muni.cz (Pavel Kadeřávek), vojtis@mail.muni.cz (Vojtěch Zapletal), fiala@chemi.muni.cz (Radovan Fiala), srb@chemi.muni.cz (Pavel Srb), padrta@chemi.muni.cz (Petr Padrta), maria.soltesova@gmail.com (Mária Šoltésová), jozef.kowalewski@mmk.su.se (Jozef Kowalewski), gw@organ.su.se (Göran Widmalm), chmelik@biomed.cas.cz (Josef Chmelík), sklenar@chemi.muni.cz (Vladimír Sklenář), lzidek@chemi.muni.cz (Lukáš Žídek*)

been developed and their performance tested. Applicability of the proposed protocols is documented in two case studies, spectral density mapping of a uniformly labeled RNA hairpin and of a selectively labeled disaccharide exhibiting highly anisotropic tumbling. Combination of auto- and cross-correlated relaxation data acquired at three magnetic fields was applied in the former case in order to separate effects of fast motions and conformational or chemical exchange. An approach using auto-correlated relaxation rates acquired at five magnetic fields, applicable to anisotropically moving molecules, was used in the latter case. The results were compared with a more advanced analysis of data obtained by interpolation of auto-correlated relaxation rates measured at seven magnetic fields, and with the spectral density mapping of cross-correlated relaxation rates. The results showed that sufficiently accurate values of auto- and cross-correlated spectral density functions at zero and ^{13}C frequencies can be obtained from data acquired at three magnetic fields for uniformly ^{13}C -labeled molecules with a moderate anisotropy of the rotational diffusion tensor. Analysis of auto-correlated relaxation rates at five magnetic fields represents an alternative for molecules undergoing highly anisotropic motions.

Keywords: Nuclear magnetic resonance, relaxation, spectral density function, magnetic field, nucleic acids, carbohydrates

1. Introduction

Nuclear magnetic resonance (NMR) not only serves as an important source of structural data, but also provides insight into molecular motions on a time scale ranging from 10^{-12} to 10^3 s. As fluctuations of local magnetic fields are the only sources of relaxation for spin-1/2 nuclei, stochastic motions causing the magnetic fields to fluctuate can be effectively studied by measuring and analyzing the NMR relaxation rates, most typically of ^{15}N , ^{13}C , and ^2H nuclei. Fluctuations of individual magnetic fields and their interference are described by auto-correlation and cross-correlation functions, respectively. The semi-classical theory of spin relaxation in isotropic liquids [1, 2, 3, 4, 5] shows that relaxation rates are given by linear combinations of Fourier-transformed correlation functions, known as *spectral density functions*, evaluated at specific *eigenfrequencies*. Hence, the measured relaxation rates directly reflect stochastic dynamics of the molecule.

Numerous NMR experiments have been designed in order to measure

relaxation of various quantities. In principle, values of spectral density functions at individual eigenfrequencies can be extracted from the relaxation rates, such process is known as *spectral density mapping*. From the practical point of view, each measured relaxation rate represents one known experimental value and each spectral density value represents an unknown parameter to be determined. Obviously, the procedure requires the number of measured relaxation rates to be equal or greater than the number of spectral density values contributing to them. The major challenge of the spectral density mapping is the fact that various types of magnetic fields contribute to relaxation in real systems. As a consequence, values of multiple auto- and cross-correlated spectral density functions at several eigenfrequencies contribute to the measured relaxation rates, which greatly increases the number of unknown parameters in the spectral density mapping analysis. Therefore, the practical analysis of relaxation rates requires a certain level of simplification.

One possibility is to a priori define *particular functional form(s)* of the spectral density function. Such an approach is the essence of various versions of the *model-free* analysis [6, 7, 8, 9, 10]. The aim is to choose a form described by less parameters than the number of spectral density values contributing to the relaxation rates. The experimental relaxation rates are then used to determine the parameters describing the assumed functional form of the spectral density function instead of calculating its individual values. This elegant idea made the NMR relaxation a widely used method of motional analysis of biomacromolecules.

In spite of numerous successful applications, the model-free analysis has its limitation. From the statistical point of view, the correct choice of the number of relevant motional modes is a difficult problem, considering the low amount of experimental data and high non-linearity of the target function. From the physical point of view, the model-free approach is well applicable to relatively rigid molecular fragments whose internal motions can be described reliably by a single motional mode. However, higher motional richness requires more complex functional forms of the spectral density functions described by more parameters and the simplifying potential of the model-free approach disappears. More importantly, the assumptions of the statistical independence and symmetry of the individual motions are not valid in general, and an unjustified use of the model free approach may lead to incorrect results. Various advanced approaches have been proposed [11, 12, 13, 14, 15, 16, 17] to overcome the limitations, but they are consid-

erably more computationally demanding.

Another possibility is to find a setup of the relaxation experiments and/or analysis when the number of the spectral density values to be determined does not exceed the number of available experimental values. It can be achieved by (i) assuming higher symmetry of molecular motions, (ii) reducing the number of eigenfrequencies (*reduced spectral density mapping* [18, 19]), or (iii) extending the set of relaxation rates measured. The first option is frequently used but applicable only to certain types of motions (usually isotropic). The second possibility is typically exploited in ^{15}N relaxation studies, when the high difference between ^{15}N and ^1H relaxation rates permits to replace high-field values of the given spectral density function with a single effective value without introducing a too large error. The third route is experimentally more demanding, but it has a potential to overcome limitations of the first two approaches. It can include both new types of experiments and measurements repeated at multiple magnetic fields.

In this paper, we examine the potential of extending the number of experimental data for reduced spectral density mapping by using multiple magnetic fields and including cross-correlated relaxation rates. We focus on relaxation of protonated ^{13}C nuclei, where some simplifications routinely applied to peptide-bond ^{15}N are not well justified (large difference of ^{15}N and ^1H magnetogyric constants, collinearity of the ^{15}N - ^1H bond and the main axis of the ^{15}N chemical shift tensor). Several methods of a combined analysis of relaxation rates obtained at multiple fields are described. Systematic errors are simulated and propagation of random experimental errors is analyzed in order to estimate when application of individual methods may be beneficial. Finally, analyses of relaxation data of two real molecules are presented. The tested samples included a uniformly ^{13}C , ^{15}N -labeled pppG-GCACUUCGGUGCC, an RNA hairpin containing the UUCG tetraloop, and methyl β -D-glucopyranosyl-(1 $\rightarrow$ 6)- α -D-[6- ^{13}C] mannopyranoside, a β -(1 $\rightarrow$ 6) linked disaccharide with a selectively ^{13}C labeled bridging methylene group. The RNA sample documents that the multiple-field mapping is applicable to relatively large uniformly labeled molecules. Also, it allowed us to test the influence of a significant conformational or chemical exchange. The disaccharide served as an example of a molecule exhibiting fast and highly anisotropic motions. The selective labeling allowed us to collect a large set of relaxation rates at seven magnetic fields, and to compare the performance of various methods of spectral density mapping in a systematic manner.

2. Methods

2.1. Sample preparation

2.1.1. *UUCG hairpin*

Fully ^{13}C - and ^{15}N -labeled sample of RNA oligomer pppGGCACUUCG-GUGCC was synthesized in-vitro from ^{13}C and ^{15}N -labeled NTPs. T7 RNA polymerase was used for the transcription from a DNA template. The synthesized oligomer was purified by a gel electrophoresis [20, 21]. A 3.0 mM oligomer sample was prepared in 99.95% D_2O at pH^* 6.7 (uncorrected reading). The sample contained 0.2 mM EDTA and 10 mM sodium phosphate buffer. A small amount of sodium azide was added to the sample. A detailed description of the sample preparation was published elsewhere [22].

2.1.2. $\beta\text{-D-Glcp-(1}\rightarrow\text{6)-}\alpha\text{-D-Manp-OMe}$

The sample was prepared by dissolving 9.5 mg of freeze-dried methyl $\beta\text{-D-glucopyranosyl-(1}\rightarrow\text{6)-}\alpha\text{-D-[6-}^{13}\text{C]}$ mannopyranoside [23] in 367 μl DMSO-d_6 (99.96 % ^2H , Euriso-Top) and 204 μl D_2O (99.96 % ^2H , Aldrich). The solution in a 4 mm NMR tube was degassed by three cycles of freezing, application of a mild vacuum, and melting. Then the evacuated NMR tube was heat-sealed.

2.2. NMR experiments

2.2.1. *UUCG hairpin*

NMR experiments were carried out at the temperature of 298.2 K on the 400 MHz Varian Unity spectrometers with a double resonance (^1H , ^{13}C) room-temperature probe, and on 500 MHz and 600 MHz Varian Unity spectrometers with triple resonance (^1H , ^{13}C , ^{15}N) room-temperature probes. The temperature was calibrated based on the chemical shift differences of pure methanol peaks. Delays of the polarization transfer periods were set for a 200 Hz ^{13}C - ^1H scalar coupling. Two-dimensional data were processed with the cosine square apodization and four-fold zero filling in the program NMRPIPE[24]. The phase in the direct dimension was manually adjusted to pure absorption. The spectra were analyzed and the peak heights were evaluated in the program SPARKY[25]. The resonances were assigned according to the literature [26]. The published [27] NMR pulse programs were used for the measurements of the R_1 and $R_{1\rho}$ relaxation rates with the R_1 relaxation delays of 5, 30*, 60, 100, 145, 200, 280*, and 420 ms at 400 MHz; 5, 35, 65, 100, 135, 175, 220, 275, 335, 410, 585, and 835 ms at 500 MHz; 5, 25*, 50, 80, 110, 150, 205, 265, 350, and 495 ms at 600 MHz and with the $R_{1\rho}$ relaxation delays

of 3, 9*, 21, 33, 48, 66, 93*, and 138 ms at 400 MHz; 3, 9, 15, 24, 33, 48, 63, 81, 108, and 150 ms at 500 MHz; 3, 9*, 15, 24, 33, 48, 63, 81, 108*, and 150 ms at 600 MHz. The asterisk denotes the spectra recorded twice. Relaxation rates were obtained by fitting peak intensities to a mono-exponential decay using the program OCTAVE[28]. The R_2 relaxation rate was calculated from the measured R_1 and $R_{1\rho}$ rates as $R_2 = (\Delta\nu_0^2 + \nu_1^2)R_{1\rho}/\nu_1^2 - \Delta\nu_0^2 R_1/\nu_1^2$, where $\Delta\nu_0$ is the difference between a particular spin resonance frequency and spin lock carrier frequency and ν_1 is the spin lock field strength, determined based on the linearity of the power amplifier and verified by calibration. The field strengths of the spin lock were 2784, 1964, and 4808 Hz at 400 MHz, 500 MHz, and 600 MHz spectrometers, respectively. The ^{13}C - ^1H steady-state NOE [27] at 500 MHz was measured twice to test the effect of various length of the relaxation period (3.5 and 5.0 s) and ^1H irradiation length (3.0 and 1.5 s) used to achieve a steady state. Both experiments provided values which did not differ significantly. NOE at 400 MHz and 600 MHz was measured with 3.0 s relaxation period and 3.0 s ^1H irradiation period to achieve a steady state. The reference spectra were measured interleaved together with the spectra under the steady state conditions. The transverse cross relaxation rate Γ_2 was measured using constant time HSQC coupled in the indirect dimension [29] with the constant time evolution delay $T = 30$ ms, and evaluated as $(\ln(I_1/I_0))/2T$, where I_0 and I_1 are the intensities of the coupled peaks. The error of transverse cross relaxation rate was determined from the comparison of results of measurements with $T = 30$ ms and 15 ms, and errors of the other measurements were recalculated based on the experimental noise. The measured experimental data were deposited in the Biological Magnetic Resonance Data Bank (<http://www.bmrb.wisc.edu>) as entry 18616.

2.2.2. β -D-Glcp-(1 $\rightarrow$ 6)- α -D-Manp-OMe

The NMR experiments were carried out at 293.2 K at the following spectrometers and probeheads: 300 MHz Bruker Avance III with a room-temperature BBO-F probe, 400 MHz Bruker Avance III with a room-temperature BBI probe, 500 MHz Bruker Avance III with a cryogenic Prodigy BBO probe, 600 MHz Bruker Avance III with a cryogenic TCI probe, 700 MHz Bruker Avance III with a room-temperature TXI probe, 850 MHz Bruker Avance III with a cryogenic TXO probe, and 950 MHz Bruker Avance III with a cryogenic TCI probe. The 1 s recycle delay was used in all experiments except the heteronuclear NOE measurement, run with a 2 s recycle delay. The broadband proton decoupling was achieved by a WALTZ-16 scheme with a $80\mu\text{s}$

length of the 90° pulse. Carbon carrier frequency was placed on-resonance to the observed signal, the spectral width of 60 ppm and 1 s acquisition time was used in all experiments. The spectra were processed in the program NMRPIPE[24] by applying 10 Hz line broadening, four-fold zero filling, Fourier transform, adjusting phase to obtain pure absorption lineshape, and using a zero-order polynomial function to correct the baseline. The longitudinal relaxation rate of ^{13}C was measured using the inversion recovery experiment with 1, 5, 10*, 20, 30, 50, 80*, 140, 220, 350*, 500, and 650 ms delays. The transverse relaxation rate was measured using $R_{1\rho}$ experiment at a spin-lock frequency of 1 kHz (300 MHz, 400 MHz, 500 MHz, and 600 MHz) or 2 kHz (700 MHz, 850 MHz, and 950 MHz) and with the following relaxation delays: 1*, 5, 10*, 20, 35, 50, 70*, 100, 140, 200*, and 300 ms (asterisk denotes spectra recorded twice). In order to account for the sample heating during long relaxation delays, fitting of the relaxation rates was repeated with variable number of data points, with the longest used relaxation delay ranging from 70 ms to 300 ms, and the obtained relaxation rates were extrapolated to a zero length of the relaxation delay. The $R_{1\rho}$ relaxation dispersion was also monitored at 600 MHz by repeating the $R_{1\rho}$ experiments with 28 different spin-lock field strengths (from 300 Hz to 2 kHz) to check for possible slow exchange contribution. The longitudinal and transverse cross-correlated relaxation rates were measured without decoupling as described earlier [30]. The inversion recovery for determination of longitudinal cross-correlated relaxation was measured with relaxation delays of 1*, 5*, 10, 15*, 20, 25*, 30, 35*, 40, 45*, 50, 55*, 60, 70, 80, 90, 1000*, 1500*, and 2000* ms. The coupled $R_{1\rho}$ experiment, used for the determination of the transverse cross-correlated relaxation rate, was performed with a 1 kHz (300 MHz, 400 MHz, 500 MHz, and 600 MHz) or 2 kHz (700 MHz, 850 MHz, and 950 MHz) spin-lock field applied for 1, 5*, 10, 15*, 20, 25, 30*, 35, 40, 45*, 50, 55*, 60, 70, 80, 90 ms (asterisk denotes spectra recorded twice). The relaxation rates of left ($W_{i,2}$), middle ($W_{i,1}$), and right ($W_{i,0}$) lines of the triplet in coupled spectra were evaluated and the longitudinal ($i = 1$) and transverse ($i = 2$) cross-correlated relaxation rates $\Gamma_i = (W_{i,2} - W_{i,0})/2$ and $\Xi_i = (W_{i,2} - 2W_{i,1} + W_{i,0})/4$ were determined [30] from data obtained with ($i = 2$) and without ($i = 1$) applying the spin-lock field. Here, the symbols Γ_i refer to the cross-correlated relaxation rates involving interference of the dipole-dipole and chemical shielding anisotropy (DD-CSA), while Ξ_i imply interference of two dipolar interactions. The ^{13}C - ^1H heteronuclear Overhauser enhancement was calculated from the ratio of intensities in reference and saturated spectra measured by the dy-

dynamic NOE technique [31]. The dynamic NOE experiment was repeated five times and other experiments three times. Bootstrap re-sampling was applied to determine the experimental errors.

2.3. Relaxation rate analysis

In order to present the relaxation rate analysis in a compact form, the following notation has been introduced. Frequencies $\omega_{n,f}$ are labeled with indices n , identifying the nucleus, and f , identifying the external magnetic field (labeled $B_{0,f}$). The index $n = 0$ refers to the observed nucleus (with the magnetogyric ratio labeled γ_0). Indices n are also used to specify the relaxation mechanism in the following text, $n = 0$ denoting relaxation of the observed nucleus due to its chemical shift anisotropy (CSA) of the observed nucleus and $n > 0$ denoting relaxation of the observed nucleus due to the dipole-dipole interaction with the n -th nucleus. Furthermore, the relaxation rates R_1 , R_2 , Γ_1 , Γ_2 , Ξ_1 , Ξ_2 , and steady-state NOE were converted to a form that allowed us to express their combinations in a more compact fashion:

$$\delta_f = \frac{2R_2(B_{0,f}) - R_1(B_{0,f})}{\sum_{n=1}^N d_n^2(\gamma_n = \gamma_1)}, \quad (1)$$

$$\rho_f = \frac{R_1(B_{0,f})}{\sum_{n=1}^N d_n^2(\gamma_n = \gamma_1)}, \quad (2)$$

$$\sigma_f = \left(\frac{I_{ss}(B_{0,f})}{I_{ref}(B_{0,f})} - 1 \right) \frac{\gamma_0}{\gamma_n} \frac{R_1(B_{0,f})}{\sum_{n=1}^N d_n^2(\gamma_n = \gamma_1)}, \quad (3)$$

$$\mu_f = \frac{b_f \Gamma_2(B_{0,f})}{c_0 B_{0,f} \sum_{n \in C} d_n p_{0,n}} = \frac{2b_f \Xi_2(B_{0,f})}{\sum_{n \in C} \sum_{n' \in C} (1 - \delta_{n,n'}) d_n d'_{n'} p_{n,n'}}, \quad (4)$$

$$\lambda_f = \frac{b_f \Gamma_1(B_{0,f})}{2c_0 B_{0,f} \sum_{n \in C} d_n p_{0,n}} = \frac{b_f \Xi_1(B_{0,f})}{\sum_{n \in C} \sum_{n' \in C} (1 - \delta_{n,n'}) d_n d'_{n'} p_{n,n'}}, \quad (5)$$

where

$$b_f = \left(c_0^2 B_{0,f}^2 + \sum_{n=1}^N d_n^2 \right) / \sum_{n=1}^N d_n^2(\gamma_n = \gamma_1), \quad (6)$$

$$c_0 = \frac{\gamma_0 \Delta_0}{3\sqrt{5}} \sqrt{1 + \frac{\eta_0^2}{3}}, \quad (7)$$

$$d_n = -\frac{\gamma_0}{2\sqrt{5}} \frac{\mu_0 h \gamma_n}{8\pi^2 r_{0,n}^3}, \quad (8)$$

$$p_{0,n} = \frac{2 - (3 + \eta_0) \cos^2 \theta_{0,n}^- - (3 - \eta_0) \cos^2 \theta_{0,n}^+}{2\sqrt{1 + \eta_0^2/3}}, \quad (9)$$

$$p_{n,n'} = (3 \cos^2 \theta_{n,n'} - 1)/2, \quad (10)$$

I_{ss} and I_{ref} are peak intensities in the steady-state NOE and reference spectra, respectively, C is the set of nuclei with a scalar coupling to the observed nucleus evolving in the cross-correlated relaxation experiments, γ_1 is the magnetogyric ratio of proton, N is the number of all interacting nuclei, Δ_0 and η_0 are anisotropy and asymmetry of the chemical shift tensor of the observed nucleus, respectively, $r_{0,n}$ is the distance between the observed nucleus and the n -th interacting nucleus, $\theta_{0,n}^\pm$ are the angles between the direction of the internuclear vector $\vec{r}_{0,n}$ (connecting the observed nucleus with nucleus n) and the x and y eigenvectors of the chemical shielding tensor of the observed nucleus, respectively, $\theta_{n,n'}$ is the angle between internuclear vectors $\vec{r}_{0,n}$ and $\vec{r}_{0,n'}$, μ_0 is the permeability of vacuum, and h is the Planck's constant. Internuclear distances, bond angles, and chemical shift tensors used in the data analysis were taken from the literature [32, 33, 34]. Note that the second equalities in Eqs. 4 and 5 do not imply a generally valid relation between Γ_i and Ξ_i , they only describe two possible definitions μ_f or λ_f used in the approximative treatment of isotropically moving molecules discussed in Section 3.1

Following a convention introduced in our previous study [35], we refer to individual spectral density mapping protocols by the types of relaxation rates used, abbreviated L for the longitudinal auto-relaxation rate R_1 , T for the transverse auto-relaxation rate R_2 , N for the heteronuclear steady-state NOE, X for the transverse cross-correlated relaxation rate Γ_2 , and by the number of magnetic fields employed. For example, analysis of R_1 , R_2 , and steady-state NOE acquired at a single magnetic field is abbreviated LTN1.

The spectral density functions were normalized so that the autocorrelated spectral density function $J^{n,n}(\omega) = 6D/(36D^2 + \omega^2)$ for a rigid spherical particle of a rotational diffusion coefficient D .

3. Results and Discussion

3.1. General concept

General forms of equations describing relaxation rates in terms of contributions of the spectral density values, presented in Appendix A, are relatively complex. Complexity of the spectral density mapping greatly reduces if all motions contributing to the relaxation are isotropic and/or if the individual interactions can be described by collinear vectors. In such cases, a single spectral density function $J(\omega)$ can be used to account for all interactions. The analysis is further simplified if the relaxation of the observed ^{13}C or ^{15}N nucleus is influenced only by protons. In such cases, five values of the same spectral density function, $J(0)$, $J(\omega_{0,f})$, $J(\omega_{1,f})$, $J(\omega_{1,f} + \omega_{0,f})$, and $J(\omega_{1,f} - \omega_{0,f})$, are sufficient to describe various relaxation rates. Eqs. A.1–A.6 can be then recast into

$$\delta_f = 2\xi_f + 8b_f J(0) + 12J(\omega_{1,f}), \quad (11)$$

$$\rho_f = 6b_f J(\omega_{0,f}) + 12J(\omega_{1,f} + \omega_{0,f}) + 2J(\omega_{1,f} - \omega_{0,f}), \quad (12)$$

$$\sigma_f = 12J(\omega_{1,f} + \omega_{0,f}) - 2J(\omega_{1,f} - \omega_{0,f}), \quad (13)$$

$$\mu_f = 8b_f J(0) + 6b_f J(\omega_{0,f}), \quad (14)$$

$$\lambda_f = 6b_f J(\omega_{0,f}), \quad (15)$$

where

$$\xi_f = R_{\text{ex}}(B_{0,f}) / \sum_{n=1}^N d_n^2 (\gamma_n = \gamma_1). \quad (16)$$

If the ρ_f , σ_f , μ_f , and λ_f values are available from measurements of R_1 , R_2 , steady-state NOE, and Γ_2 or Ξ_2 , calculation of four spectral density values $J(0)$, $J(\omega_{0,f})$, $J(\omega_{1,f} - \omega_{0,f})$, and $J(\omega_{1,f} + \omega_{0,f})$ from four Equations 12–15 at each magnetic field is a trivial task. If the exchange contribution ξ_f is negligible or known from an independent experiment, experimental δ_f (derived from R_2 and R_1) can be also included in order to determine $J(\omega_{1,f})$.

While solving Eqs. 12–15 is straightforward from the theoretical point of view, precision of the obtained values might be compromised by high experimental error of some relaxation rates. For example, sensitivity of experiments for reliable measurement of longitudinal cross-correlated relaxation rates, providing λ_f , is rather low. If sufficiently precise λ_f values are not

available, additional data must be acquired or approximations must be used to reduce the number of unknowns, i.e., of the spectral density values.

The most common approximative approach utilizing R_1 , R_2 , and steady-state NOE data acquired at a single magnetic field (abbreviated LTN1 in this paper) is the method known as *reduced spectral density mapping* [18, 19, 36]. The set of Eqs. 11–13 is solved assuming that $J(\omega_{1,f} - \omega_{0,f}) \approx J(\omega_{1,f}) \approx J(\omega_{1,f} + \omega_{0,f})$ and neglecting the exchange contribution. Systematic errors introduced by such an approximation, referred to as *reduction bias* in this paper, have been examined in our recent study for the isolated N-H bond in the backbone of ^{15}N -labeled disordered proteins [35]. Simulations of the systematic errors for Lorentzian spectral density functions $L(\omega) = \tau/(1 + \omega^2\tau^2)$ showed that the assumption $J(\omega_{1,f} - \omega_{0,f}) \approx J(\omega_{1,f}) \approx J(\omega_{1,f} + \omega_{0,f})$ is well acceptable when analyzing the ^{15}N relaxation of both ordered and disordered proteins (data not shown here, cf. Fig. S1 in Ref. [35]). The reduction bias of spectral density values obtained by alternative protocols employing cross-correlated relaxation rates (LNX1 and LTX1) was also low [35].

A similar simulation for ^{13}C is presented in Fig. 1. The solid lines correspond to the most sensitive case, when dipole-dipole interactions represent the only source of relaxation. The dashed lines describe systematic errors when the relative contribution of dipole-dipole interactions is reduced by a relatively large (170 ppm) chemical shift anisotropy. The reduction bias of the LTX1 protocol does not exceed 6 % for the Lorentzian spectral density function evaluated at all three frequencies (green lines in Fig. 1). However, the reduction bias of all three values calculated by the LTN1 protocol (i.e., the original reduced spectral density mapping) was greater than 15 % for short correlation times (red lines in Fig. 1). Moreover, the spectral density value at the ^{13}C frequency ($L(\omega_{0,1})$) was overestimated by approximately 5 % even for large CSA and correlation times corresponding to the tumbling of proteins and nucleic acids (dashed red line in Fig. 1b). The reduction bias of the zero-frequency values calculated by the LNX1 protocol was also high, exceeding 10 % (blue lines in Fig. 1a, note that the other values, plotted in Fig. 1b,c are identical to those obtained by the LTN1 analysis). In summary, the simulations show that systematic errors of the reduced spectral density mapping at a single field are high in the case of ^{13}C , especially for motions described by short correlation times.

Several methods correcting the reduction bias have been described in the literature [36, 37]. These methods involve description of spectral density

Figure 1: Simulation of relative reduction bias of $J(0)$ **(a)**, $J(\omega_{0,1})$ **(b)**, and $J(\omega_{1,1})$ **(c)** obtained by reduced spectral density mapping of an isolated ^{13}C - ^1H pair at a single magnetic field ($B_{0,1} = 11.75$ T). Relative errors of the $J(\omega)$ values are described by functions $\Delta L(\omega)/L(\omega)$, where $\Delta L(\omega)$ is a deviation of the back-calculated $L(\omega)$ from its correct value. The red curve corresponds to the analysis of R_1 , R_2 , and NOE values (LTN1), the green curve corresponds to the analysis of R_1 , R_2 , and Γ_2 values (LTX1), and the blue curve corresponds to the analysis of R_1 , Γ_2 , and NOE values (LNX1). The solid and dashed lines correspond to errors calculated for $\Delta_0 = 0$ and $\Delta_0 = 170$ ppm, respectively. Since the $L(\omega_{0,1})$ and $L(\varepsilon\omega_{1,1})$ are identical for LTN1 and LNX3, the blue curves (identical with the red ones) are not plotted in Panels **(b)** and **(c)**.

functions in a certain frequency region by an approximative analytical form and determine frequency dependence of the approximated regions of the spectral density function from data obtained at multiple magnetic fields. In this study, we decided to employ relaxation rates measured at multiple fields in a somewhat different manner, and to explore the possibility to obtain exact spectral density values regardless of the actual analytical form of the spectral density function.

In the ideal case of isolated ^{13}C - ^1H spin systems in isotropically moving molecules, number of the spectral density values to be determined can be reduced without introducing any systematic error and for any form of the spectral density function if the magnetic fields are chosen so that certain eigenfrequencies match *different* eigenfrequencies at another magnetic field. For example, four rescaled relaxation rates δ_1 , δ_3 , ρ_2 , and σ_2 , obtained from R_2 measured at $B_{0,1}$ and $B_{0,3}$, steady-state NOE measured at $B_{0,1}$, and R_1 measured at all three fields, can be combined in four different ways:

$$\frac{6\delta_3 - \delta_1 - 6\sigma_2}{8(6b_3 - b_1)} = J(0) + \frac{6\xi_3 - \xi_1}{4(6b_3 - b_1)} + \frac{3}{2} \frac{6\psi_3 - \psi_1}{6b_3 - b_1}, \quad (17)$$

$$\frac{2(b_1\delta_3 - b_3\delta_1) + (6b_3 - b_1)\rho_2 - (6b_3 + b_1)\sigma_2}{6b_2(6b_3 - b_1)} = J(\omega_{0,2}) - 2\frac{b_3\xi_1 - b_1\xi_3}{3b_2(6b_3 - b_1)} - 4\frac{b_3\psi_1 - b_1\psi_3}{b_2(6b_3 - b_1)}, \quad (18)$$

$$\frac{b_3\delta_1 - b_1\delta_3 + b_1\sigma_2}{2(6b_3 - b_1)} = J(\omega_{1,1}) + \frac{b_3\xi_1 - b_1\xi_3}{6b_3 - b_1} - b_1\frac{6\psi_3 - \psi_1}{6b_3 - b_1}, \quad (19)$$

$$\frac{b_3\delta_1 - b_1\delta_3 + 6b_3\sigma_2}{12(6b_3 - b_1)} = J(\omega_{1,3}) + \frac{b_3\xi_1 - b_1\xi_3}{6(6b_3 - b_1)} - b_3\frac{6\psi_3 - \psi_1}{6b_3 - b_1}, \quad (20)$$

where $\psi_1 = J(\omega_{1,1}) - J(\omega_{1,2} - \omega_{0,2})$ and $\psi_3 = J(\omega_{1,3}) - J(\omega_{1,2} + \omega_{0,2})$.

Left-hand sides of Eqs. 17–20 can be viewed as recipes for calculating the four values of the spectral density function expressed explicitly. The terms with ξ_f and ψ_f in Eqs. 17–20 represent exchange contribution and reduction bias, respectively.

Obviously, $6\psi_3 - \psi_1$ is equal to zero if

$$\omega_{1,1} = \omega_{1,2} - \omega_{0,2} \quad (21)$$

and

$$\omega_{1,3} = \omega_{1,2} + \omega_{0,2}, \quad (22)$$

Conditions 21 and 22 are thus sufficient to obtain $J(0)$, $J(\omega_{0,2})$, $J(\omega_{1,1})$, $J(\omega_{1,2})$, and $J(\omega_{1,3})$ free of any reduction bias. These conditions are fulfilled exactly if the ratio of magnetic fields $B_{0,1}/B_{0,2}$ matches $1 - \gamma_0/\gamma_1$ and the ratio $B_{0,3}/B_{0,2}$ matches $1 + \gamma_0/\gamma_1$. The optimal ratio of magnetic fields is very close to 3:4:5 for the ^{13}C - ^1H pair and to 11:10:9 for the ^{15}N - ^1H pair (note the decreasing field strength in the latter case due to the negative sign of the magnetogyric ratio of ^{15}N). While the latter ratio is difficult to find among spectrometers on the market, the former one is well available (e.g., 300 MHz, 400 MHz, and 500 MHz).

The effect of exchange in Eqs. 17–20 cannot be eliminated by the choice of magnetic fields. However, it can be avoided by replacing δ_f with μ_f in the analysis. In this case, the reduction bias is completely eliminated if

$$\omega_{1,1} + \omega_{0,1} = \omega_{1,3} - \omega_{0,3} \quad (23)$$

(see Appendix B). This condition is fulfilled exactly for the magnetic field ratio $B_{0,3}/B_{0,1} = (\gamma_1 + \gamma_0)/(\gamma_1 - \gamma_0)$, e.g., 300 MHz and 500 MHz for ^{13}C . Note that an almost optimal ratio of magnetic fields for ^{15}N can be achieved by using routinely available 500 MHz and 600 MHz spectrometers.

Complete protocols based on various combinations of relaxation rates are presented in Appendix B.

In order to test performance of individual protocols and their limitations, they were applied to molecules not representing ideal samples for relaxation studies. In one case, the test molecule was uniformly labeled which introduced additional contributions to relaxation rates, not included in Eqs. 11–15. In the other case, the studied molecule tumbled highly anisotropically, which excluded the possibility to describe all interactions by a single spectral density function.

3.2. Case study I: UUCG hairpin

The first case study represents an example of spectral density mapping of a typical subject of relaxation studies, a well ordered macromolecule (RNA hairpin). The UUCG hairpin was chosen as a well described model RNA molecule [38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51].

The motional probe used in this case study was the ^{13}C nucleus in C8-H8 and C6-H6 groups of purines and pyrimidines, respectively. This choice is typical in relaxation studies of nucleic acids because ^{15}N relaxation experiments are usually limited to imino groups of guanine and uracil/thymine,

whose signals might be too broad due to the exchange contribution in unpaired bases. The C-H bonds are more frequent in nucleic acid bases and their peaks are well resolved and not influenced by proton exchange. The following protocols were applied to obtain $J(\omega)$ values for individual nucleotides, assuming that the molecular motions are close to isotropic.

3.2.1. Auto-correlated mapping: LTN3

Application of the LTN3 protocol to the UUCG hairpin required minor modifications of Eqs. 17–20. Since the studied molecule was uniformly labeled, Eqs. 11–13 do not fully describe the measured relaxation rates. The complete equations, listed in Appendix A, contain additional terms with frequencies of ^{13}C , ^{15}N , and their combinations. However, inspection of bond lengths and magnetogyric ratios reveals that the d_n constants describing dipole-dipole interactions with ^{13}C and ^{15}N nuclei, are rather low. Therefore, the spectral density values of these interactions at frequencies different from 0, $\omega_{0,f}$, $\omega_{1,f}$, and $(\omega_{0,f} \pm \omega_{1,f})$ can be neglected without introducing a significant error, and Eqs. 11 and 12 can be replaced by

$$\delta_f = 2\xi_f + 8b_f J(0) + 12b_0 J(\omega_{0,f}) + 12J(\omega_{1,f}), \quad (24)$$

$$\begin{aligned} \rho_f &= 2b_0 J(0) + 6b_f J(\omega_{0,f}) \\ &+ 12J(\omega_{1,f} + \omega_{0,f}) + 2J(\omega_{1,f} - \omega_{0,f}), \end{aligned} \quad (25)$$

where

$$b_0 = \sum_{n=1}^N d_n^2(\gamma_n = \gamma_0) / \sum_{n=1}^N d_n^2(\gamma_n = \gamma_1) \quad (26)$$

The relatively small term $12b_0 J(\omega_{0,f})$ in Eq. 24 can be safely neglected and $J(0)$ and $J(\omega_{0,2})$ expressed as

$$J(0) = \frac{6\delta_3 - \delta_1 - 6\sigma_2}{8(6b_3 - b_1)} - \frac{6\xi_3 - \xi_1}{4(6b_3 - b_1)} - \frac{3}{2} \frac{6\psi_3 - \psi_1}{6b_3 - b_1}, \quad (27)$$

$$\begin{aligned} J(\omega_{0,2}) &= \frac{(8b_1 - 6b_0)\delta_3 - (8b_3 - b_0)\delta_1 + 4(6b_3 - b_1)\rho_2 - (24b_3 + 4b_1 - 6b_0)\sigma_2}{24b_2(6b_3 - b_1)} \\ &- \frac{(8b_1 - 6b_0)\xi_3 - (8b_3 - b_0)\xi_1}{12b_2(6b_3 - b_1)} - \frac{2\psi_3(4b_1 - 3b_0) + \psi_1(b_0 - 8b_3)}{2b_2(6b_3 - b_1)}. \end{aligned} \quad (28)$$

Like in Eq. 17 and 18, ψ_1 and ψ_3 , representing the reduction bias, disappear if $B_{0,1}/B_{0,2}$ matches $1 - \gamma_0/\gamma_1$ and $B_{0,3}/B_{0,2}$ matches $1 + \gamma_0/\gamma_1$. The $J(\omega_{1,f})$ values can be calculated in a similar manner, but they were not determined in this study because their accuracy is compromised by relatively large systematic errors in uniformly labeled molecules (vide infra).

3.2.2. Exchange-free mapping: LNX2

If a slow exchange contributes to R_2 , apparent spectral density values calculated by the LTN3 analysis include the ξ_f terms. The exchange bias is eliminated if Γ_2 is used instead of R_2 (approach LNX). Then, the spectral density values are calculated from a set of ρ_f , σ_f , and μ_f , derived from R_1 , NOE, and Γ_2 , none of which is influenced by the slow exchange. Combining Eqs. 25, 13, and 14 for $B_{0,1}$ and $B_{0,3}$ gives

$$J(0) + \frac{12\psi_2}{4(6b_3 - b_1) - 5b_0} = \frac{\rho_1 + \sigma_1 - \mu_1 - 6\rho_3 + 6\sigma_3 + 6\mu_3}{8(6b_3 - b_1) - 10b_0}, \quad (29)$$

$$J(\omega_{0,1}) - \frac{16\psi_2}{4(6b_3 - b_1) - 5b_0} = \frac{4b_1(6\rho_3 - 6\sigma_3 - 6\mu_3 - \rho_1 - \sigma_1) + (24b_3 - 5b_0)\mu_1}{6b_1(24b_3 - 4b_1 - 5b_0)}, \quad (30)$$

$$J(\omega_{0,3}) - \frac{16\psi_2}{4(6b_3 - b_1) - 5b_0} = \frac{4b_3(6\rho_3 - 6\sigma_3 - \rho_1 - \sigma_1 + \mu_1) - (4b_1 + 5b_0)\mu_3}{6b_3(24b_3 - 4b_1 - 5b_0)}, \quad (31)$$

where $\psi_2 = J(\omega_{1,1} + \omega_{0,1}) - J(\omega_{1,3} - \omega_{0,3})$ disappears for the magnetic field ratio $B_{0,3}/B_{0,1} = (\gamma_1 + \gamma_0)/(\gamma_1 - \gamma_0)$.

3.2.3. Information on exchange: LTX3

Spectral density values can be also calculated from the R_1 , R_2 , and Γ_2 rates (LTX3). Combinations of δ_1 , δ_3 , ρ_2 , and μ_2 provide

$$J(0) + \frac{6\xi_3 + \xi_1}{4(b_1 + 6b_2 + 6b_3) - 6b_0} + 3\frac{6\psi_3 + \psi_1}{2(b_1 + 6b_2 + 6b_3) - 3b_0} = \frac{\delta_1 + 6\mu_2 - 6\rho_2 + 6\delta_3}{8(b_1 + 6b_2 + 6b_3) - 12b_0}, \quad (32)$$

$$J(\omega_{0,2}) - 2 \frac{6\xi_3 + \xi_1}{6(b_1 + 6b_2 + 6b_3) - 9b_0} - 4 \frac{6\psi_3 + \psi_1}{2(b_1 + 6b_2 + 6b_3) - 3b_0} = \frac{(2b_1 + 12b_3 - 3b_0)\mu_2 - 2b_2(\delta_1 - 6\rho_2 + 6\delta_3)}{12b_2(b_1 + 6b_2 + 6b_3) - 18b_0b_2}. \quad (33)$$

Such an approach is inferior from the statistical point of view, but may be useful in some cases. It allows one to avoid NOE measurements if accurate values of the NOE enhancement are difficult to obtain [52]. Also, a strong sensitivity of the LTX3 mapping may be used for a visual identification of a slow exchange. Comparison of the exchange terms in Eqs. 27, 28, 32, and 33 shows a difference between LTX3 and another exchange-dependent protocol, LTN3. In the case of LTX3, the slow exchange contributes to $J(\omega_{0,f})$ and $J(0)$ always in the ratio $-4 : 3$. On the other hand, in the case of LTN3, the slow exchange contributes to $J(\omega_{0,2})$ much less than to $J(0)$. Hence, the effects of fast and slow dynamics are well distinguished in the LTX3 data, but can compensate each other in the LTN3 data. Therefore, the LTX3 approach is better suited for a visual identification of a slow exchange. Note however, that the orientational bias affects LTX3 in a similar manner as the exchange. Therefore, possible deviations from isotropy of molecular motions must be taken into account when interpreting the LTX3 results.

3.2.4. Systematic errors

Before presenting results of the analysis of the hairpin motions, three possible sources of inaccuracy should be discussed.

First, asymmetry and orientation of the ^{13}C chemical shielding tensor and additional dipole-dipole interactions do not allow us to assume that all relaxation rates can be expressed using a single spectral density function in all cases; this assumption is only valid for isotropically tumbling molecules. In principle, the errors of spectral density values due to the anisotropy of the overall rotational diffusion tensor can be calculated if the structure of the molecule and the diffusion tensor are known and if the contributions of local motions can be neglected. Such calculations were performed using the published structure of the RNA hairpin, size, and orientation of its diffusion tensor [49]. The simulation showed (Table S1 in Supplementary material) that the bias of the $J(0)$ and $J(\omega_{0,f})$ values due to the tumbling anisotropy is minor for the studied relatively small and rigid molecule, whose global motion can be described [49] by an axially symmetric diffusion tensor with $D_{\parallel}/D_{\perp} = 1.35$ and by an average correlation time of 2.85 ns (recalculated for

tumbling in $^2\text{H}_2\text{O}$). The deviation of the $J(0)$ and $J(\omega_{0,2})$ values calculated by the LTN3 and LNX2 protocols did not exceed 0.04 ns rad^{-1} and 0.15 ns rad^{-1} for C5 and A4, respectively (residues with the orientation of the studied C-H bond most sensitive to the motional anisotropy).

Second, ^{13}C R_1 , R_2 , Γ_2 , and steady-state ^{13}C - ^1H NOE were measured on a uniformly ^{13}C , ^{15}N -labeled sample as described in Methods (Section 2.1). This relatively cheap labeling scheme introduces further complications because the measured ^{13}C relaxation is influenced by ^{13}C and/or ^{15}N nuclei directly bonded to the observed ^{13}C 6/8 nucleus. The resulting contributions of spectral density values at the ω_{N} , $\omega_{\text{C}} \pm \omega_{\text{N}}$, $2\omega_{\text{C}}$ frequencies could not be evaluated due to the lack of experimental data. Fortunately, the neglected $J(\omega_{\text{N}})$, $J(\omega_{\text{C}} \pm \omega_{\text{N}})$, and $J(2\omega_{\text{C}})$ contributions are smaller than five $J(\omega)$ values defining relaxation of the ^{13}C 6- ^1H 6 or ^{13}C 8- ^1H 8 pairs due to longer internuclear distances and lower magnetogyric ratios (vide supra). As a consequence, only the high-frequency spectral density values are significantly biased by neglecting $J(\omega_{\text{N}})$, $J(\omega_{\text{C}} \pm \omega_{\text{N}})$, and $J(2\omega_{\text{C}})$ (Fig. S1 in Supplementary material). The remaining contributions of the additional nuclei are accounted for by the modified LTN3, LNX2, and LTX3 protocols, and their systematic error does not exceed 3 %.

Third, the relaxation rates were measured at three spectrometers with a magnetic field ratio 4:5:6 (400 MHz, 500 MHz, and 600 MHz, distinguished by subscripts 1, 2, and 3, respectively, in the following text). Such combination represents an easily accessible set of spectrometers with fields close enough to the optimal ratio 3:4:5. If the high-frequency spectral density values $J(\omega)$ are approximately proportional to ω^{-2} (i.e., if $\omega^2\tau^2 \gg 1$), accuracy of the calculated $J(\omega)$ values is further improved by replacing the high-frequency values with the effective values $J(\varepsilon_f\omega_{1,f})$ and by optimizing ε_f . The optimization of ε_f is analogous to Method 2 proposed by Farrow et al. [36]. Simulations showed (Fig. S2 in Supplementary material) that the suboptimal magnetic field ratio has only a marginal effect on the values of $J(0)$, $J(\omega_{0,2})$, and $J(\omega_{0,3})$ (systematic error less than 4 % in the limit of a single motional mode).

3.2.5. Propagation of random errors

The fact that relaxation rates can be measured at magnetic fields required by Eqs. 21, 23, and 22 makes the complete elimination of the reduction bias possible in practice. However, this ultimate accuracy is achieved at the price of calculating $J(\omega_{0,f})$ and $J(\omega_{1,f})$ by subtracting relaxation rates containing

the $J(0)$ term. Therefore, the relative random error of the determined $J(\omega_{0,f})$ and $J(\omega_{1,f})$ values is significantly larger than the experimental error of the measured relaxation rates if $J(\omega_{n,f}) \ll J(0)$ for $n = 0$ and 1. Evaluation of propagated experimental errors of real data showed that LTN3, LNX2, and LTX3 provided $J(\omega_{0,2})$, $J(\omega_{0,3})$, and $J(\omega_{0,2})$ values, respectively, with an acceptable precision (Fig. 2). On the other hand, $J(\omega_{0,1})$ from the LNX2 analysis and $J(\omega_{0,2})$ from the LNX3 extension of the protocol (Equation B.4) was considerably less precise, and errors of the $J(\omega_{1,f})$ values were very high (data not shown). It documents that the spectral density mapping at multiple fields should be limited to the zero- and low-frequency values in uniformly labeled molecules because both accuracy and precision of the high-frequency values are poor.

3.2.6. Interpretation of the spectral density values

Results of the spectral density mapping are displayed in a form of $J(0)$ vs. $J(\omega_{0,f})$ plots in this paper (Fig. 2). Such presentation of data depends only on the knowledge of shielding tensors and local geometry, and on errors of measurement and systematic errors of spectral density mapping, discussed above; it is not influenced by any assumption of the nature and independence of molecular motions. Nevertheless, it is instructive to consider certain limit cases before describing the general interpretation of the results.

In the limit of rigid-body isotropic tumbling, values of $J(0)$, $J(\omega_{0,f})$ of all residues are represented by a single point at the solid curve in Fig. 2 (blue circle in Fig. 2d). Position of the point is given by the correlation time of the tumbling, τ_0 .

In the limit of the simple Lipari-Szabo approach [8, 9], dynamics is influenced by an additional local motion, described by a mono-exponential correlation function independent of the tumbling. Values of $J(0)$, $J(\omega_{0,f})$ of individual residues (white circle in Fig. 2d) are then weighted averages of values represented by the blue and red circles in Fig. 2d. The weights of the overall tumbling and of the local motion are equal to the order parameter S^2 and to $1 - S^2$, respectively. Position of the red circle is given by the effective correlation time τ_1 (with the correlation time of the local motion being $(\tau_1^{-1} - \tau_0^{-1})^{-1}$). The values of S^2 in different regions of the plot are represented by the background color in Fig. 2d, ranging from dark red for $S^2 = 0$ to blue for $S^2 = 1$ above the white line. Motions slower than the tumbling (conformational or chemical exchange) further shift the white circle in a direction depending on the particular spectral density map-

Figure 2: Apparent $J(0)$ vs. $J(\omega_{0,2})$ values obtained by LTN3 **(a)**, $J(0)$ vs. $J(\omega_{0,3})$ values obtained by LNX2 **(b)**, and $J(0)$ vs. $J(\omega_{0,2})$ values obtained by LTX3 **(c)**, applied to the relaxation data of the UUCG RNA hairpin. The ellipses indicate the experimental errors. Data are labeled with residue numbers. Color-coding of the LTX3 spectral density values is defined in Panel **(d)**. The value of the overall correlation time τ_0 , defining the blue limit, was estimated by fitting the experimental Γ_2/R_1 ratio of residues 4, 5, 10–14 to the ratio given by Eqs. 25 and 14, with $J(\omega)$ approximated by $\tau_0/(1 + (\tau_0\omega)^2)$. The white circle represents the weighted average of the red and blue circles for $S^2 = 0.7$. The green arrow indicates that the actual experimental spectral density values are shifted to the black circle due to an exchange contribution.

ping protocol. The green arrow in Fig. 2d shows the shift for LTX3. In the absence of experimental errors, values below the white line clearly indicate an exchange contribution if the molecule moves isotropically. The size of the exchange term for $\tau_1 = 0$ is indicated by the content of green color in the region below the white line in Fig. 2d.

When the Lipari-Szabo approach is not applicable, S^2 , τ_0 , and τ_1 cannot be determined. However, a straightforward general interpretation of the plotted values is still possible. Values in the blue region above the white line correspond to residues with dynamics dominated by the overall tumbling. Increasing contribution of fast local motions is manifested by shifts of the data towards the dark red boundary. Such shifts are identical in all protocols. On the other hand, shifts in directions depending on the type of analysis carry information about the exchange on a slow time scale. Shift of values provided by the LTX3 analysis below the white line in Fig. 2d indicate conformational or chemical exchange (color-coded by increasing content of green in the background). A quantitative estimation of the exchange contribution can be obtained by comparing $J(0)$ values from the LTN3 and LNX2 protocols, which differ by a factor of $(6\xi_3 - \xi_1)/(24b_3 - 4b_1)$ (cf. Eqs. B.1 and B.3).

3.2.7. Experimental results

The $J(0)$, $J(\omega_{0,2})$ values obtained from the LTN3 analysis are plotted in Fig. 2a. The exchange-free $J(0)$, $J(\omega_{0,3})$ values calculated by LNX2 are presented in Fig. 2b. The $J(0)$, $J(\omega_{0,2})$ values provided by the LTX3 analysis are displayed in Fig. 2c. The less precise and inaccurate spectral density values are not presented.

The displayed spectral density values were determined with a good precision, with the exception of G1, G2, and G9. Signal-to-noise ratio of peaks of the mentioned residues was very low in the spectra, resulting in a large experimental error of the obtained values. Spectral density values of the remaining residues were interpreted as described in the preceding section.

The following picture of the hairpin dynamics was obtained. Bases of the loop residues U7 and C8 exhibit higher flexibility on the ns-ps time scale and large exchange contributions on the μ s-ms time scale, with the fast motions more pronounced at U7 and slow motions more pronounced at C8. Identification of the strong exchange in C8 was based solely on the LTX3 analysis because Γ_2 was not measurable for $^{13}\text{C}6$ of C8 at $B_{0,3}$ due to a peak overlap in the coupled spectrum. Low signal intensities reflected

by large experimental errors do not allow for reliable interpretation of $J(\omega)$ values for another loop base, G9. Nevertheless, the observed line broadening serves as an indirect evidence that G9 is also influenced by motions on a slow time scale. The same applies to the terminal base of G1. The $J(\omega)$ values and line broadening show that G2 is also affected by some sort of a slow exchange. The remaining stem residues gave $J(\omega)$ values close to the limit of overall tumbling. Nevertheless, the comparison of $J(0)$ values provided by LTN3 (Fig. 2a) and LNX2 (Fig. 2b) reveals that the stem residues also exhibit signs of a slow exchange. These small exchange contributions are also visible in the LTX3 plots (Fig. 2c). A structure of the RNA hairpin colored according to Fig. 2d is presented in Fig. 3.

In principle, the effects interpreted as exchange contribution cannot be distinguished from the error due to neglecting anisotropy of the molecular motions. However, comparison of the differences observed in Fig. 2 with simulations presented in Table S1 in Supplementary material showed that the influence of the anisotropic motions is smaller than the shifts observed in the plots. Therefore, most of the differences between $J(0)$ plotted in Fig. 2a and 2b and shifts in Fig. 2c can be attributed to the exchange contribution. However, it should be emphasized that rotational diffusion of the studied molecule only moderately deviates from isotropic tumbling. Higher anisotropy of the molecular motions increases the error introduced by combining the (in principle different) $J^{n,n'}(\omega)$ spectral density functions. In the case of molecules undergoing highly anisotropic rotational diffusion, LNX2 and LTX3 approaches are not applicable. Spectral density mapping of such a molecule is discussed in the next section.

3.3. Case study II: β -D-Glcp-(1 $\rightarrow$ 6)- α -D-Manp-OMe

In order to examine effects of the large motional anisotropy and of short timescales of the motions, a strongly anisotropically tumbling disaccharide was investigated as the second test molecule. Thanks to the selective labeling, the studied ^1H - ^{13}C - ^1H group represents an isolated AMX spin system. Presence of a single ^{13}C nucleus in the studied molecule allowed us to apply fast one-dimensional ^{13}C -detected experiments and to collect large amount of experimental data in a short time.

R_1 , R_2 , Ξ_1 , Ξ_2 , and NOE values for the studied molecule were already reported by Zerbetto et al. [23] at 400 MHz, 600 MHz, and 900 MHz. However, this combination of magnetic fields is not well suited for the multiple-field spectral density mapping protocols tested in this paper. Therefore, we

Figure 3: Stereoview of the RNA hairpin (PDB structure 1KOC) with carbon atoms whose relaxation data were analyzed highlighted by spheres colored according to Fig. 2d. Slow exchange that was obvious from line broadening but could not be described quantitatively due to a high experimental error is indicated by the green color.

started our study by measuring a new set of relaxation data, namely R_1 , R_2 , Γ_1 , Γ_2 , Ξ_1 , Ξ_2 , and steady-state NOE at seven magnetic fields. The ^{13}C and ^1H frequencies at various magnetic fields are referred to as $\omega_{0,f}$ and $\omega_{1,f}$, respectively, in this paper, with $f = 1, 2, 3, 4, 5, 6, 7$ for data acquired at 300 MHz, 400 MHz, 500 MHz, 600 MHz, 700 MHz, 850 MHz, and 950 MHz spectrometers, respectively. The obtained relaxation rates, listed in Table S2 in Supplementary material, are in a good agreement with the previously published values [23]. The measured ^{13}C relaxation rates are dominated by dipole-dipole interactions with two attached protons, while the CSA contribution is relatively small [34]. The shape of the disaccharide indicates that its motions are likely to be largely anisotropic, as confirmed in previous studies [23, 53, 54]. Relaxation rates of such a molecule cannot be described by the same spectral density function. Therefore, the auto- and cross-correlated relaxation rates were analyzed separately.

3.3.1. Cross-correlated spectral density mapping

Quantities $J^\Xi(\omega)$ and $J^\Gamma(\omega)$, proportional to the spectral density function $J^{1,2}(\omega)$ and to the combination $(J^{0,1}(\omega) + J^{0,2}(\omega))/2$, respectively, were calculated from the cross-correlated relaxation rates separately for each magnetic field as

$$J^\Gamma(0) = \frac{3\Gamma_1(B_{0,f}) - 6\Gamma_2(B_{0,f})}{32c_0d_1B_{0,f}}, \quad (34)$$

Table 1: Values of spectral density functions, expressed in units of ps/rad, calculated from cross-correlated relaxation data of β -D-Glcp-(1 $\rightarrow$ 6)- α -D-Manp-OMe for ideal tetrahedral geometry and axial CSA tensor with the main axis coinciding with the C6–O bond. Errors of the last digits, expressed as standard deviations, are given in the parentheses.

$B_{0,f}/\text{T}$	$J^\Gamma(0)$	$J^\Gamma(\omega_{0,f})$	$J^\Xi(0)$	$J^\Xi(\omega_{0,f})$
7.05	932(286)	813(145)	69(79)	187(33)
9.39	809(258)	695(090)	119(51)	193(19)
11.74	914(100)	602(029)	140(25)	166(09)
14.09	977(060)	565(027)	140(20)	178(09)
16.44	907(097)	532(046)	73(49)	207(14)
19.97	859(028)	461(010)	112(14)	204(05)
22.31	922(028)	412(006)	119(15)	209(06)

$$J^\Gamma(\omega_{0,f}) = -\frac{\Gamma_1(B_{0,f})}{8c_0d_1B_{0,f}}, \quad (35)$$

$$J^\Xi(0) = \frac{3\Xi_1(B_{0,f}) - 6\Xi_2(B_{0,f})}{16d_1^2}, \quad (36)$$

$$J^\Xi(\omega_{0,f}) = -\frac{\Xi_1(B_{0,f})}{4d_1^2}. \quad (37)$$

The coefficients in Eqs. 34–37 correspond to ideal tetrahedral geometry and to axial CSA tensor with the main axis coinciding with the C6–O bond [34], and $J^\Xi(\omega)$ and $J^\Gamma(\omega)$ are normalized to approach $J(\omega)$ in the limit of isotropic motions.

Since the calculations did not require combination of data acquired at several spectrometers with given magnetic field ratios, data probing the ^{13}C frequency in a broad range (75–238 MHz) was obtained. The results are presented in Table 1 and Figure 4. Precision of the measurement strongly depended on the sensitivity of the spectrometer. The experimental error was relatively high at low magnetic fields, while highly precise spectral density values were calculated from the high-field data. The number of magnetic fields employed allowed us to monitor the frequency dependence of $J^\Gamma(\omega)$ and $J^\Xi(\omega)$ in detail. Large differences between $J^\Gamma(\omega)$ and $J^\Xi(\omega)$ values and their dependence on the frequency clearly indicated that the molecular motions are highly anisotropic (vide infra).

3.3.2. Auto-correlated spectral density values

LTN3 represents the simplest protocol providing spectral density values free of the reduction bias if a slow exchange does not affect the relaxation and if the CSA contribution is negligible ($b_f = 1$ for any f). Therefore, relaxation rates acquired at the lowest magnetic fields (300 MHz, 400 MHz, and 500 MHz in our study) are best suited for such an analysis. Also, the ratio of magnetic fields required by the LTN3 protocol is fulfilled almost exactly for relaxation rates measured at 300 MHz, 400 MHz, and 500 MHz ($f = 1, 2, 3$, respectively). In the absence of the CSA contribution ($\Delta_0 = 0$), relaxation of an anisotropically moving molecule can be described in terms of values of the combination $J^\Delta(\omega) = (J^{1,1}(\omega) + J^{2,2}(\omega))/2$. The definition of $J^\Delta(\omega)$ was chosen so that the $J^\Delta(\omega)$ values approach values $J(\omega)$ of Eqs. 11–15 in the limit of isotropic motion. The $J^\Delta(\omega)$ values are provided by Eqs. 17–20 with the $J(\omega)$ terms replaced by $J^\Delta(\omega)$. $R_{1\rho}$ relaxation dispersion at 600 MHz did not reveal a significant exchange contribution ($R_{\text{ex}}(B_{0,4}) < 0.1 \text{ s}^{-1}$, data not shown). Therefore, the exchange terms were neglected in the analysis. Results of the analysis are listed in Tables 2 and 3 (line 1).

Another combination of magnetic fields in a ratio close to the requirements of LTN3 is provided by the 500 MHz, 700 MHz, and 850 MHz spectrometers ($f = 3, 5, 6$, respectively). Analysis of the rates evaluated at the mentioned magnetic fields (line 2 in Table 2) provided $J^\Delta(0)$ only slightly higher than $J^\Delta(0)$ calculated from rates at measured at 300 MHz, 400 MHz, and 500 MHz (line 1 in Table 2). In addition, $J^\Delta(\omega_{1,3})$ values, determined for both sets, were very similar. It shows that the effect of the CSA contribution is small even at the higher fields.

In presence of the CSA contribution, $J^\Delta(0)$ and $J^\Delta(\omega_{0,f})$ are mixed with $J^{0,0}(0)$ and $J^{0,0}(\omega_{0,f})$, respectively. Therefore, $J(0)$ and $J(\omega_{0,f})$ in Eqs. 11 and 12 must be replaced by their combination, defined as

$$J_f^\Sigma(\omega) = \frac{1}{b_f} \left(\frac{c_0^2 B_{0,f}^2}{2d_1^2} J^{0,0}(\omega) + J^\Delta(\omega) \right). \quad (38)$$

Similarly to $J^\Delta(\omega)$, the values of $J^\Sigma(\omega)$ converge to the isotropic $J(\omega)$ values if the anisotropy of molecular motions is negligible. The fact that the zero-frequency term $J_f^\Sigma(0)$ depends on the magnetic field $B_{0,f}$ prohibits to apply the LTN3 protocol to the data obtained at three magnetic fields in a rigorous manner. Considering the low value of Δ_0 for the studied molecule, one possible approach is to ignore the inequality $J_f^\Sigma(\omega) \neq J^\Delta(\omega)$ at the zero

Table 2: Values of spectral density functions at zero frequency, expressed in units of ps/rad, calculated from auto-correlated relaxation data of β -D-Glcp-(1 $\rightarrow$ 6)- α -D-Manp-OMe using various LTN protocols. Errors of the last digits, expressed as standard deviations, are given in the parentheses.

	Protocol	$J^{0,0}(0)$	$J^\Delta(0)$	$J_f^\Sigma(0)$	
				$f=2$	$f=5$
1	LTN3 ^{a,b}		520(18)	520(18)	
2	LTN3 ^{b,c}		536(16)		536(16)
3	LTN3 ^{a,d}	505(18)	505(18)	505(18)	
4	LTN3 ^{c,d}	493(15)	493(15)		493(15)
5	LTN5 ^e	276(459)	512(31)	508(23)	500(11)
6	LTN5 ^f	215(150)	511(11)	506(09)	505(08)
7	LTN4 ^g	407(1934)	508(73)	507(53)	
8	LTN1 ^{h,i}			636(17)	
9	LTN1 ^{i,j}				527(16)
10	LTN1 ^{h,k}			570(26)	
11	LTN1 ^{j,k}				496(26)

^a using data from 300 MHz, 400 MHz, and 500 MHz spectrometers

^b for $\Delta_0 = 0$.

^c using data from 500 MHz, 700 MHz, and 850 MHz spectrometers

^d $J^{0,0}(0)$ and $J^\Delta(0)$ are assumed to be identical

^e using data from 300 MHz, 400 MHz, 500 MHz, 700 MHz, and 850 MHz spectrometers

^f using interpolations of data from all spectrometers

^g using data from 300 MHz, 400 MHz, 500 MHz, and 700 MHz spectrometers

^h using data from 400 MHz spectrometer

ⁱ $J^\Sigma(0) = (5\delta_f - 6\sigma_f)/40b_f$ and $J_f^\Sigma(\omega_{0,f}) = (5\rho_f - 7\sigma_f)/30b_f$

^j using data from 700 MHz spectrometer

^k $J^\Sigma(0) = (5\delta_f - 6\sigma_f)/40b_f + 21s\omega_{0,f}/10b_f$ and $J_f^\Sigma(\omega_{0,f}) = (5\rho_f - 7\sigma_f)/30b_f + 8s\omega_{0,f}/5b_f$, where $s = 0.1(\sigma_{f+1} - \sigma_f)/(\omega_{1,f+1} - \omega_{1,f})$

Table 3: Selected values of spectral density functions at ^{13}C and ^1H frequencies, expressed in units of ps/rad, calculated from auto-correlated relaxation data of $\beta\text{-D-Glcp-(1}\rightarrow\text{6)-}\alpha\text{-D-Manp-OMe}$ using various LTN protocols. Errors of the last digits, expressed as standard deviations, are given in the parentheses.

	Protocol	$J_f^\Sigma(\omega_{0,f})$		$J^\Delta(\omega_{1,f})$				
		$f=2$	$f=5$	$f=1$	$f=2$	$f=3$	$f=5$	$f=6$
1	LTN3 ^{a,b}	425(20)		228(26)	189(21)	124(15)		
2	LTN3 ^{b,c}		374(11)			114(14)	53(21)	53(14)
3	LTN3 ^{a,d}	413(20)		235(26)	194(21)	126(15)		
4	LTN3 ^{c,d}		343(10)			134(14)	65(21)	56(14)
5	LTN5 ^e	416(37)	348(24)	232(29)	191(23)	125(16)	59(20)	55(12)
6	LTN5 ^f	418(07)	350(01)	239(10)		128(02)		55(01)
7	LTN4 ^g	415(46)		233(43)	192(32)	125(18)		
8	LTN1 ^{h,i}	499(03)						
9	LTN1 ^{i,j}		402(03)					
10	LTN1 ^{h,k}	449(02)						
11	LTN1 ^{j,k}		379(04)					

^a using data from 300 MHz, 400 MHz, and 500 MHz spectrometers

^b for $\Delta_0 = 0$.

^c using data from 500 MHz, 700 MHz, and 850 MHz spectrometers

^d $J^{0,0}(0)$ and $J^\Delta(0)$ are assumed to be identical

^e using data from 300 MHz, 400 MHz, 500 MHz, 700 MHz, and 850 MHz spectrometers

^f using interpolations of data from all spectrometers

^g using data from 300 MHz, 400 MHz, 500 MHz, and 700 MHz spectrometers

^h using data from 400 MHz spectrometer

ⁱ $J^\Sigma(0) = (5\delta_f - 6\sigma_f)/40b_f$ and $J_f^\Sigma(\omega_{0,f}) = (5\rho_f - 7\sigma_f)/30b_f$

^j using data from 700 MHz spectrometer

^k $J^\Sigma(0) = (5\delta_f - 6\sigma_f)/40b_f + 21s\omega_{0,f}/10b_f$ and $J_f^\Sigma(\omega_{0,f}) = (5\rho_f - 7\sigma_f)/30b_f + 8s\omega_{0,f}/5b_f$, where $s = 0.1(\sigma_{f+1} - \sigma_f)/(\omega_{1,f+1} - \omega_{1,f})$

frequency and to apply the LTN3 approach to rates measured at three fields in spite of the motional anisotropy (lines 3 and 4 in Tables 2 and 3). In such a case, $J^\Delta(0)$ is calculated with a systematic error, but the spectral density values at other frequencies are much less influenced.

A more rigorous approach is to treat $J^{0,0}(0)$ and $J^\Delta(0)$ separately (Eqs. 39, 40–42). In such a case, combinations described by Equation 17 must be calculated for *two sets* of three magnetic fields $B_{0,1}$, $B_{0,2}$, $B_{0,3}$ and $B_{0,4}$, $B_{0,5}$, $B_{0,6}$ (protocol LTN6), both in the ratio $(\gamma_1 - \gamma_0) : 1 : (\gamma_1 + \gamma_0)$ in order to separate $J^{0,0}(0)$ and $J^\Delta(0)$.

$$J^{0,0}(0) = \frac{6\delta_6 - 6\sigma_5 - \delta_4 - 6\delta_3 + 6\sigma_2 + \delta_1}{8(6b_6 - b_4 - 6b_3 + b_1)}, \quad (39)$$

$$J^\Delta(0) = \frac{(6b_6 - b_4 - 5)(6\delta_3 - 6\sigma_2 - \delta_1) - (6b_3 - b_1 - 5)(6\delta_6 - 6\sigma_5 - \delta_4)}{40(6b_6 - b_4 - 6b_3 + b_1)}, \quad (40)$$

$$J_f^\Sigma(\omega_{0,f}) = \frac{6\rho_f - \delta_{f-1} - 6\delta_{f+1} + 8(6b_{f+1} + b_{f-1} - 7)J^{0,0}(0) + 56J^\Delta(0)}{36b_f}, \quad (41)$$

where f is 2 or 5. Note that the low-frequency values are obtained only as combinations $J_f^\Sigma(\omega_{0,f})$ which cannot be further separated.

The high frequency values can be calculated as

$$J^\Delta(\omega_{1,f}) = \frac{\delta_f}{12} - \frac{2}{3}((b_f - 1)J^{0,0}(0) + J^\Delta(0)). \quad (42)$$

In theory, the number of measurements can be reduced if $B_{0,3}$ is equal to $B_{0,4}$ or to $B_{0,5}$ (protocol LTN5). However, spectrometers operating at such magnetic fields are not easily available. A simpler alternative is to measure relaxation data at four magnetic fields (protocol LTN4) chosen so that the ratios $B_{0,1} : B_{0,2} : B_{0,3}$ and $B_{0,2} : B_{0,3} : B_{0,4}$ are close to $(\gamma_1 - \gamma_0) : 1 : (\gamma_1 + \gamma_0)$ (note that this requirement cannot be fulfilled exactly). Spectral density values can be then obtained from equations 39–42 with the subscripts 4, 5, 6 replaced by 2, 3, 4, respectively.

In this study, relaxation rates were measured at five magnetic fields in a ratio very close to that required by the LTN5 protocol (at 300 MHz, 400 MHz, 500 MHz, 700 MHz, and 850 MHz). It allowed us to test the LTN5 protocol without introducing substantial systematic errors. The obtained spectral

density values are presented in Tables 2 and 3 (line 5). Comparison of the LTN3 and LTN5 analyses showed a good agreement for lower magnetic fields, when the CSA contribution is negligible (lines 1 and 2 in Tables 2 and 3).

An alternative approach to the analysis of relaxation rates at given magnetic fields was also examined, taking advantage of the large number of magnetic fields used. The R_1 , R_2 , and $I_{ss}/I_{ref} - 1$ values measured at various fields were fitted to arbitrary functions $F_i(B_0) = (\alpha_{1,i} + \alpha_{2,i}B_0^2)/(1 + \alpha_{3,i}B_0^2)$, where $\alpha_{j,i}$ are the fitted parameters and subscripts i distinguish R_1 , R_2 , and $I_{ss}/I_{ref} - 1$ (note that F_i has the same form as equations defining the relaxation rates with the spectral density functions expressed as series of Lorentzian functions and with the terms higher than B_0^2 neglected). The functions F_i allowed us to interpolate the relaxation data to any value of B_0 . Therefore, we were able to perform the LTN5 mapping for magnetic fields in the exact ratios regardless of the commercial availability of the magnets. Moreover, correct interpolation should result in the following linear relationship

$$Y = J^\Delta(0) + J^{0,0}(0)X, \quad (43)$$

where

$$X = c_0^2(6B_+^2 - B_-^2)/10d_1^2, \quad (44)$$

$$Y = (12R_2(B_+) - 6R_1(B_+) - 2R_2(B_-) + R_1(B_-) - 6R_0(B_0)) / 80d_1^2, \quad (45)$$

$$R_0(B_0) = \left(\frac{I_{ss}(B_0)}{I_{ref}(B_0)} - 1 \right) \frac{\gamma_0}{\gamma_1} R_1(B_0), \quad (46)$$

and

$$B_\pm = (1 \pm \gamma_0/\gamma_1)B_0. \quad (47)$$

Equation 43 provides not only estimates of $J^\Delta(0)$ and $J^{0,0}(0)$, but also a check of the internal consistency of the relaxation data and of the model used.

The LTN5 analysis of interpolated relaxation data was performed for frequencies probed by the experimentally determined relaxation rates. The

bootstrap re-sampling [55] of peak intensities was employed to estimate variance of determination of the spectral density values. In order to narrow the estimation of $J^{0,0}(0)$, individual values of the distribution obtained by the bootstrap re-sampling were weighted by the relative error of the slope of the linear fit described by Equation 43 when the mean value and its standard deviation was calculated. The complete list of obtained values is presented in Tables 2 and 3 (line 6, only values calculated for magnetic fields within the range used for fitting are listed). The calculated $J^\Delta(0)$, $J_2^\Sigma(\omega_{0,2})$, $J_5^\Sigma(\omega_{0,5})$, $J^\Delta(\omega_{1,1})$, $J^\Delta(\omega_{1,3})$, and $J^\Delta(\omega_{1,6})$ values were very well defined and close to the results of the LTN5 analysis applied directly to experimental data (line 5 in Table 3). Only the standard deviation of $J^{0,0}(0)$ was high, reflecting low contribution of $J^{0,0}(\omega)$ to the relaxation due to the low Δ_0 of the studied molecule. It should be stressed that the high uncertainty of determination of $J^{0,0}(0)$ has a little effect on precision of evaluation of $J_f^\Sigma(0)$. The values of $J_2^\Sigma(0) = (0.506 \pm 0.009)$ ns/rad and $J_5^\Sigma(0) = (0.505 \pm 0.008)$ ns/rad obtained from the interpolated relaxation rates were close to the values calculated from the experimental data $((0.508 \pm 0.023)$ ns/rad and (0.500 ± 0.011) ns/rad, respectively).

The results of the LTN5 analyses were compared to approximative approaches as well. The LTN4 approach was applied to data acquired at 300 MHz, 400 MHz, 500 MHz, and 600 MHz spectrometers. Somewhat lower $J_2^\Sigma(0)$ and $J_2^\Sigma(\omega_{0,2})$ were obtained (line 7 in Table 3) and the decomposition of $J_2^\Sigma(0)$ into $J^{0,0}(0)$ and $J^\Delta(0)$ failed (line 7 in Table 2). Finally, the single-field reduced spectral density mapping (LTN1) was performed for each magnetic field separately. The reduction bias of the LTN1 procedure resulted in large deviations of the obtained spectral density values from the results of the LTN5 protocols (lines 8 and 9 in Tables 2 and 3). Deviations of $J_f^\Sigma(0)$ and $J_f^\Sigma(\omega_{0,f})$ were partially suppressed by applying a correction based on approximating $J^\Delta(\omega_{1,f})$ with a linear function, the slope of which was calculated from NOE data obtained at two magnetic fields [36, 35] (lines 10 and 11 in Tables 2 and 3).

3.3.3. Comparison of auto- and cross-correlated spectral density values

The broad range of magnetic fields used in this study allowed us to map spectral density functions for angular frequencies from 0 to 6 rad/ns (Figure 4). Comparison of various types of the spectral density functions ($J^{0,0}(\omega)$, $J^\Delta(\omega)$, $J^\Gamma(\omega)$, and $J^\Xi(\omega)$) clearly show that different interactions cannot be described by a single spectral density function. It documents that isotropic

Figure 4: Values of $J^{0,0}(\omega)$ (black), $J^{\Delta}(\omega)$ (blue), $J_f^{\Sigma}(\omega)$ (cyan), $J^{\Gamma}(\omega)$ (green), and $J^{\Xi}(\omega)$ (red) calculated from relaxation rates of β -D-Glcp-(1 $\rightarrow$ 6)- α -D-Manp-OMe. The cross-correlated values were calculated using Eqs. 34–37 for each field separately. The $J^{\Delta}(\omega)$ and $J_f^{\Sigma}(\omega)$ values were obtained by applying the LTN5 protocol to R_1 , R_2 , and I_{ss}/I_{ref} measured at 300 MHz, 400 MHz, 500 MHz, 700 MHz, and 850 MHz. The plotted $J^{0,0}(0)$ value is an average of values calculated from interpolated R_1 , R_2 , and I_{ss}/I_{ref} measured at all magnetic fields, weighted by the relative error of the slope of the fit described by Equation 44.

spectral density mapping or model-free analysis cannot be used to describe highly anisotropic motions of the molecule.

The differences in the spectral density functions are most evident at low frequencies. It can be interpreted easily if the spectral density functions are decomposed into series of Lorentzian functions

$$J^j(\omega) = \sum_i a_i^j \tau_i / (1 + (\omega \tau_i)^2), \quad \sum_i a_i^j = 1. \quad (48)$$

For $\omega \ll \tau^{-1}$, the series in Equation 48 reduces to the weighted average of correlation times $J^j(\omega) \approx \sum_i a_i^j \tau_i$, dominated by the longest correlation times, describing the overall rotational diffusion. Their weights a_i^j depend on (very different) orientations of the interaction tensors (directions of ^{13}C - ^1H

bonds and of the axis of the ^{13}C CSA tensor) with respect to the diffusion tensor [56, 57]. On the other hand, the shortest correlation times of fast internal motions dominate for $\omega \gg \tau^{-1}$ since $J^j(\omega) \approx \sum_i a_i^j \omega^{-2} \tau_i^{-1}$. It explains why the differences in spectral density values, reflecting anisotropy of the overall tumbling, decrease as the frequency and contribution of short correlation times increases.

Finally, we compared $J(\omega)$ values determined in this study to the values obtained in the course of the analysis published by Zerbetto et al. [23]. The latter values were retrieved by using the program C++OPPS [23] with the parameters reported by Zerbetto et al. C++OPPS provided $J^\Delta(\omega)$ values higher by approximately 10 % than those calculated by the LTN3 and LTN5 protocols (lines 3, 5, and 6 in Table 3). The $J^\Xi(\omega)$ values did not differ significantly. On the other hand, the C++OPPS analysis resulted in significantly lower $J^\Gamma(\omega)$ values (0.51 and 0.30 ns/rad at 0 and 1.5 rad/ns, respectively) than those obtained this study. The difference may reflect the fact that Γ_1 and Γ_2 were not measured and the CSA contributions were neglected in the previous study [23].

4. Conclusion

Various combinations of ^{13}C relaxation rates examined in this study offer a possibility to calculate spectral density values without the reduction bias and to separate exchange contributions from fast dynamics. The described protocols rely on combining experimental data acquired at several magnetic fields, whose ratios match the magnetogyric constants of ^{13}C and ^1H . In an ideal case of ^{13}C - ^1H spin systems in isotropically moving molecules, exact $J(\omega)$ values can be obtained. Two case studies presented in this article document that the proposed spectral density mapping protocols are applicable to real samples.

Approaches combining auto-correlated and cross-correlated relaxation rates are applicable if rotational diffusion is close to isotropic. The uniformly ^{13}C , ^{15}N labeled RNA hairpin, investigated in the first case study, represents an example of such a molecule. Three different spectral density mapping protocols (LTN3, LNX2, LTX3) were tested. The results showed that values of spectral density functions at zero and ^{13}C frequencies can be obtained with a good accuracy and precision even under non-ideal circumstances (suboptimal magnetic field ratios, additional spin-1/2 nuclei). Precision of the LTX3 analysis was sufficient to reveal fast and slow internal motions in a single plot.

However, the analysis should be strictly limited to zero- and low-frequency $J(\omega)$ values, unless a selective labeling is applied.

The case study of a selectively ^{13}C labeled disaccharide documented that multiple magnetic fields make reliable spectral density mapping possible even if highly anisotropic motions greatly increase the number of unknown parameters. In such cases, combination of auto-correlated and cross-correlated relaxation rates introduces a large systematic error. Therefore, auto- and cross-correlated data were analyzed separately. Analysis of auto-correlated relaxation rates requires data measured at more than three magnetic fields if the CSA contribution is significant. Two variants of the LTN5 protocol were tested. The first variant was a direct mapping of the experimental data acquired at five magnetic fields, the second one used interpolations of rates measured at seven magnetic fields. Both variants provided identical values of spectral density functions. Interpolation reduced the standard deviation of the calculated values, which is critical for estimation of the very weak contribution of the chemical shift anisotropy ($J^{0,0}(0)$). Analysis of the cross-correlated relaxation rates confirmed that individual spectral density functions differ dramatically and that the LNX2 and LTX3 protocols were not applicable.

Acknowledgements

This work was supported by the projects "CEITEC - Central European Institute of Technology" (grant CZ.1.05/1.1.00/02.0068) from European Regional Development Fund (P. P., R. F., and V. S.), by project INBIOR (grant number CZ.1.07/2.3.00/20.0042) from the European Social Fund and the state budget of the Czech Republic (L. Ž.), by project "Employment of Newly Graduated Doctors of Science for Scientific Excellence" (grant number CZ.1.07/2.3.00/30.0009) from the European Social Fund and the state budget of the Czech Republic (P. S.), and by the Swedish Research Council (J. K. and G. W.). The access to the 400 MHz spectrometer was supported by the project LO1509 of the Ministry of Education, Youth and Sports of the Czech Republic and by Operational Program Prague – Competitiveness project (CZ.2.16/3.1.00/24023) supported by EU). The authors thank Zdeněk Moravec of Masaryk University for a technical help, Mirco Zerbetto of University of Padova for providing the C++OPPS program and assistance with the analysis, and Dinshaw J. Patel of Memorial Sloan-Kettering Cancer Center for permission to use relaxation data measured in his laboratory.

Appendix A. Relaxation rates

Values of the relaxation rates determined at magnetic fields $B_{0,f}$ are given by the following sums of contributions of all relevant interaction mechanisms [1, 2, 3, 4]:

$$\begin{aligned}
 R_1(B_{0,f}) &= 6c_0^2 B_{0,f}^2 J^{0,0}(\omega_{0,f}) \\
 &+ \sum_{n=1}^N d_n^2 (6J^{n,n}(\omega_{0,f}) + 12J^{n,n}(\omega_{n,f} + \omega_{0,f}) + 2J^{n,n}(\omega_{n,f} - \omega_{0,f})),
 \end{aligned} \tag{A.1}$$

$$\begin{aligned}
 R_2(B_{0,f}) &= R_{\text{ex}}(B_{0,f}) + c_0^2 B_{0,f}^2 (4J^{0,0}(0) + 3J^{0,0}(\omega_{0,f})) \\
 &+ \sum_{n=1}^N d_n^2 (4J^{n,n}(0) + 3J^{n,n}(\omega_{0,f})) \\
 &+ \sum_{n=1}^N d_n^2 (6J^{n,n}(\omega_{n,f}) + 6J^{n,n}(\omega_{n,f} + \omega_{0,f}) + J^{n,n}(\omega_{n,f} - \omega_{0,f})),
 \end{aligned} \tag{A.2}$$

$$\frac{I_{\text{ss}}(B_{0,f})}{I_{\text{ref}}(B_{0,f})} = 1 + \frac{1}{R_1(B_{0,f})} \sum_{n \in \text{S}} \frac{\gamma_n}{\gamma_0} d_n^2 (12J^{n,n}(\omega_{n,f} + \omega_{0,f}) - 2J^{n,n}(\omega_{n,f} - \omega_{0,f})), \tag{A.3}$$

$$\Gamma_1(B_{0,f}) = \sum_{n \in \text{C}} 6c_0 d_n B_{0,f} (J^{0,n}(\omega_{0,f}) + J^{n,0}(\omega_{0,f})), \tag{A.4}$$

$$\Xi_1(B_{0,f}) = \sum_{n \in \text{C}} \sum_{n' \in \text{C}} (1 - \delta_{n,n'}) 6d_n d_{n'} J^{n,n'}(\omega_{0,f}), \tag{A.5}$$

$$\Gamma_2(B_{0,f}) = \sum_{n \in \text{C}} c_0 d_n B_{0,f} (4J^{0,n}(0) + 4J^{n,0}(0) + 3J^{0,n}(\omega_{0,f}) + 3J^{n,0}(\omega_{0,f})), \tag{A.6}$$

$$\Xi_2(B_{0,f}) = \sum_{n \in \text{C}} \sum_{n' \in \text{C}} (1 - \delta_{n,n'}) d_n d_{n'} (4J^{n,n'}(0) + 3J^{n,n'}(\omega_{0,f})), \tag{A.7}$$

where $\delta_{n,n'}$ is the Kronecker's delta.

The cross-relaxation rate of the operator $\hat{I}_{0,z}$ was calculated from the steady-state heteronuclear Overhauser enhancement due to saturation of all protons, assuming that cross-relaxation rates of operators $\hat{I}_{n,z}$ are much lower.

Note that Equation A.3 is valid only for magnetically nonequivalent protons, examined in this study. Treatment of magnetically equivalent AX_2 and AX_3 systems is described elsewhere [56].

Appendix B. Basic LTN3, LNX2, and LTX3 protocols

The LTN3, LNX2, and LTX3 protocols of spectral density mapping for nuclei influenced only by interactions with protons in isotropically moving molecules (or of isolated spin-pairs with axially symmetric CSA tensors aligned along the internuclear vector) are defined as follows.

Protocol LTN3:

$$\begin{aligned}
& \begin{bmatrix} J(0) \\ J(\omega_{0,2}) \\ J(\omega_{1,1}) \\ J(\omega_{1,3}) \end{bmatrix} + \frac{1}{12(6b_3 - b_1)} \left(\begin{bmatrix} 3(6\xi_3 - \xi_1) \\ 8(b_1\xi_3 - b_3\xi_1)/b_2 \\ -12(b_1\xi_3 - b_3\xi_1) \\ -2(b_1\xi_3 - b_3\xi_1) \end{bmatrix} + \begin{bmatrix} 18(6\psi_3 - \psi_1) \\ 48(b_1\psi_3 - b_3\psi_1)/b_2 \\ -12b_1(6\psi_3 - \psi_1) \\ -12b_3(6\psi_3 - \psi_1) \end{bmatrix} \right) \\
&= \frac{1}{24(6b_3 - b_1)} \begin{bmatrix} -3 & 18 & 0 & -18 \\ -8b_3/b_2 & 8b_1/b_2 & 4(6b_3 - b_1)/b_2 & -4(6b_3 + b_1)/b_2 \\ 12b_3 & -12b_1 & 0 & 12b_1 \\ 2b_3 & -2b_1 & 0 & 12b_3 \end{bmatrix} \begin{bmatrix} \delta_1 \\ \delta_3 \\ \rho_2 \\ \sigma_2 \end{bmatrix} \quad (\text{B.1})
\end{aligned}$$

If R_2 is measured at $B_{0,2}$ too, $J(\omega_{1,2})$ can be also calculated:

$$J(\omega_{1,2}) + \frac{(6b_3 - b_1)\xi_2 - b_2(6\xi_3 - \xi_1)}{6(6b_3 - b_1)} - b_2 \frac{6\psi_3 - \psi_1}{6b_3 - b_1} = \frac{6b_2\sigma_2 - b_2(6\delta_3 - \delta_1) + (6b_3 - b_1)\delta_2}{12(6b_3 - b_1)}. \quad (\text{B.2})$$

Protocol LNX2:

$$\begin{bmatrix} J(0) \\ J(\omega_{0,1}) \\ J(\omega_{0,3}) \\ J(\omega_{1,1} - \omega_{0,1}) \\ J(\omega_{1,1} + \omega_{0,1}) \\ J(\omega_{1,3} + \omega_{0,3}) \end{bmatrix} + \frac{1}{6b_3 - b_1} \begin{bmatrix} 3 \\ -4 \\ -4 \\ 6b_1 \\ b_1 \\ b_3 \end{bmatrix} \psi_2$$

$$= \frac{1}{24(6b_3 - b_1)} \begin{bmatrix} 3 & 3 & -3 & -18 & 18 & 18 \\ -4 & -4 & 24b_3/b_1 & 24 & -24 & -24 \\ -4 & -4 & 4 & 24 & -24 & -4b_1/b_3 \\ 36b_3 & 12b_1 - 36b_3 & -36b_3 & -36b_1 & 36b_1 & 36b_1 \\ 6b_3 & 6b_3 & -6b_3 & -6b_1 & 6b_1 & 6b_1 \\ b_3 & b_3 & -b_3 & -b_1 & 12b_3 - b_1 & b_1 \end{bmatrix} \begin{bmatrix} \rho_1 \\ \sigma_1 \\ \mu_1 \\ \rho_3 \\ \sigma_3 \\ \mu_3 \end{bmatrix} \quad (\text{B.3})$$

If Γ_2 is measured at an additional field $B_{0,f}$ too (approach LNX3), $J(\omega_{0,f})$ can be also calculated:

$$J(\omega_{0,f}) - \frac{4\psi_2}{6b_3 - b_1} = \frac{b_f(6(\rho_3 - \sigma_3 - \mu_3) - \rho_1 - \sigma_1 + \mu_1) + (6b_3 - b_1)\mu_f}{6b_f(6b_3 - b_1)}. \quad (\text{B.4})$$

Protocol LTX3:

$$\begin{aligned} & \begin{bmatrix} J(0) \\ J(\omega_{0,2}) \\ J(\omega_{1,1}) \\ J(\omega_{1,3}) \end{bmatrix} + \frac{1}{12(b_1 + 6b_2 + 6b_3)} \left(\begin{bmatrix} 3(6\xi_3 + \xi_1) \\ -4(6\xi_3 + \xi_1) \\ -12(b_1\xi_3 - (b_2 + b_3)\xi_1) \\ 2((6b_2 + b_1)\xi_3 - b_3\xi_1) \end{bmatrix} + \begin{bmatrix} 18 \\ -24 \\ -12b_1 \\ -12b_3 \end{bmatrix} (6\psi_3 + \psi_1) \right) \\ &= \frac{1}{24(b_1 + 6b_2 + 6b_3)} \begin{bmatrix} 3 & 18 & -18 & 18 \\ -4 & -24 & 24 & 4(6b_3 + b_1)/b_2 \\ 12b_2 + 12b_3 & -12b_1 & 12b_1 & -12b_1 \\ -2b_3 & 12b_2 + 2b_1 & 12b_3 & -12b_3 \end{bmatrix} \begin{bmatrix} \delta_1 \\ \delta_3 \\ \rho_2 \\ \mu_2 \end{bmatrix} \quad (\text{B.5}) \end{aligned}$$

If Γ_2 and/or R_2 and R_1 are measured at another $B_{0,f}$ too, the following spectral density values can be also calculated:

$$J(\omega_{0,f}) - \frac{6\xi_3 + \xi_1}{3(b_1 + 6b_2 + 6b_3)} - 2\frac{6\psi_3 - \psi_1}{b_1 + 6b_2 + 6b_3} = \frac{(b_1 + 6b_2 + 6b_3)\mu_f - b_f(\delta_1 + 6\mu_2 - 6\rho_2 + 6\delta_3)}{6b_f(b_1 + 6b_2 + 6b_3)}, \quad (\text{B.6})$$

$$J(\omega_{1,f}) - \frac{b_f(6\xi_3 + \xi_1) - (b_1 + 6b_2 + 6b_3)\xi_f}{6(b_1 + 6b_2 + 6b_3)} - b_f\frac{6\psi_3 - \psi_1}{b_1 + 6b_2 + 6b_3} = \frac{(b_1 + 6b_2 + 6b_3)\delta_f - b_f(\delta_1 + 6\mu_2 - 6\rho_2 + 6\delta_3)}{12(b_1 + 6b_2 + 6b_3)}. \quad (\text{B.7})$$

Appendix C. Supplementary material

References

- [1] R. Wangsness, F. Bloch, The Dynamical Theory of Nuclear Induction, *Phys. Rev. Lett.* 89 (1953) 728–739. doi:10.1103/PhysRev.89.728.
- [2] A. Abragam, The principles of nuclear magnetism, Oxford: Clarendon Press, 1961.
- [3] A. G. Redfield, The theory of relaxation processes, *Adv. Magn. Reson.* 1 (1965) 1–32.
- [4] J. Cavanagh, W. Faibrother, A. Palmer III, M. Rance, N. Skelton, Protein NMR spectroscopy: principles and practise, Elsevire Academic Press, 2007.
- [5] A. Kowalewski, L. Mäler, Nuclear spin relaxation in liquids: theory, experiments, and applications, New York: Taylor & Francis, 2006.
- [6] H. Wennerstroem, B. Lindman, O. Soederman, T. Drakenberg, J. B. Rosenholm, Carbon-13 magnetic relaxation in micellar solutions. Influence of aggregate motion on T1, *J. Am. Chem. Soc.* 101 (1979) 6860–6864. doi:10.1021/ja00517a012.
- [7] B. Halle, T. Andersson, S. Forsén, B. Lindman, Protein hydration from water oxygen-17 magnetic-relaxation, *J. Am. Chem. Soc.* 103 (1981) 500–508. doi:10.1021/ja00393a004.
- [8] G. Lipari, A. Szabo, Model-free approach to the interpretation of nuclear magnetic-resonance relaxation in macromolecules 1. Theory and range of validity, *J. Am. Chem. Soc.* 104 (1982) 4546–4559. doi:10.1021/ja00381a009.
- [9] G. Lipari, A. Szabo, Model-free approach to the interpretation of nuclear magnetic-resonance relaxation in macromolecules 2. Analysis of experimental results, *J. Am. Chem. Soc.* 104 (1982) 4559–4570. doi:10.1021/ja00381a010.
- [10] B. Halle, The physical basis of model-free analysis of NMR relaxation data from proteins and complex fluids, *J. Chem. Phys.* 131 (2009) 224507. doi:10.1063/1.3269991.

- [11] A. V. Buevich, J. Baum, Dynamics of unfolded proteins: Incorporation of distributions of correlation times in the model free analysis of NMR relaxation data, *J. Am. Chem. Soc.* 121 (1999) 8671–8672. doi:0.1021/ja9910412.
- [12] A. V. Buevich, U. P. Shinde, M. Inouye, J. Baum, Backbone dynamics of the natively unfolded pro-peptide of subtilisin by heteronuclear NMR relaxation studies, *J. Biomol. NMR* 20 (2001) 233–349. doi:10.1023/A:1011243116136.
- [13] A. Polimeno, A many-body stochastic approach to rotational motions in liquids, *Adv. Chem. Phys.* 83 (1993) 89–206.
- [14] A. Polimeno, J. H. Freed, Slow motional ESR in complex fluids - the slowly relaxing local-structure model of solvent cage effects, *J. Phys. Chem.* 99 (1995) 10995–11006. doi:10.1021/j100027a047.
- [15] E. Meirovitch, Y. E. Shapiro, A. Polimeno, J. H. Freed, Structural dynamics of bio-macromolecules by NMR: The slowly relaxing local structure approach, *Prog. Nucl. Magn. Reson. Spectrosc.* 56 (2010) 360–405. doi:10.1016/j.pnmrs.2010.03.002.
- [16] E. Meirovitch, A. Polimeno, J. H. Freed, Comment on "The physical basis of model-free analysis of NMR relaxation data from proteins and complex fluids", *J. Chem. Phys.* 132 (2010) 207101. doi:10.1063/1.3429599.
- [17] J. J. Prompers, R. Bruschweiler, General framework for studying the dynamics of folded and nonfolded proteins by NMR relaxation spectroscopy and MD simulation, *J. Am. Chem. Soc.* 124 (2002) 4522–4534. doi:10.1021/ja012750u.
- [18] R. Ishima, K. Nagayama, Quasi-spectral-density function analysis for nitrogen-15 nuclei in proteins, *J. Magn. Reson. Ser. B* 108 (1995) 73–76. doi:10.1006/jmrb.1995.1104.
- [19] R. Ishima, K. Nagayama, Protein backbone dynamics revealed by quasi spectral density function analysis of amide N-15 nuclei, *Biochemistry* 34 (1995) 3162–3171. doi:10.1021/bi00010a005.

- [20] E. P. Nikonowicz, A. Sirr, P. Legault, F. M. Jucker, L. M. Baer, A. Pardi, Preparation of ^{13}C and ^{15}N labelled RNAs for heteronuclear multi-dimensional NMR studies, *Nucleic Acids Res.* 20 (1992) 4507–4513. doi:10.1093/nar/20.17.4507.
- [21] R. T. Batey, M. Inada, E. Kujawinski, J. D. Puglisi, J. R. Williamson, Preparation of isotopically labeled ribonucleotides for multidimensional NMR spectroscopy of RNA, *Nucleic Acids Res.* 20 (1992) 4515–4523. doi:10.1093/nar/20.17.4515.
- [22] F. Jiang, R. Fiala, D. Live, R. A. Kumar, D. J. Patel, RNA folding topology and intermolecular contacts in the AMP-RNA aptamer complex, *Biochemistry* 35 (1996) 13250–13266. doi:10.1021/bi961345q.
- [23] M. Zerbetto, A. Polimeno, D. Kotsyubynskyy, L. Ghalebani, J. Kowalewski, E. Meirovitch, U. Olsson, G. Widmalm, An integrated approach to NMR spin relaxation in flexible biomolecules: Application to β -D-glucopyranosyl-(1 $\rightarrow$ 6)- α -D-mannopyranosyl-OMe, *J. Chem. Phys.* 131. doi:10.1063/1.3268766.
- [24] F. Delaglio, S. Grzesiek, G. W. Vuister, G. Zhu, J. Pfeifer, A. Bax, NMRPipe - A multidimensional spectral processing system based on UNIX pipes, *J. Biomol. NMR* 6 (1995) 277–293. doi:10.1007/BF00197809.
- [25] T. D. Goddard, D. G. Kneller, Sparky (T.D.Goddard and D.G.Kneller, UCSF, San Francisco, CA) , UCSF, San Francisco, CA.
- [26] B. Fürtig, C. Richter, W. Bermel, H. Schwalbe, New NMR experiments for RNA nucleobase resonance assignment and chemical shift analysis of an RNA UUCG tetraloop, *J. Biomol. NMR* 28 (2004) 69–79. doi:10.1023/B:JNMR.0000012863.63522.1f.
- [27] T. Yamazaki, R. Muhandiram, L. E. Kay, NMR experiments for the measurement of carbon relaxation properties in highly enriched, uniformly ^{13}C , ^{15}N -labeled proteins - application to $^{13}\text{C}^{\alpha}$ carbons, *J. Am. Chem. Soc.* 116 (1994) 8266–8278. doi:10.1021/ja00097a037.
- [28] J. W. Eaton, et al., Octave 3.4.3, (<http://www.gnu.org/software/octave/>), University of Wisconsin.

- [29] N. Tjandra, A. Bax, Solution NMR measurement of amide proton chemical shift anisotropy in ^{15}N -enriched proteins. Correlation with hydrogen bond length, *J. Am. Chem. Soc.* 119 (1997) 8076–8082. doi:10.1021/ja970876e.
- [30] L. Ghalebani, P. Bernatowicz, S. N. Aski, J. Kowalewski, Cross-correlated and conventional dipolar carbon-13 relaxation in methylene groups in small, symmetric molecules, *Concepts Magn. Reson. Part A* 30A (2007) 100–115. doi:10.1002/cmr.a.20074.
- [31] J. Kowalewski, A. Ericsson, R. Vestin, Determination of NOE factors using dynamic Overhauser enhancement technique combined with a non-linear least-squares-fitting procedure, *J. Magn. Reson.* 31 (1978) 165–169. doi:10.1016/0022-2364(78)90179-8.
- [32] R. Fiala, J. Czernek, V. Sklenář, Transverse relaxation optimized triple-resonance NMR experiments for nucleic acids, *J. Biomol. NMR* 16 (2000) 291–302. doi:10.1023/A:1008388400601.
- [33] M. K. Pandey, S. Vivekanandan, S. Ahuja, K. Pichumani, S. Im, L. Waskell, A. Ramamoorthy, Determination of N-15 Chemical Shift Anisotropy from a Membrane-Bound Protein by NMR Spectroscopy, *J. Phys. Chem. B* 116 (2012) 7181–7189. doi:10.1021/jp3049229.
- [34] L. Ghalebani, D. Kotsyubynskyy, J. Kowalewski, NMR relaxation interference effects and internal dynamics in γ -cyclodextrin, *J. Magn. Reson.* 195 (2008) 1–8. doi:10.1016/j.jmr.2008.07.025.
- [35] P. Kadeřávek, V. Zapletal, A. Rabatinová, L. Krásný, V. Sklenář, L. Žídek, Spectral density mapping protocols for analysis of molecular motions in disordered proteins, *J. Biomol. NMR* 58 (2014) 193–207. doi:10.1007/s10858-014-9816-4.
- [36] N. A. Farrow, O. Zhang, A. Szabo, D. A. Torchia, L. E. Kay, Spectral density function mapping using ^{15}N relaxation data exclusively, *J. Biomol. NMR* 6 (1995) 153–162.
- [37] R. B. Hill, C. Bracken, W. F. DeGrado, A. G. Palmer, Molecular motions and protein folding: Characterization of the backbone dynamics and folding equilibrium of $\alpha_2\text{D}$ using ^{13}C NMR spin relaxation, *J. Am. Chem. Soc.* 122 (2000) 11610–11619. doi:10.1021/ja001129b.

- [38] W. B. T. Cruse, P. Saludjian, E. Biala, P. Strazewski, T. Prangé, O. Kennard, Structure of a mispaired RNA double helix at 1.6-Å resolution and implications for the prediction of RNA secondary structure, *Proc. Natl. Acad. Sci. U. S. A.* 91 (1994) 4160–4164. doi:10.1073/pnas.91.10.4160.
- [39] E. Ennifar, A. Nikulin, S. Tishchenko, A. Serganov, N. Nevskaya, M. Garber, B. Ehresmann, C. Ehresmann, S. Nikonov, P. Dumas, The crystal structure of UUCG tetraloop, *J. Mol. Biol.* 304 (2000) 35–42. doi:10.1006/jmbi.2000.4204.
- [40] S. Eswaramoorthy, S. T. Rao, B. C. Pan, M. Sundaralingam, Structure of the dodecamer r(GAUCACUUCGGU) with four 5′-overhang nucleotides, *Acta Crystallogr. Sect. D-Biol. Crystallogr.* 60 (2004) 8–12. doi:10.1107/S0907444903027860.
- [41] C. J. Cheong, G. Varani, I. Tinoco, Solution structure of an unusually stable RNA hairpin, 5′GGAC(UUCG)GUCC, *Nature* 346 (1990) 680–682. doi:10.1038/346680a0.
- [42] G. Varani, C. J. Cheong, I. Tinoco, Structure of an unusually stable RNA hairpin, *Biochemistry* 30 (1991) 3280–3289. doi:10.1021/bi00227a016.
- [43] J. V. Hines, S. M. Landry, G. Varani, I. Tinoco, Carbon-proton scalar couplings in RNA - 3D heteronuclear and 2D isotope-edited NMR of a ¹³C-labeled extra-stable hairpin, *J. Am. Chem. Soc.* 116 (1994) 5823–5831. doi:10.1021/ja00092a037.
- [44] S. R. Lynch, J. G. Pelton, I. Tinoco, NMR assignment of a 2′-hydroxyl proton from the UUCG tetraloop through long-range correlations with ¹³C, *Magn. Reson. Chem.* 34 (1996) S11–S17. doi:10.1002/(SICI)1097-458X(199612)34:13<S11::AID-OMR17>3.0.CO;2-X.
- [45] M. Abdelkafi, M. Ghomi, P. Y. Turpin, V. Baumruk, C. H. duPenhoat, O. Lampire, N. BouchemalChibani, P. Goyer, A. Namane, C. Gouyette, T. HuynhDinh, L. Bednárová, Common structural features of UUCG and UACG tetraloops in very short hairpins determined by UV absorption, Raman, IR and NMR spectroscopies, *J. Biomol. Struct. Dyn.* 14 (1997) 579–593.

- [46] S. Nozinovic, B. Fürtig, H. R. A. Jonker, C. Richter, H. Schwalbe, High-resolution NMR structure of an RNA model system: the 14-mer cUUCGg tetraloop hairpin RNA, *Nucleic Acids Res.* 38 (2010) 683–694. doi:10.1093/nar/gkp956.
- [47] A. Y. Denisov, R. N. Hannoush, K. Gehring, M. J. Damha, A novel RNA motif based on the structure of unusually stable 2',5'-linked r(UUCG) loops, *J. Am. Chem. Soc.* 125 (2003) 11525–11531. doi:10.1021/ja036207k.
- [48] M. Akke, R. Fiala, F. Jiang, D. Patel, A. G. Palmer, Base dynamics in a UUCG tetraloop RNA hairpin characterized by ^{15}N spin relaxation: Correlations with structure and stability, *RNA-Publ. RNA Soc.* 3 (1997) 702–709.
- [49] E. Duchardt, H. Schwalbe, Residue specific ribose and nucleobase dynamics of the cUUCGg RNA tetraloop motif by NMR ^{13}C relaxation, *J. Biomol. NMR* 32 (2005) 295–308. doi:10.1007/s10858-005-0659-x.
- [50] P. Vallurupalli, L. E. Kay, A suite of ^2H NMR spin relaxation experiments for the measurement of RNA dynamics, *J. Am. Chem. Soc.* 127 (2005) 6893–6901. doi:10.1021/ja0427799.
- [51] J. Rinnenthal, C. Richter, S. Nozinovic, B. Fürtig, J. J. Lopez, C. Glaubitz, H. Schwalbe, RNA phosphodiester backbone dynamics of a perdeuterated cUUCGg tetraloop RNA from phosphorus-31 NMR relaxation analysis, *J. Biomol. NMR* 45 (2009) 143–155. doi:10.1007/s10858-009-9343-x.
- [52] Q. Gong, R. Ishima, $^{15}\text{N}\{-^1\text{H}\}$ NOE experiment at high magnetic field strengths, *J. Biomol. NMR* 37 (2007) 147–157. doi:10.1007/s10858-006-9125-7.
- [53] R. Pendrill, E. Sävén, G. Widmalm, Conformation and dynamics at flexible glycosidic linkage revealed by NMR spectroscopy and molecular dynamics simulations: Analysis of $\beta\text{-L-Fucp-(1}\rightarrow\text{6)-}\alpha\text{-D-Glcp-OMe}$ in water solution, *J. Phys. Chem. B* 117 (2013) 14709–14722. doi:dx.doi.org/10.1021/jp409985h.
- [54] D. S. Patel, R. Pendrill, S. S. Mallajosyula, G. Widmalm, A. D. M. Jr, Conformational properties of α - or β -(1 $\rightarrow$ 6)-linked

oligosaccharides: Hamiltonian replica exchange MD simulations and NMR experiments, *J. Phys. Chem. B* 118 (2014) 2851–2871. doi:dx.doi.org/10.1021/jp412051v.

- [55] B. Efron, Bootstrap methods: another look at the jackknife, *Ann. Statist.* 7 (1979) 1–26. doi:10.1214/aos/1176344552.
- [56] L. G. Werbelow, D. M. Grant, Intramolecular dipolar relaxation in multispin systems, *Adv. Magn. Reson* 9 (1977) 189–299.
- [57] D. M. Korzhnev, M. Billeter, A. S. Arseniev, V. Y. Orekhov, NMR studies of Brownian tumbling and internal motions in proteins, *Prog. Nucl. Magn. Reson. Spectrosc.* 38 (2001) 197–266. doi:10.1016/S0079-6565(00)00028-5.