NMR Spectroscopy

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Recovering Invisible Signals by Two-Field NMR Spectroscopy

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Abstract: *Nuclear magnetic resonance (NMR) studies have benefited tremendously from the steady increase in the strength of magnetic fields. Spectacular improvements in both sensitivity and resolution have enabled the investigation of molecular systems of rising complexity. At very high fields, this progress may be jeopardized by line broadening, which is due to chemical exchange or relaxation by chemical shift anisotropy. In this work, we introduce a two-field NMR spectrometer designed for both excitation and observation of nuclear spins in two distinct magnetic fields in a single experiment. NMR spectra of several small molecules as well as a protein were obtained, with two dimensions acquired at vastly different magnetic fields. Resonances of exchanging groups that are broadened beyond recognition at high field can be sharpened to narrow peaks in the low-field dimension. Two-field NMR spectroscopy enables the measurement of chemical shifts at optimal fields and the study of molecular systems that suffer from internal dynamics, and opens new avenues for NMR spectroscopy at very high magnetic fields.*

The ability of nuclear magnetic resonance (NMR) to probe the chemical and physical properties of matter at atomic resolution renders it a universally applicable spectroscopic method for molecular chemistry, material science, structural biology, and medicine. The ubiquity of NMR spectroscopy has greatly benefited from the enhanced resolution and sensitivity offered by high magnetic fields and the introduction of two- and multidimensional NMR experiments.[1] Severe line broadening, which is due to micro- to millisecond dynamics (known as "chemical exchange"), occurs in many chemical and biological systems.[2] Such line-broadening effects are often exacerbated at high fields. Hence, chemists and biologists face a dilemma: They must choose between good sensitivity and resolution at high fields and more favorable linewidths and relaxation rates at lower fields.

Magnetic-field-dependent properties can be probed by a broad range of NMR techniques that explore two or more fields in a single experiment, as in fast field-cycling relaxometry,[3] zero-field NMR,[4] dynamic nuclear polarization,[5] and other methods.[6] Such systems have enabled the characterization of a host of properties of magnetic resonance imaging,[7] and particularly of contrast agents,[8] materials,[9] as well as macromolecules.[10] However, to the best of our knowledge, no experiment has ever been proposed that allows one to correlate chemical shifts at two different fields. The requirement that both fields should be homogeneous has thus far been a formidable obstacle. Herein, we introduce a two-field NMR spectrometer and illustrate its potential for high-resolution two-field NMR spectroscopy. The benefits of high fields (sensitivity and resolution) and low fields (line narrowing despite chemical exchange) can thus be combined. This approach can yield high-resolution chemical-shift correlations between high and low fields, for example, in heteronuclear (e.g., ^{1}H–^{13}C) spin systems in small molecules and proteins alike. We show that signals of methyl groups that cannot be detected at 14.1 T because of chemical exchange can be recovered by two-field correlation spectroscopy. This work shows how a wide range of molecules and biomolecules prone to excessive broadening by chemical exchange can be studied by NMR spectroscopy. Two-field NMR spectroscopy paves

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the way to a new generation of NMR spectrometers, where multiple fields can be explored in the course of a single experiment to achieve an optimal combination of sensitivity, resolution, and spectral information.

Our two-field NMR spectrometer is based on a commercial NMR spectrometer at 14.1 T (600 MHz for protons) with a series of new accessories (Figure 1). First, a system was

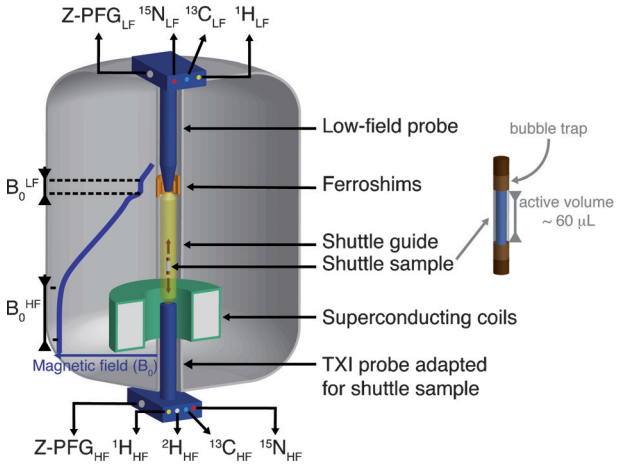

Figure 1. The two-field NMR spectrometer. Ferroshims that are placed in the bore of the magnet provide a plateau at $B_0^{LF} = 0.33$ T. In addition to a classical probe positioned at the high-field center, $B_0^{HF} = 14.1$ T, a second probe is introduced from the top of the bore and positioned at the plateau B_0^{LF}. The sample is shuttled between the two magnetic centers by a pneumatic system.

designed to obtain a reasonably homogeneous field "plateau" at 0.33 T in the stray field of the 14.1 T superconducting magnet, with a homogeneity of about 10 ppm over the 2 cm length of the sample (see the Supporting Information, Figure S1). A patterned structure of soft magnetic materials, known as ferroshims, was optimized.[5a,11] A low-field triple resonance probe (^{15}N, ^{13}C, ^{1}H) equipped with a single-axis gradient was designed and combined with radiofrequency (rf) synthesizers and amplifiers operating at 1.42, 3.52, and 14.1 MHz. Finally, a pneumatically driven sample shuttle[3f] ensures fast transfer between the two magnetic centers. The time required for transfer between the high and low fields is about 100 ms.

High-resolution zero-quantum spectra can be obtained for heteronuclear systems provided that the effective chemical shift evolution is carefully tailored.[12] Herein, we introduce a two-field heteronuclear zero-quantum correlation experiment (2F-HZQC), where a zero-quantum ^{13}C–^{1}H coherence evolves under the difference of the offsets of ^{13}C and ^{1}H with respect to the relevant carrier frequencies at low field, while single-quantum ^{1}H coherences are detected at high field. The experiment is based on a simple sequence for heteronuclear multiple quantum coherence (HMQC)[13] and related to methyl HZQC experiments[14] with the following modifications (Figure 2a): A two-spin order $2H_zC_z$ is generated before each transfer between the high- and low-field centers, a zero-quantum ^{13}C–^{1}H coherence is selected by phase cycling at low field,[15] and a 180° pulse is used to scale

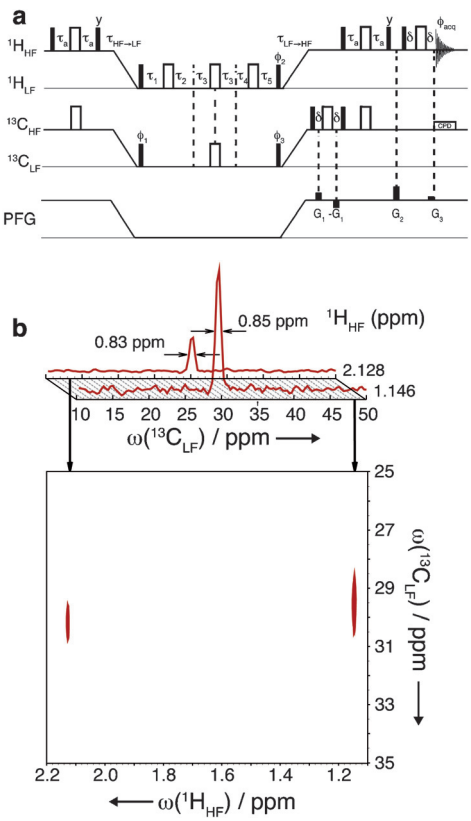

Figure 2. Two-field heteronuclear correlation spectrum. a) Pulse sequence for 2F-HZQC. For details see the Supporting Information. Narrow black and wide open rectangles represent 90° and 180° pulses, respectively. All pulses were applied along the *x* axis of the rotating frame unless otherwise indicated. The phases are cycled as follows: $\phi_1 = x, -x$; $\phi_2 = 4\{x\}, 4\{y\}$; $\phi_3 = 2\{x\}, 2\{-x\}, 2\{y\}, 2\{-y\}$; with the receiver phase $\phi_{acq} = x, -x, -x, x$. The phase ϕ_3 was incremented following the TPPI scheme.[17] The delays were $\tau_a = 1/(4J_{CH})$ with $J_{CH} = 125$ Hz. δ comprises the length of the gradient and the gradient recovery delay, $\tau_1 = (\tau_0 + n_1\Delta t_1)\,c$, $\tau_2 = (\tau_0 + n_1\Delta t_1)\,(1-c)$, $\tau_3 = \tau_0(2c-1) + n_1\Delta t_1(c-0.5)$, $\tau_4 = \tau_0(1-c)$, and $\tau_5 = \tau_0 c$. Δt_1 is the time increment in the indirect dimension, and n_1 is the index of the time increment. $c = (\gamma_C/\gamma_H + 1)/2 = 0.6257$. The gradients G_1, G_2, and G_3 of 0.9 ms duration were applied along the *z* axis with respective amplitudes of 10 G cm^{-1}, 15 G cm^{-1}, and $G_3 = 2(\gamma_C/\gamma_H)G_1$. GARP composite pulse decoupling[18] was applied on the ^{13}C channel during detection with $\omega_{1GARP}/2\pi = 2.08$ kHz. b) Natural-abundance $\{^{13}C_{LF}, ^{1}H_{HF}\}$ 2F-HZQC spectrum of a sample containing 0.5 M acetone and 0.5 M *tert*-butyl alcohol. Low-frequency one-dimensional ^{13}C spectra (vertical cross-sections) extracted from the 2F-HZQC spectrum at $\delta(^{1}H_{HF}) = 1.146$ ppm (*tert*-butyl alcohol) and $\delta(^{1}H_{HF}) = 2.128$ ppm (acetone). A shearing transformation was used to obtain the $^{13}C_{LF}$ chemical shift in the indirect dimension.

the ^{1}H offset down by the ratio of the gyromagnetic ratios, $\gamma_C/\gamma_H = 0.2514$, in the indirect dimension. The evolution of the coherence at low field is thus determined by a combination of the ^{13}C and ^{1}H chemical shifts: $\omega_{LF}^{eff} = \omega_{LF}(^{13}C) - (\gamma_C/\gamma_H)\,\omega_{LF}(^{1}H)$. This combination is immune to line broadening that is due to magnetic-field inhomogeneities.

The contributions of the latter to the linewidth in the indirect dimension is significantly reduced by the tailored zero-quantum sequence of Figure 2a (Figure 2b). In the natural-abundance high-resolution $[^{1}H,^{13}C]$ 2F-HZQC spec-

trum of 0.5 M *tert*-butanol and 0.5 M acetone in D_2O (Figure 2b), the methyl signals of both butanol and acetone have full linewidths at half height below 1 ppm. The single-quantum ^{13}C chemical shifts at low field can be obtained after a shearing transformation of the spectrum, as the proton chemical shifts do not depend on the magnetic field. The spectrum has a similar appearance as a conventional high-field heteronuclear single-quantum coherence (HSQC) correlation[16] spectrum (Figure S3).

The generality of two-field NMR spectroscopy was further demonstrated with a protein in solution (Figure 3).

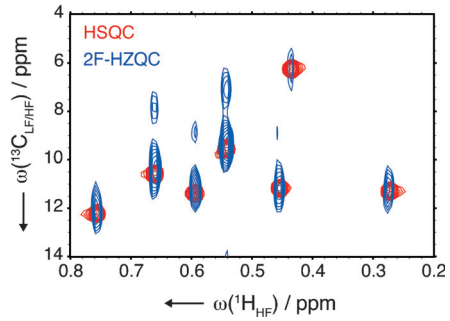

Figure 3. 2D ^{1}H, ^{13}C correlation spectrum human ubiquitin labeled with $^{13}C^{1}H_3$ groups on the isoleucine δ_1 positions. An HSQC spectrum recorded at 14.1 T (red) was superimposed onto the two-field HZQC spectrum recorded on the two-field spectrometer (blue) with the sequence of Figure 2a (additional pulsed field gradients flank all 180° pulses at both high and low field).

A high-resolution 2F-HZQC spectrum was obtained for a sample of human ubiquitin (1.5 mM, pH 4.5) with specific isotope labeling on the δ_1 $^{13}C^{1}H_3$ methyl groups of its seven isoleucine residues with a ^{2}H,^{12}C background.[19] The narrow linewidths in the indirect dimension are likely due to relaxation interference effects, in the manner of transverse optimized relaxation spectroscopy (TROSY).[20] The sensitivity is sufficient to obtain such a spectrum in nine hours. Weak artifacts appear 2.7 ppm away from the most intense peaks in the indirect dimension. The details of this spectrum will be the subject of a forthcoming study.

Most chemical and biological systems suffer from exchange broadening. In many organic molecules and biomolecules, this effect can be subtle and merely gives rise to slight perturbations in the NMR spectra. In other instances, for example, when labile protons undergo exchange with a solvent, or in so-called molten globules,[21] chemical exchange may lead to dramatic spectral broadening, which can prevent any NMR measurement. The line broadening that is due to R_{ex} strongly depends on the magnetic field B_0, so that reducing the field may have dramatic effects on the linewidths.

Here, we combined the excellent sensitivity and resolution achieved at high fields with the dramatic reduction of exchange-induced line broadening at low fields. At 14.1 T, the ^{13}C signals of the two methyl groups of the dimethyl triazene compound[22] shown in Figure 4a are in slow exchange at 21 °C. Line-shape analysis based on Markov chain Monte

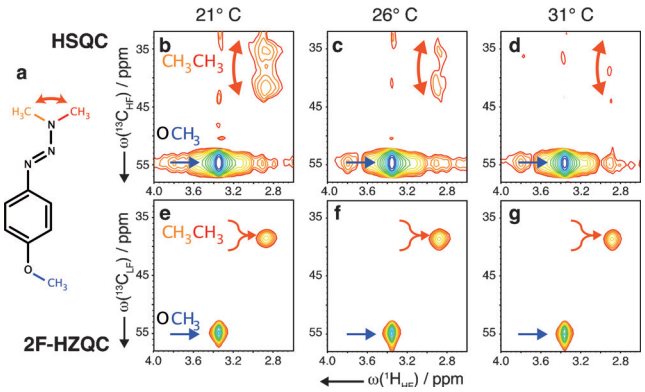

Figure 4. a) The two exchanging methyl groups of the triazene compound shown in orange and red swap their positions on a sub-millisecond timescale at 31 °C. The non-exchanging methoxy group shown in blue provides a reference signal. Top row (b–d): Conventional high-field HSQC spectra with increasingly broad lines at 21, 26, and 31 °C. The lowest contour corresponds to 1/403 of the intensity of the methoxy signal at 31 °C to show the weak signal of the exchanging methyl groups. Bottom row (e–g): Two-field HZQC spectra with sharp lines regardless of the temperature. The lowest contour corresponds to 1/8 of the intensity of the methoxy signal at 31 °C.

Carlo (MCMC) methods[23] provides a k_{ex}(21 °C) value of 1700 s^{-1} (Figure 4b and Figure S5). The system approaches coalescence at 26 °C (Figure 4c). At 31 °C, the resonances of the exchanging methyl groups are broadened beyond detection (Figure 4d). For comparison, the peak of the resonance of the non-exchanging methoxy group is at least 1000 times more intense (Figure S6). The decay rate of the ^{13}C coherence was estimated to be about 1000 s^{-1} at 14.1 T for an estimated k_{ex}(31 °C) value of 3400 s^{-1}. At 0.33 T, the exchange contribution is predicted to be dramatically reduced to 1.9 s^{-1}, corresponding to a line narrowing by a factor of 500. Remarkably, the signal that is due to the exchanging methyl groups is readily observable in the two-field correlation spectra and barely affected over the whole temperature range (Figure 4e–g). These spectra were recorded with a 2F-HZQC sequence (see Figure S2).

Very high fields above 1 GHz will soon become available, but their possible drawbacks are hard to predict. Chemical exchange can lead to severe line broadening, and carbon-13 relaxation by chemical shift anisotropy (CSA) can become a major limitation. Many multidimensional NMR experiments include delays for evolution under chemical shifts that may not be optimal at one and the same field for all nuclei. In some cases, the best fields may be extremely high (e.g., to reduce the effects of second-order quadrupolar couplings).[24] For single-quantum evolution of carbonyl ^{13}C nuclei in large proteins, the optimal magnetic field lies below 14 T.[25] The transverse ^{13}C relaxation rates in proteins have been predicted to be minimal between about 2 and 5 T.[26] On the other hand, the optimal field may be close to 1 GHz for TROSY of amide ^{15}N–^{1}H pairs.[27] Two-field NMR spectroscopy offers the possibility of manipulating spins such as ^{15}N, ^{13}C, or ^{31}P at a field that is most appropriate for them, and detecting signals of other nuclei such as ^{1}H at a higher field where the best resolution and sensitivity can be achieved. The effects of field

inhomogeneities can be greatly reduced by exploiting zero-quantum coherences. The superb resolution and sensitivity that can be achieved at high fields can thus be combined with the favorable properties offered by low fields. Most high-field NMR systems may be turned into two-field spectrometers with a plateau at 0.33 T. The design of the ferroshims requires a simple optimization for each type of superconducting magnet. Two-field systems with a low-field center above 0.5 T should rely on other technologies that are currently available[28] to obtain a second magnetic-field plateau. This study marks the beginning of a new generation of multiple-field NMR experiments, and opens the way to the characterization of a wide variety of systems.

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