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# Extending the Scope of Singlet-State Spectroscopy

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*Different decoupling sequences are tested—using various shaped radio-frequency (RF) pulses—to achieve the longest possible lifetimes of singlet-state populations over the widest possible bandwidths, that is, ranges of offsets and relative chemical shifts of the nuclei involved in the singlet states. The use of sinc or refocusing broadband universal rotation pulses (RE-BURP) for decoupling during the intervals where singlet-state populations are preserved allows one to extend the useful bandwidth with respect to prior state-of-the-art methods based on composite-pulse WALTZ decoupling. The improved sinc decoupling sequences afford a more reliable and sensitive measure of the lifetimes of singlet states in pairs of spins that have widely different chemical shifts, such as the two aromatic protons $H^5$ and $H^6$ in uracil. Similar advantages are expected for nucleotides in RNA and DNA. Alternative approaches, in particular frequency-modulated decoupling sequences, also appear to be effective in preserving singlet-state populations, even though the profiles of the apparent relaxation rate constants as a function of the offset are somewhat perturbed. The best decoupling sequences prove their utility in sustaining longer lifetimes of singlet states than previously achieved for the side-chain tyrosine protons in bovine pancreatic trypsin inhibitor (BPTI) at 600 MHz (14.1 T), where the differences of chemical shifts between coupled protons are a challenge.*

## Introduction

The recent discovery of long-lived spin states[1,2] opens new horizons for the investigation of slow dynamic processes by means of NMR. Singlet-state spectroscopy has proven its utility for studies of diffusion,[3,4] slow exchange,[5] and relaxation by dipole–dipole and chemical shift anisotropy interactions.[6] A wide range of applications can be contemplated, provided adequate methods can be developed to increase the versatility of the excitation and preservation of singlet-state populations. In high fields, these long-lived states are preserved by applying an irradiating field $B_1$, the amplitude of which is critical to sustaining their lifetimes.[7] It has been shown experimentally[5] that singlet states can be sustained over a reasonably broad bandwidth by using composite-pulse decoupling sequences, such as WALTZ-16.[8] With a constant radio-frequency (RF) amplitude $\nu_1=\omega_1/(2\pi)=1.3$ kHz, singlet-state populations can be maintained by means of WALTZ-16 decoupling during intervals $\tau_m$ as long as 60 s and over a range of chemical shifts of about $\pm 1$ kHz with respect to the RF carrier frequency. With modern instruments it is feasible to use WALTZ-16 decoupling with amplitudes as high as 3.9 kHz without excessive heating of the sample and probe, and without interfering with the field-frequency lock circuit. The frequency interval over which the singlet states are preserved using WALTZ-16 can be extended from $\pm 1$ to $\pm 5$ kHz when $\nu_1$ is extended from 1.3 to 3.9 kHz. Note that this goes beyond what would be expected from the usual Fourier relationship (which predicts an increase in bandwidth in proportion to the RF amplitude). Here, we show that the peak RF amplitude can be increased to about 15 kHz by using adequate decoupling methods with shaped pulses, and that the frequency band over which singlet states are sustained can be extended to about $\pm 12$ kHz.

These methods open the way to applications to molecules containing pairs of coupled spins I and S that feature either 1) a wide range of offsets $\Delta\nu=\nu_{av}-\nu_{RF}$ between the average chemical shift of the spins, $\nu_{av}=(\nu_I+\nu_S)/2$, and the carrier frequency $\nu_{RF}$ or 2) large differences in the chemical shifts $\Delta\nu_{IS}=\nu_I-\nu_S$. The ability of the decoupling sequence to sustain singlet states depends critically on these two parameters (Figure 1 A). Herein, we systematically study the efficiency of decoupling schemes as a function of $\Delta\nu$ and show that the resulting decoupling sequences are also effective in preserving long-lived states in systems with large differences in chemical shifts $\Delta\nu_{IS}$. Wide ranges of offsets may be encountered in studies of exchange processes involving several molecular conformations, where each conformation is associated with a characteristic set of chemical shifts, while large spreads in chemical shifts are

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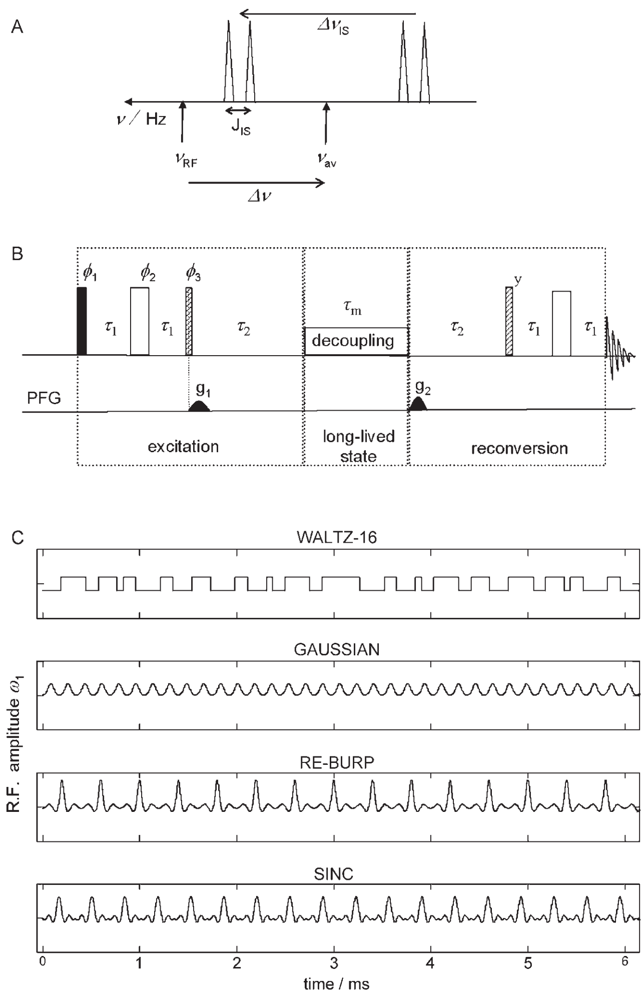


**Figure 1.** A) Experimental parameters that are relevant for exciting and sustaining long-lived (singlet) state populations in two-spin (sub)systems. B) Typical pulse sequence designed for singlet-state spectroscopy, where the long-lived state is sustained during a protracted decoupling interval $\tau_m$ that can be on the order of 10 to 100 s. The hatched, filled, and open rectangles represent $\pi/4$, $\pi/2$, and $\pi$ pulses, respectively. The phases of the pulses are along the *x* axis unless otherwise indicated. The recommended phase cycle is $\phi_1 = x, -x$, $\phi_2 = 2(x), 2(-x)$, $\phi_3 = 4(y), 4(-y)$, and $\phi_{rec} = 2(x,-x), 2(-x,x)$. C) Decoupling sequences used in this work. The time axis spans the length of one full WALTZ-16 cycle, which can be written as $Q\bar{Q}\bar{Q}Q$, with amplitudes of alternating signs, that is, $Q = \{270_{-x}\ 360_{+x}\ 180_{-x}\ 270_{+x}\ 90_{-x}\ 180_{+x}\ 360_{-x}\ 180_{+x}\ 270_{-x}\}$. The pulse durations $\tau_p$ and amplitudes of the shaped pulses have typical values, namely (from top to bottom), $\tau_p = 64$, 175, 400, and 340 μs for rectangular, Gaussian, RE-BURP, and sinc pulses, which have maximum amplitudes of $\nu_1^{max} = 3.9$, 7, 15.7, and 12.4 kHz, respectively.

likely to occur when the two coupled spins belong to nuclear species such as $^{13}C$. Both parameters increase in proportion to the strength of the static field.

Singlet-state exchange spectroscopy starts with an *excitation* sequence (Figure 1 B), which is usually composed of nonselective "hard" pulses and delays designed to maximize the population of a singlet state or—more accurately—in the parlance recently introduced by Levitt and co-workers,[9] to maximize the difference between singlet- and triplet-state populations, described by a long-lived state (LLS) operator $Q_{LLS} = -N\left[I_x S_x + I_y S_y + I_z S_z\right] = -N\vec{I} \cdot \vec{S}$, with a norm $N = 2/3^{1/2}$. The relaxation rate constant $R_{LLS}$ of the long-lived state described by $Q_{LLS}$ should be kept to a minimum during the protracted decoupling interval $\tau_m$. This can be achieved by suitable pulse sequences which, in effect, suppress the effects of the chemical shifts and thus transform the weakly coupled two-spin I–S system into a pair of magnetically equivalent spins.[1] At the end of the interval $\tau_m$, decoupling is switched off, so that the remaining singlet-state population is converted into a superposition of zero-quantum coherence and longitudinal two-spin order. A *reconversion* sequence is then used to transform either (or both) of these terms into detectable single-quantum coherences. Here, we have used the pulse sequence of Figure 1 B, which is equivalent to sequence II described by Sarkar et al.,[5] in combination with various decoupling schemes. We have shown[5] that the use of WALTZ-16 decoupling[8] during the $\tau_m$ period in Figure 1 B allows one to increase the useful bandwidth by a factor of about ten with respect to continuous-wave (CW) irradiation,[7] using the same RF amplitude $\nu_1$ A theoretical analysis of the relaxation rate constant $R_{LLS}$ of a long-lived state in a system under CW irradiation[7] revealed that $R_{LLS}$ increases with increasing $(\Delta\nu/\nu_1)^2$, where $\nu_1$ is the RF amplitude, and the average offset $\Delta\nu$ is defined in Figure 1 A. An increased RF decoupling amplitude $\nu_1$ is therefore necessary with increasing $\Delta\nu$. However, to avoid heating effects, the amplitude $\nu_1$ must obviously be limited during the intervals $\tau_m$, which can be as long as 100 s. We have therefore evaluated the use of amplitude- and frequency-modulated "shaped" decoupling pulses to cover a broad range of frequencies. Examples of decoupling sequences used during $\tau_m$ are detailed in Figure 1 C.

## Results and Discussion

To test the efficiency of various decoupling methods, we have used a partially deuterated saccharide[5] (see Figure 2). The two diastereotopic protons attached to the same carbon atom have a relative shift $\Delta\nu_{IS}$ of 0.19 ppm (75 Hz at 400 MHz or 9.4 T). Similar partially deuterated saccharides could be incorporated into various nucleic acids. Different amplitude-modulated shaped pulses (Figure 1 C) were tested as a function of the offset $\Delta\nu$ (Figure 1 A). If $\Delta\nu$ lies within a range where the decoupling sequence remains effective, the observed relaxation rate constants of the long-lived states, $R_{LLS}$, will remain close to the minimum observed for $\Delta\nu = 0$. An abrupt increase in the apparent value of $R_{LLS}$ is observed beyond this frequency range. To establish the maximum amplitude $\nu_1^{max}$ that can be used with various decoupling sequences, each of the pulse trains was tested for $\tau_m = 30$ s, progressively increasing $\nu_1^{max}$, with a relaxation delay of 1 s between two consecutive scans. The amplitudes $\nu_1^{max}$ that were considered safe correspond to an attenuation of 1 dB below the amplitudes that induced a detectable perturbation of the lock level during the test. It was observed that the amplitudes calibrated with this protocol were safe to use both for a room-temperature probe at 400 MHz and for a cryoprobe at 600 MHz. A sequence of contiguous Gaussian $\pi$ pulses,[10] with peak RF amplitudes $\nu_1^{max} = 7$ kHz truncated at 1% of their maximum intensity and pulse

  

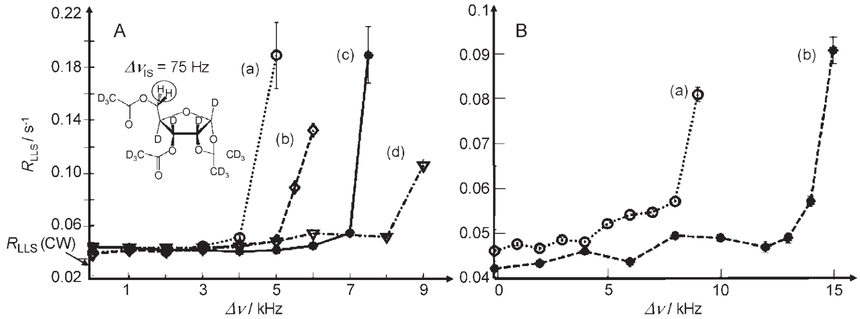


**Figure 2.** A) Comparison of the experimental relaxation rate constants $R_{LLS} = 1/T_{LLS}$ of long-lived (singlet) state populations at 400 MHz in a partially deuterated saccharide (see insert), where the two coupled spins I and S are protons $H_5'$ and $H_5''$, which exhibit a difference between the chemical shifts $\Delta\nu_{IS} = 75$ Hz, as a function of the average offset $\Delta\nu = (\nu_I + \nu_S)/2 - \nu_{RF}$, observed for different decoupling schemes applied during the interval $\tau_m$: Curve (a) sequence of contiguous Gaussian $\pi$ pulses with a length $\tau_p = 175$ μs and peak RF amplitudes $\nu_1^{max} = 7$ kHz, truncated at 1 % of their maximum; curve (b) WALTZ-16 scheme using "hard" pulses with a constant RF amplitude $\nu_1 = 3.9$ kHz (i.e., the length of each $\pi/2$ pulse was $\tau_p = 64$ μs); curve (c) sequence of contiguous RE-BURP pulses with a length $\tau_p = 400$ μs and peak RF amplitudes $\nu_1^{max} = 15.7$ kHz; curve (d) sequence of contiguous sinc-shaped $\pi$ pulses (truncated at the second nul-passage on either side of the peaks) with a duration $\tau_p = 340$ μs and peak RF amplitudes $\nu_1^{max} = 12.4$ kHz. The "true" relaxation rate constant $R_{LLS}$ (CW), indicated by an arrow on the left, was measured experimentally using CW decoupling with $\Delta\nu = 0$ and a constant RF amplitude $\nu_1^{max} = 1.3$ kHz. B) Experimental relaxation rate constants $R_{LLS}$ as a function of the offset $\Delta\nu$ using a decoupling sequence consisting of: curve (a) contiguous CHIRP pulses, each with a duration $\tau_p = 1$ ms, with frequency sweep of the RF carrier over a range of 22 kHz and a maximum amplitude $\nu_1^{max} = 4.4$ kHz, apodized[13] by quarter sine waves in the first and last 10% of the sweeps, and curve (b) TanhTan pulses with a duration $\tau_p = 1$ ms, a maximum amplitude $\nu_1^{max} = 4.4$ kHz, and a frequency sweep range of 44 kHz.

lengths $\tau_p = 175$ μs (Figure 1 C), was found to give a profile of $R_{LLS}$ that is very similar to that of a WALTZ-16 sequence with a constant RF amplitude $\nu_1^{max} = 3.9$ kHz [Figure 2 A, curves (a) and (b)]. A sequence of contiguous refocusing broadband universal rotation pulses (RE-BURP), with peak RF amplitudes $\nu_1^{max} = 15.7$ kHz, pulse lengths $\tau_p = 400$ μs, and shapes defined by the summation of 15 sine and cosine modulated pulses[11] was found to cover a bandwidth of $\pm 7$ kHz [Figure 2 A, curve (c)]. A twofold improvement of the bandwidth was observed when the pulse length of the RE-BURP shape was decreased by a factor of two and the value of $\nu_1^{max}$ was increased by the same factor (data not shown). Different pulses from the BURP family (I-BURP, U-BURP) were also tested, but gave less satisfactory results. The profile of a decoupling sequence using contiguous sinc-shaped pulses[12] was found to be even wider than that of the RE-BURP sequence [Figure 2 A, curve (d)]. Frequency-modulated CHIRP[13] or TanhTan[14] pulses afforded offset profiles of the rate constants $R_{LLS}(\Delta\nu)$ that were remarkably wide, though not as uniform as for amplitude-modulated pulses (Figure 2 B). At small offsets $\Delta\nu$, the apparent lifetimes of the long-lived states sustained during $\tau_m$ by using frequency-modulated pulses correlate with the amplitudes of the pulses, which means that frequency-modulated pulses with low amplitudes still afford large bandwidths but lead to higher $R_{LLS}$ values. The bandwidth of the $R_{LLS}(\Delta\nu)$ profile increases with the frequency sweep range of the pulses. The possibility of sustaining long-lived states with frequency-modulated pulses is encouraging in view of their possible applications in magnetic resonance imaging (MRI).

Among the two parameters that critically affect the efficiency of a decoupling sequence, namely $\Delta\nu$ and $\Delta\nu_{IS}$, we could only systematically vary the first one experimentally. In the following, we show some results obtained in systems with large chemical-shift differences $\Delta\nu_{IS}$. The experiments confirm the intuition that decoupling sequences optimized for large $\Delta\nu$ values also perform well in cases where $\Delta\nu_{IS}$ represents a challenge.

## Applications to Uracil

The protons $I = H^5$ and $S = H^6$ in uracil dissolved in $D_2O$ (Figure 3) provide an example of a coupled two-spin system with a modest scalar coupling constant $J_{IS} = 7.7$ Hz and a large chemical-shift difference $\Delta\nu_{IS} = 1.7$ ppm, that is, 693 or 1040 Hz at 400 or 600 MHz, respectively ($B_0 = 9.4$ or 14.1 T). This is a challenging test for decoupling sequences intended to sustain singlet-state populations. Indeed, in a static field of 9.4 T, an attempt to preserve singlet states by using WALTZ-16 decoupling with moderate amplitude ($\nu_1^{max} = 1.3$ kHz) resulted in scattered signal intensities as a function of time, with an approximate relaxation rate constant of $0.18 \pm 0.01$ s$^{-1}$, when fitted to an exponential decay function. When the amplitude was increased to $\nu_1^{max} = 3.9$ kHz, the signal intensities featured a monoexponential decay as a function of $\tau_m$, which could be fitted to an exponential function with a decay rate $R_{LLS} = 0.116 \pm 0.004$ s$^{-1}$.

We have attempted, for experiments carried out at 600 MHz, to use a sequence consisting of contiguous sinc pulses (Figure 2 A). Using a WALTZ-16 decoupling sequence, the experimental signal intensities of uracil in Figure 3 are slightly scattered around an ideal exponential decay. We attributed this behavior to the fact that the necessary condition for sustaining singlet states is not properly fulfilled, since the RF amplitude should ideally be much higher than the separation of signals, $\nu_1 \gg \Delta\nu_{IS}$, while in this case $\nu_1 = 2.6$ kHz and $\Delta\nu_{IS} \approx 1$ kHz. The use of sinc pulses diminished scattering in the measurements (Figure 3 A). The fitted relaxation rate constants were $R_{LLS} = 0.14$ s$^{-1}$ with both sequences, with errors of 1.5 and 1.1 % for the WALTZ-16 and sinc sequences, respectively. Thus, the decoupling sequence that has the larger bandwidth in terms of the average offset $\Delta\nu$ also has the best ability of sustaining singlet-state populations in molecules with large differences in the chemical shifts $\Delta\nu_{IS}$.

  

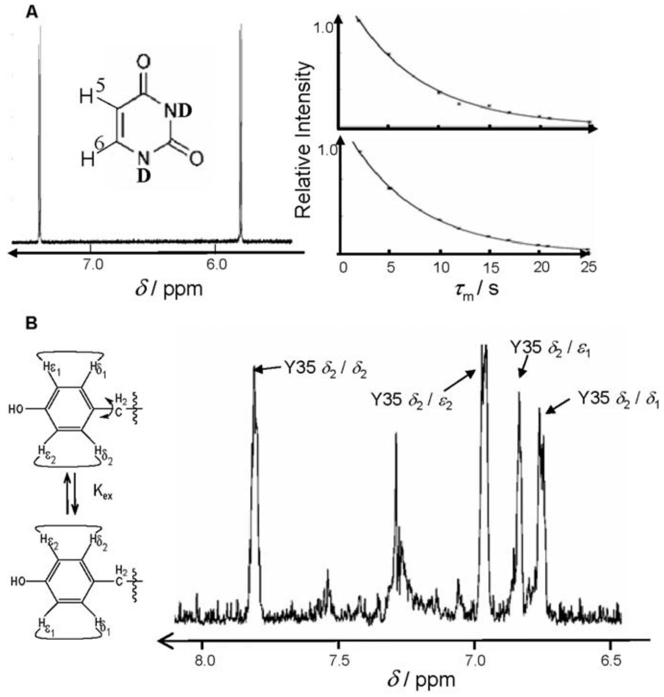


**Figure 3.** A) Left: signals of the $H^5$ and $H^6$ protons in a sample of uracil (shown in the inset) dissolved in $D_2O$ recorded with a cryoprobe at 14.1 T (600 MHz) with the pulse sequence of Figure 1 B (using $\tau_m = 12$ s and WALTZ-16 decoupling). Right: fits of the decays of the summed intensities of the four peaks shown on the left to the function $\exp(-\tau_m/T_{LLS}) = \exp(-\tau_m R_{LLS})$, using WALTZ-16 decoupling with a constant RF amplitude of $\nu_1 = 2.6$ kHz (top) and contiguous sinc pulses with a peak RF amplitude of $\nu_1^{max} = 5.6$ kHz and a pulse length of 500 μs (bottom). The error bars (of the size of the symbols) reflect the difference in intensities of two identical experiments recorded with the same delay $\tau_m$. B) Ring flips of tyrosine residues in proteins (which lead to an exchange $H^{\delta 1} \leftrightarrow H^{\delta 2}$ and $H^{\varepsilon 1} \leftrightarrow H^{\varepsilon 2}$), and a cross-section through a 2D SS-EXSY spectrum of BPTI, taken at the chemical shift of $H^{\delta 2}$, $\omega_1 = 7.84$ ppm. The 2D spectrum was recorded at 600 MHz by using the 2D SS-EXSY sequence[5] with a Thrippleton–Keeler zero-quantum filter[15] and a mixing time of $\tau_m = 300$ ms, during which decoupling was applied using contiguous sinc pulses with a duration of 400 μs, and peak amplitudes of 7.3 kHz.

### Singlet-State Exchange Spectroscopy of BPTI at High Field

A second application where broadband decoupling with shaped pulses has proven to be particularly effective is two-dimensional correlation spectroscopy for the study of the exchange resulting from tyrosine ring flips in bovine pancreatic trypsin inhibitor (BPTI). Singlet states can be created for the coupled pairs of protons on both sides of the Tyr rings in this protein (i.e. the pairs $H^{\delta 1}$-$H^{\varepsilon 1}$ and $H^{\delta 2}$-$H^{\varepsilon 2}$, both with a J-coupling constant of $\approx 8$ Hz, shown in Figure 3 B). Zero-quantum coherences must be eliminated by proper filtration at the beginning and the end of the $\tau_m$ interval.[15] Chemical exchange between the two singlet states ($H^{\delta 1} \leftrightarrow H^{\delta 2}$ and $H^{\varepsilon 1} \leftrightarrow H^{\varepsilon 2}$) occurs as a result of the slow rotation of the Tyr rings around the $C^{\beta}$-$C^{\gamma}$ axis (e.g., with $K_{ex} \approx 30\ s^{-1}$ at 309 K for Tyr-35).[16] Tyr-35 is the most challenging case because of the dispersion of its chemical shifts (the protons $H^{\delta 2}$ and $H^{\varepsilon 2}$ are separated by 0.87 ppm, that is, 520 Hz at 600 MHz). Four signals are observed at the chemical shift of $H^{\delta 2}$ in the $\omega_1$ domain, resonating in the $\omega_2$ domain at the chemical shifts of the following protons: $H^{\delta 2}$ (diagonal peak), $H^{\varepsilon 2}$ (attributed to the distribution of coherence over the two spins involved in the singlet state at the end of $\tau_m$), and $H^{\varepsilon 1}$ and $H^{\delta 1}$ (both of which are due to slow exchange). The singlet states could be sustained—and the exchange probed—for $\tau_m = 300$ ms by using a sequence of sinc pulses. When WALTZ-16 decoupling was used under similar conditions, with $\nu_1 = 3.8$ kHz, the signals for Tyr-35 barely emerged above the noise level.

## Experimental Section

The experiments for testing the sequences were carried out at 400 MHz ($B_0 = 9.4$ T) and 300 K on a monosaccharide containing a five-membered furanose ring, which was synthesized in such a way that all the hydrogen atoms, except those in the positions $H_5'$ and $H_5''$, were substituted by deuterium atoms (as described in more detail elsewhere).[5] The concentration of this sample was $\approx 10$ mM. Four transients were acquired using a relaxation delay of 30 s, a spectral width of 80 ppm, and an acquisition lasting 1.02 s. The amplitudes of the decoupling pulses are given in the caption to Figure 2. All decoupling sequences, except for WALTZ-16, which is composed of pulses along $x$ and $-x$,[8] were formed of blocks of 20 shaped $\pi$ pulses phased according to the five-step cycle of Tycko et al.[17] {0°, 150°, 60°, 150°, 0°}, nested within an MLEV-4 supercycle.[18]

The uracil spectra were recorded at 600 MHz ($B_0 = 14.1$ T) by using a cryoprobe and accumulating four transients for each experiment. The spectral window was 10 ppm, the acquisition lasted 2.72 s, and the relaxation delay was 20 s. The concentration of the sample was $\approx 10$ mM and the temperature was 300 K.

The 2D-EXSY spectrum of BPTI was recorded at 600 MHz ($B_0 =$ 14.1 T), acquiring 224 transients and 64 points in the indirect dimension, for a spectral width of 1.7 ppm. The carrier was placed at 7.3 ppm, and the acquisition and the relaxation delays were set to 0.61 s and 1.1 s, respectively. Filtering of zero-quantum coherences with the method of Thrippleton and Keeler[15] was achieved by using 12 ms Chirp pulses with a maximum amplitude of 2.3 kHz, apodized by quarter sine waves in the first and last 10% of the sweep and with a sweep width of 80 kHz in conjunction with rectangular gradients of 27 G cm$^{-1}$. The concentration of the BPTI sample was $\approx 2$ mM, and the temperature was 309 K. The matrix of $4096 \times 64$ points was Fourier transformed to $4096 \times 256$ points.

## Conclusions

To characterize exchange rate constants when observing individual resonances in large molecules, where significant overlap may occur, or to measure slow exchange constants under various conditions (e.g. variations of temperature, pressure, etc.), it is necessary to perform experiments at high field. We have shown here that it is possible to use singlet-state exchange spectroscopy (SS-EXSY) at high fields, where the chemical-shift differences $\Delta\nu_{IS}$ and the ranges of chemical shifts $\Delta\nu$ with respect to the RF carrier are large compared to the decoupling amplitudes that can typically be attained during protracted mixing times. Long-lived states can be sustained at high fields through the use of decoupling sequences that employ shaped pulses to reduce heating of the coil and the sample. The higher the RF amplitude $\nu_1^{max}$ that can be tolerated by the probe, the larger the bandwidth of singlet-state spectroscopy. Currently, it is possible to study molecules with differences in chemical shifts of up to $\Delta\nu_{IS} \approx 1000$ Hz. The study of slow exchange phenomena, where the exchanging sites may display widely different chemical shifts, should benefit from the decoupling methods presented herein. These methods may also be useful to study long-lived states in multiple-spin systems,[19,20] as the range of frequencies that must be covered by the RF field during the $\tau_m$ interval may increase with the number of spins involved in the long-lived state. Decoupling with weak frequency-modulated adiabatic pulses opens the way to using long-lived states of heteronuclei, such as $^{13}$C, in MRI.

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**Keywords:** NMR spectroscopy · nucleotides · proteins · singlet states · slow conformational exchange

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