

Singlet-State Exchange NMR Spectroscopy for the Study of Very Slow Dynamic Processes

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Abstract: Singlet states with lifetimes that are longer than spin–lattice relaxation times $T_S \gg T_1$ offer unique opportunities for studying very slow dynamic processes in solution-state NMR. A set of novel experiments can achieve broadband excitation of singlet states in pairs of coupled spins. The most elaborate of these experiments, two-dimensional singlet-state exchange spectroscopy (SS-EXSY), is independent of the offsets of the two spins, their relative chemical shifts, and their scalar couplings. The new methods open the way to study very slow chemical exchange or translational diffusion using mixing times $\tau_m \approx T_S \gg T_1$. The lifetimes T_S of singlet states of pairs of protons in a partially deuterated saccharide are shown to be longer than the longitudinal proton relaxation times T_1 in the same compound by a factor of ca. 37.

Introduction

In conventional NMR studies of slow translational diffusion, chemical exchange, and refolding of biomolecules such as proteins or nucleic acids, the upper limit of the accessible time scale is normally determined by the longitudinal relaxation time constant T_1 , which is usually regarded as the maximum lifetime of the memory of nuclear spins. Correlations between states that are connected through very slow processes with a characteristic time constant longer than T_1 could not be monitored by NMR so far. However, recent work by Carravetta and Levitt^{1–3} has shown that it is possible to excite and observe so-called singlet states in systems containing pairs of scalar-coupled spins. Such singlet states $|S_0\rangle = N\{|\alpha\beta\rangle - |\beta\alpha\rangle\}$ with $N = 2^{-1/2}$ are antisymmetric under a permutation of the two spins, and the singlet-state lifetime T_S is not affected by the mutual dipole–dipole interaction between these two spins. Singlet-state lifetimes T_S can be more than an order of magnitude longer than longitudinal relaxation times T_1 in two-spin systems with analogous dynamic properties.⁴ A nonvanishing population of a singlet state $|S_0\rangle$ can be obtained by first exciting a zero-quantum coherence ZQ_x , i.e., a coherent superposition of two states $|\alpha\beta\rangle$ and $|\beta\alpha\rangle$ with a suitable phase, which is then converted into a population of the singlet state $|S_0\rangle$ by an appropriate radio frequency irradiation that suppresses the effects of the offsets. In practice, this irradiation converts a coupled two-spin IS system ($J_{IS} \neq 0$) into an I_2 system with two

magnetically equivalent spins. Alternatively, as shown below, a singlet state $|S_0\rangle$ can be populated by first creating a longitudinal two-spin order $\sigma = 2I_z S_z$, which, in contrast to a system in thermal equilibrium with $\sigma = I_z + S_z$, comprises eigenstates $|\alpha\beta\rangle$ and $|\beta\alpha\rangle$ with nonvanishing populations. Like a ZQ_x coherence, a $2I_z S_z$ term (also known as “ZZ order”) can be converted into a population of the singlet-state $|S_0\rangle$ by appropriate radio frequency irradiation, which in effect leads to decoupling of the J_{IS} interaction. The population of a singlet state can in principle be preserved indefinitely if decoupling is ideal and if all relaxation mechanisms other than the dipolar interaction between spins I and S can be neglected. In practice, nonideal decoupling leads to a reduction in the lifetime T_S ,⁵ as does relaxation of the I and/or S spins by chemical shift anisotropy (CSA) or by dipolar interactions with further spins in the vicinity that may belong to the same molecule or to neighboring (solvent) molecules.

It has been shown that singlet states can be exploited to study slow translational diffusion.⁴ Preliminary demonstrations have been carried out with a simple test molecule, 2-chloroacrylonitrile, which contains only two protons I and S with a small difference in chemical shifts $\Delta\nu_{IS} = \nu_I - \nu_S = 38$ Hz at 300 MHz (0.13 ppm) and a scalar coupling $J_{IS} = -3$ Hz.⁴ We have found that, contrary to earlier belief, proton-containing solvents do not lead to a dramatic reduction of the lifetime T_S of the singlet states; however, the removal of dissolved oxygen is recommended. Molecules such as saccharides that contain more protons have reduced lifetimes T_S compared to molecules that contain only isolated proton pairs, but partial deuteration of saccharides in all positions except for the H_5' and H_5'' protons (we have adopted the numbering appropriate for nucleic acids)

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leaves a pair of diastereotopic protons with $J_{IS} = J(H_5' H_5'') = -12.5$ Hz and a small difference in chemical shifts $\Delta\nu_{IS} = \nu_1 - \nu_S = \nu(H_5') - \nu(H_5'') = 75$ Hz at 400 MHz (0.18 ppm). (Such partial deuteration can be achieved conveniently by oxidation of a perdeuterated saccharide to an aldose and subsequent reduction.) When these saccharides are incorporated into nucleic acids such as RNA, the chemical shifts of the H_5' and H_5'' protons should be affected by conformational exchange and refolding processes.^{6,7} Such protons can therefore be used to study the kinetics of slow exchange, for example, by two-dimensional exchange spectroscopy (EXSY).^{8,9} The kinetic window of such experiments is normally limited by longitudinal relaxation to mixing times $\tau_m \approx T_1(^1\text{H})$. In ^1H -detected ^{15}N exchange spectroscopy this limitation can be somewhat relaxed since one can use mixing times $\tau_m \approx T_1(^{15}\text{N}) > T_1(^1\text{H})$. One of the objectives of this paper is to show that singlet-state (SS) variants of two-dimensional exchange spectroscopy (SS-EXSY) can be designed that extend the observable kinetic window to mixing times $\tau_m \approx T_S(^1\text{H}) > T_1(^{15}\text{N}) > T_1(^1\text{H})$.

The pulse sequences for singlet-state excitation that have been described so far^{3,4} suffer from a number of drawbacks: (i) the RF carrier ν_{RF} must be positioned halfway between the chemical shifts ν_1 and ν_S of the two nuclei, (ii) the efficiency of the sequences depends on the difference $\Delta\nu_{IS} = \nu_1 - \nu_S$ between the chemical shifts, and (iii) the efficiency also depends on the scalar coupling constant J_{IS} . Clearly, slow dynamic processes $A \leftrightarrow B$, such as chemical exchange or refolding of biomolecules like proteins and nucleic acids, must lead to changes in chemical shifts $\nu_1^A \neq \nu_1^B$ and/or $\nu_S^A \neq \nu_S^B$ to be observable by NMR. In general, the differences in chemical shifts may also be affected by chemical exchange, i.e., $\Delta\nu_{IS}^A = (\nu_1^A - \nu_S^A) \neq \Delta\nu_{IS}^B = (\nu_1^B - \nu_S^B)$. Furthermore, it is possible that the scalar couplings are also affected by chemical exchange, i.e., $J_{IS}^A \neq J_{IS}^B$. If singlet states are to be used to investigate such slow processes, the pulse sequences must be modified so as to become independent of chemical shifts and couplings.

In this work, we present new methods that make slow exchange phenomena in biological macromolecules amenable to study via singlet-state NMR spectroscopy. To begin with, we introduce ways to excite singlet states in spin pairs with different chemical shifts and J -coupling constants. Next, we show that adequate broadband decoupling preserves singlet states over a wide range of frequencies, and finally we present a 2D experiment that can correlate signals of coupled spins that are exchanging slowly between different magnetic environments that lead them to have different chemical shifts and J -couplings. Such slow exchange processes occur, for instance, during the refolding of RNA⁷ and play a key role in protein–protein interactions.¹⁰ Their study can greatly benefit from the atomic resolution that NMR can afford in native molecular conditions.

Theory

As will be shown below, there are two distinct ways of obtaining a system described by a density matrix σ in which

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the singlet state $|S_0\rangle$ has a nonvanishing population $p(S_0) = \text{Tr}\{\sigma|S_0\rangle\langle S_0|\}$. Prior to applying the RF field, one must have either a nonvanishing ZQ_x zero-quantum coherence or a nonvanishing longitudinal two-spin ZZ order $2I_zS_z$, or a combination of both ZQ_x and ZZ. There are two orthogonal forms of zero-quantum coherences, i.e., $ZQ_x = \{|\alpha\beta\rangle\langle\beta\alpha| + |\beta\alpha\rangle\langle\alpha\beta|\}/2$ and $ZQ_y = -\{i|\alpha\beta\rangle\langle\beta\alpha| - i|\beta\alpha\rangle\langle\alpha\beta|\}/2$. Only the ZQ_x component can be converted into a singlet state by decoupling. On the other hand, two-spin ZZ order can be expressed as a linear combination of four populations, i.e., $2I_zS_z = \{|\alpha\alpha\rangle\langle\alpha\alpha| - |\alpha\beta\rangle\langle\alpha\beta| - |\beta\alpha\rangle\langle\beta\alpha| + |\beta\beta\rangle\langle\beta\beta|\}/2$. Only the middle two terms can contribute to the population of the singlet state $p(S_0)$ when the RF irradiation is switched on. This population $p(S_0)$ can be determined by converting the ZQ_x and ZZ terms contained in the density operator from the product base PB into the singlet–triplet base STB. Likewise, when the RF irradiation is switched off at the end of the mixing interval τ_m , it is sufficient to convert the remaining population $p(S_0)$ from the singlet–triplet base STB into ZQ_x and ZZ terms in the product base PB. The latter can then be converted into observable terms following the usual rules of product operator calculations.¹¹

Through a simple basis transformation, any Cartesian operator product represented in the product base PB of the weakly coupled IS system can be expressed in the singlet–triplet base STB of the I_2 system when the two spins are in effect made to be magnetically equivalent. The matrix representations of various terms in the two bases are given in Table 1 of the Supporting Information. The following combinations of Cartesian operator terms in the product base PB can contribute to the populations of the singlet–triplet base STB:

$$\{E, (I_z + S_z), 2I_zS_z, ZQ_x\}_{\text{PB}} \quad (1)$$

The conversion matrix is the following:

$$\begin{pmatrix} |S_0\rangle\langle S_0| \\ |T_0\rangle\langle T_0| \\ |T_1\rangle\langle T_1| \\ |T_{-1}\rangle\langle T_{-1}| \end{pmatrix} = \begin{pmatrix} \frac{1}{4} & 0 & -\frac{1}{2} & -1 \\ \frac{1}{4} & 0 & -\frac{1}{2} & 1 \\ \frac{1}{4} & \frac{1}{2} & \frac{1}{2} & 0 \\ \frac{1}{4} & -\frac{1}{2} & \frac{1}{2} & 0 \end{pmatrix} \cdot \begin{pmatrix} E \\ I_z + S_z \\ 2I_zS_z \\ ZQ_x \end{pmatrix} \quad (2)$$

It can readily be seen that only longitudinal two-spin order $2I_zS_z$ and real zero-quantum coherence ZQ_x can be converted into singlet-state populations. The inverse transformation can be described by the following matrix:

$$\begin{pmatrix} E \\ I_z + S_z \\ 2I_zS_z \\ ZQ_x \end{pmatrix} = \begin{pmatrix} 1 & 1 & 1 & 1 \\ 0 & 0 & 1 & -1 \\ -\frac{1}{2} & -\frac{1}{2} & \frac{1}{2} & \frac{1}{2} \\ -\frac{1}{2} & \frac{1}{2} & 0 & 0 \end{pmatrix} \cdot \begin{pmatrix} |S_0\rangle\langle S_0| \\ |T_0\rangle\langle T_0| \\ |T_1\rangle\langle T_1| \\ |T_{-1}\rangle\langle T_{-1}| \end{pmatrix} \quad (3)$$

Clearly, if we ignore the unobservable unity operator E , the singlet-state populations can only be converted into longitudinal two-spin order $2I_zS_z$ and real zero-quantum coherence ZQ_x . The transformations of the 16-dimensional Liouville space are given in the Supporting Information.

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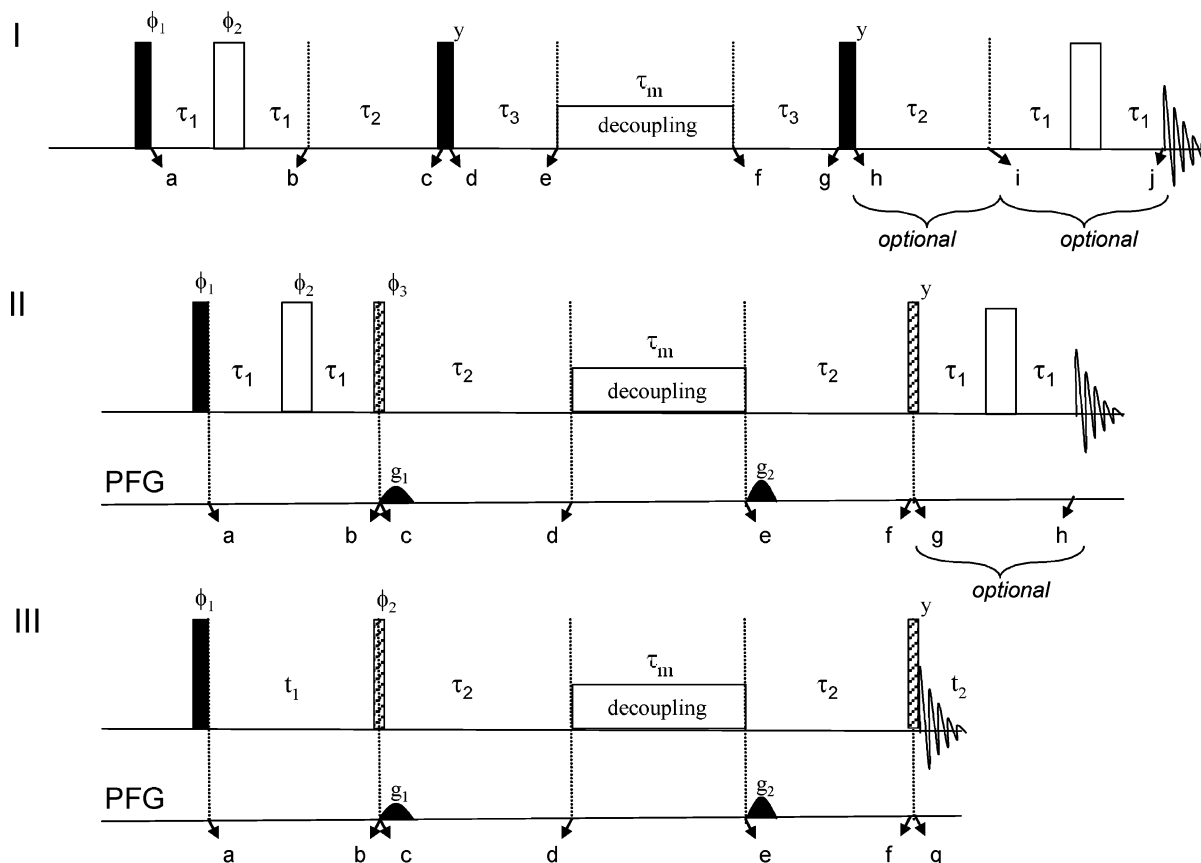


Figure 1. Pulse sequences designed for excitation of singlet-state populations and their subsequent reconversion into observable magnetization. Pulses with flip angles of π , $\pi/2$, and $\pi/4$ (45°) are indicated by open, filled, and hatched rectangles, respectively. All pulses are applied with phases along the x -axis of the rotating frame, unless otherwise indicated. The delays must be adjusted to the offsets ν_I and ν_S of spins I and S and to the coupling constant J_{IS} according to Table 1: $\tau_1 = 1/4J_{IS}$, $\tau_2 = 1/[2(\nu_I - \nu_S)]$, $\tau_3 = \tau_2/2$. Sequence I also requires that the carrier be set halfway between the two chemical shifts, $\nu_{RF} = (\nu_I + \nu_S)/2$. (I) Sequence for singlet-state excitation designed by Caravetta and Levitt,³ expanded with some optional delays for the sake of symmetry. The singlet state is excited only via ZQ coherences which precess in the τ_3 intervals. The phase cycle is $\varphi_1 = x, -x$, $\varphi_2 = 2(x), 2(-x)$, and $\varphi_{rec} = x, -x$. (II) Sequence for singlet-state excitation that uses both ZQ coherences and longitudinal two-spin order $2I_zS_z$ (“ZZ” order) in the τ_2 intervals. The phase cycle was $\varphi_1 = x, -x$, $\varphi_2 = 2(x), 2(-x)$, $\varphi_3 = 4(y), 4(-y)$, and $\varphi_{rec} = 2(x, -x), 2(-x, x)$. (III) Sequence designed for two-dimensional singlet-state exchange spectroscopy (2D SS-EXSY) to monitor correlations between different states of a spin system undergoing a slow dynamic process. The singlet state is excited via a combination of ZQ and ZZ terms in the τ_2 intervals. The phase cycle was $\varphi_1 = x, -x$, $\varphi_2 = 2(y), 2(-y)$, and $\varphi_{rec} = x, 2(-x), x$. Quadrature detection in the t_1 dimension was achieved by time-proportional phase incrementation of φ_1 (TPPI). The pulsed-field gradients (PFG) g_1 and g_2 lead to dephasing of coherences with order $p \neq 0$.

Experimental Methods

This study presents a series of new spectroscopic methods that are designed to fulfill the following demands:

(i) Broadband excitation of singlet-state populations in both 1D and 2D NMR spectroscopy to convert the equilibrium populations of the IS system into singlet-state populations of the I_2 system over a wide range of shifts and couplings. Previous methods contained intervals that had to be adapted to the coupling constants and to the offsets between the individual chemical shifts and the RF carrier, which made them impractical for molecules containing coupled spin pairs featuring a range of chemical shifts or couplings;

(ii) An evaluation of adequate broadband composite decoupling methods for optimizing the lifetime T_S of the singlet state when there is a nonvanishing offset between the average chemical shift of the two spins and the RF carrier;

(iii) Two-dimensional experiments designed to study correlations between different environments, with a mixing time period τ_m where the “memory” of the system is “stored” in the form of singlet-state populations.

Figure 1 shows a comparison between existing methods (that require prior knowledge of the chemical shifts and scalar couplings) and new proposed techniques for broadband singlet-state excitation. A step-by-step analysis of the sequences in terms of product operators is given in

the Supporting Information. In sequence I of Figure 1 (which becomes equivalent to the experiment of Carravetta and Levitt if the optional delays at the end are dropped), single-quantum in-phase coherences excited at point a are transformed into antiphase coherences between points a and b. Between points b and c, the chemical shifts lead to a conversion of the real terms $2I_zS_x + 2I_xS_z$ (which have the same phase) into imaginary terms $2I_zS_y - 2I_xS_z$ (with opposite phases). To achieve this effect, the carrier $\nu_{RF} = (\nu_I + \nu_S)/2$ has to be set precisely halfway between the chemical shifts of spins I and S. The $(\pi/2)_y$ pulse at point c excites imaginary zero-quantum coherence $ZQ_y = 2I_xS_y - 2I_yS_x$. Between points d and e, this is converted into the desired real ZQ_x coherence. As explained in more detail in the Supporting Information, this coherence is transformed into opposite contributions to the populations of the singlet and central triplet states under RF irradiation starting at point e. The triplet state populations can be neglected because they do not survive the protracted decoupling period.

The new sequence II in Figure 1 was designed for broadband excitation of singlet states in coupled spin systems. In contrast to sequence I, where the carrier must be set halfway between the two chemical shifts, i.e., $\nu_{RF} = (\nu_I + \nu_S)/2$, sequence II does not have any requirement on ν_{RF} . Consequently, it is possible to study molecules with multiple sites undergoing dynamic effects if they feature different chemical shifts in the two conformations A and B, provided the

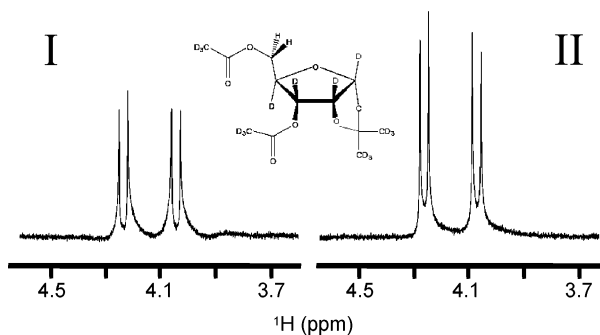


Figure 2. Comparison of signals of the H_5' and H_5'' protons in the partly deuterated saccharide shown in the inset, obtained with sequences I (left panel) and II (right panel) of Figure 1, including the “optional” elements, at 300 K and 400 MHz. Sequence II is less prone to various relaxation effects. The doublets stem from the diastereotopic protons with $J(H_5', H_5'') = -12.5$ Hz. The fixed intervals were $\tau_1 = 20$ ms, $\tau_2 = 6.7$ ms, and $\tau_m = 5$ s. The continuous decoupling field had an amplitude of ca. 1.2 kHz. To limit artifacts arising from convection induced by sample heating, a Shigemmi sample tube was used with a sample height of ca. 15 mm.

chemical shift differences between the coupled pairs of protons are preserved ($\Delta\nu_{IS}^A = \Delta\nu_{IS}^B$). This is achieved by exciting both ZQ_x coherence and longitudinal two-spin order $2I_S S_z$ using a $(\pi/4)_x$ pulse at point b. Between time points c and d, the sign of the ZQ_x coherence is reversed ($ZQ_x \rightarrow -ZQ_x$) under the effect of the difference of the chemical shifts $\Delta\nu_{IS} = \nu_1 - \nu_S$, a process which we may refer to as “ZQ reversal”. Since $\tau_2 = 1/(2\Delta\nu_{IS})$, this requires prior knowledge of the relative chemical shifts but not of the individual offsets. This ZQ reversal is necessary to prevent mutual cancellation of ZQ and ZZ contributions to the singlet state. As seen from the analysis provided in the Supporting Information, the singlet population created at point d of sequence II is equal to the singlet population created at point e of sequence I. The sensitivities of these two experiments are therefore comparable if losses through various relaxation processes can be neglected. It should be emphasized that the mechanisms are not the same: in sequence I, only a ZQ_x term contributes to the population of the singlet state, while a combination of ZQ_x and $2I_S S_z$ contributes in sequence II. In the last part of sequence II, the singlet-state population remaining at the end of the mixing time τ_m is reconverted into single-quantum coherences via symmetrical processes. If the optional delays at the end of sequence II are used, two in-phase doublets with the same sign for both spins I and S can be observed. In Figure 2 we show a comparison between signals detected using sequences I and II in Figure 1, using the pair of protons in a partly deuterated saccharide shown in the inset.

The 2D sequence III in Figure 1 does not contain any fixed $[\tau_1 - \pi - \tau_1]$ interval for generating antiphase magnetization, since such terms will build up naturally, with a coefficient $\sin(\pi J_{IS} t_1)$, in the course of the evolution time t_1 . Like other 2D experiments with antiphase multiplet structures (such as COSY, ZZ-EXSY, etc.), this requires an adequate choice of the maximum duration t_1^{\max} of the evolution period so that one achieves sufficient resolution in the ω_1 domain to resolve the antiphase J -coupled multiplets. In sequence III, the coherence transfer from both I and S spins that undergo precession in the evolution period contributes to both ZQ and ZZ terms and, hence, to the population of the singlet state during the mixing time. The reconversion of singlet-state populations into ZQ and ZZ terms and from there into observable (antiphase) coherences of spins I and S follows similar pathways as described above for sequence II.

It is noteworthy that in the case where not only the average chemical shift but also the difference $\Delta\nu_{IS} = \nu_1 - \nu_S$ changes from site to site ($\Delta\nu_{IS}^A \neq \Delta\nu_{IS}^B$) neither is it possible to ensure that the ZQ_y terms in sequence I are converted into ZQ_x , nor can one be sure that the ZQ_x components in sequences II or III are converted into $-ZQ_x$. Therefore,

it is preferable to suppress the zero-quantum terms by a filter proposed by Thrippleton and Keeler,^{12,13} since the longitudinal two-spin order $2I_S S_z$ can still be used to generate singlet-state populations. This is demonstrated in the 1D and 2D sequences IV and V of Figure 3, where two frequency-swept pulses and pulsed field gradients, both of duration τ_f , are inserted prior to and after the mixing period. The most effective suppression of ZQ_x was achieved by using a first gradient along the z -axis and a second gradient along an orthogonal (x or y) axis. The use of the filter makes the efficiency of singlet-state excitation and reconversion entirely independent not only on the chemical shift differences $\Delta\nu_{IS} = \nu_1 - \nu_S$ but also on the individual offsets ν_1 and ν_S . This extension of the range comes at a cost of 50% of the signal intensity for each interval where the ZQC is suppressed, so the 2D sequence V affords only 25% of the signal detected with the 2D sequence III (see Supporting Information). The pathways shown below the 2D sequence of Figure 3 indicate the population transfer processes that occur between the evolution and detection intervals.

In systems where the selective excitation of ZZ order suffices, say to determine the time constant T_S in the absence of exchange, it is possible to excite ZZ order directly from thermal equilibrium by selective inversion of one or several lines in the single-quantum spectrum¹⁴ albeit at the expense of a 50% loss in signal intensity which can only be recovered by using both ZZ and ZQ terms.

Table 1 summarizes to what extent the fixed delays in sequences I to V have to be adapted to the scalar coupling constant J_{IS} and to the difference in chemical shifts $\Delta\nu_{IS} = \nu_1 - \nu_S$. Only the original sequence I of Carravetta and Levitt¹ requires that the RF carrier be positioned in the center of the spectrum $\nu_{RF} = (\nu_1 + \nu_S)/2$. If there are no requirements, the sequences may be considered to be broadband with respect to the average shift $(\nu_1 + \nu_S)/2$, to the relative shift $(\nu_1 - \nu_S)$, and to the coupling constant J_{IS} .

The broadband character of the excitation and reconversion of singlet states is not the only requirement for studying pairs of spins in different environments simultaneously. In order to preserve the populations of singlet states for long time periods, suitable decoupling sequences have to be used. Figure 4 shows signals of the saccharide described below, observed using sequence II while stepping the offset between the RF carrier and the average chemical shift, using either continuous-wave (CW) decoupling or composite-pulse radio frequency irradiation. The use of the WALTZ16 sequence¹⁵ has proven to be effective in preserving the singlet state over a range of ± 1 kHz with respect to the carrier (± 2.5 ppm at 400 MHz). This is a satisfactory range for typical groups involving coupled protons in biomolecules, such as diastereotopic CH_2 groups in proteins or nucleic acids.

A mixing period τ_m with continuous decoupling, no matter how weak, may lead to undesirable heating effects that can cause convection currents. This phenomenon can lead to a shortening of the apparent lifetimes $T_S^{\text{app}} < T_S$ of the singlet-state populations if the molecules that carry these singlet states move out of the sensitive volume enclosed by the transmitter and receiver coils. The use of Shigemmi tubes with a limited sample height is recommended to combat such artifacts.

Results and Discussion

A monosaccharide with a five-membered furanose ring was synthesized in such a manner that all hydrogen atoms except those in positions H_5' and H_5'' were substituted by deuterium atoms. The H_5' and H_5'' protons have a chemical shift difference of 75 Hz at 400 MHz (0.18 ppm) and a scalar coupling constant

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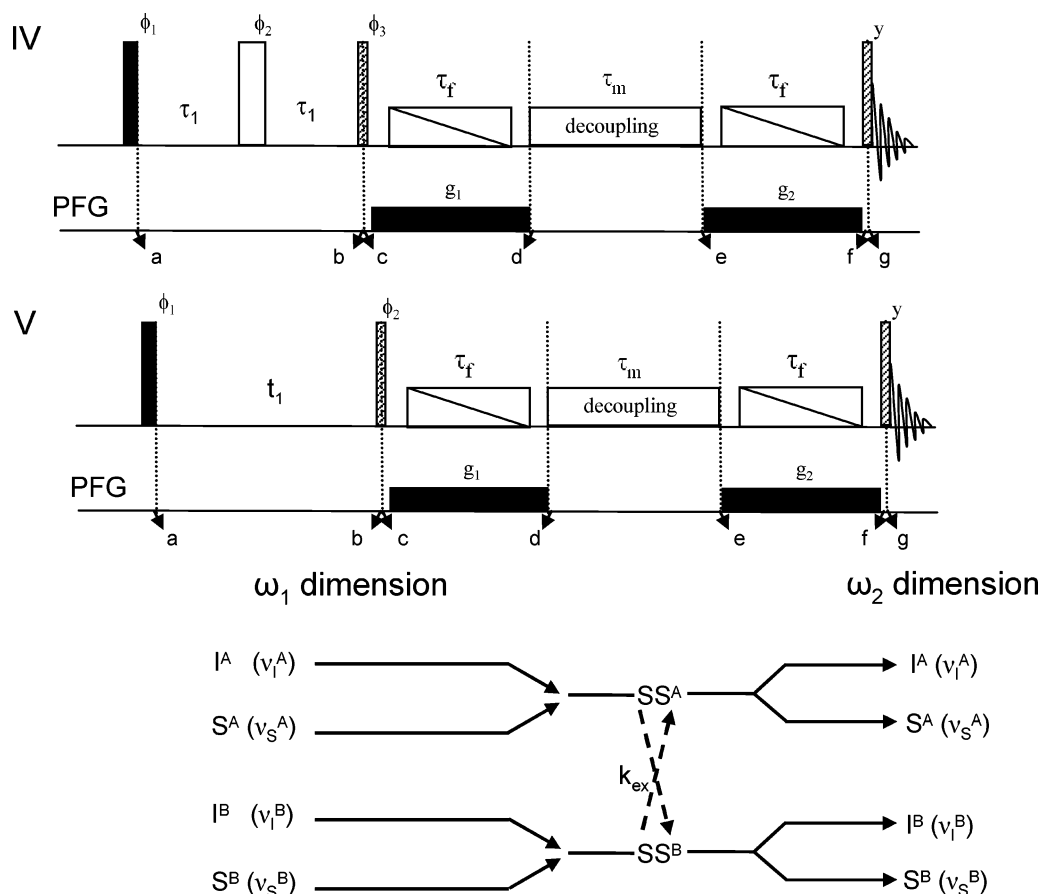


Figure 3. (IV) Sequence designed for 1D spectroscopy with excitation of singlet-state populations only via two-spin ZZ order $2I_S S_z$, using filters to suppress the ZQ terms in the τ_f intervals. The rectangles with diagonal bars represent adiabatic frequency-swept pulses applied in conjunction with pulsed field gradients (black rectangles) to suppress zero-quantum coherences. The phase cycle is the same as that for sequence II. (V) Sequence for 2D SS-EXSY using only two-spin ZZ order. The phase cycle is the same as that for sequence III. The chemical shift correlations that can be observed in the 2D spectra are shown schematically below the pulse sequence.

Table 1. Comparison of the Five Sequences Presented in Figures 1 and 3, Showing Which Delays Must Be Adapted to the Coupling Constant J_{IS} , the Relative Shift $(\nu_I - \nu_S)$, and the Average Shift $(\nu_I + \nu_S)/2^a$

adjustment with respect to	sequence I	sequence II	sequence III	sequence IV	sequence V
coupling constant J_{IS}	$\tau_1 = 1/ 4J_{IS} $	$\tau_1 = 1/ 4J_{IS} $	broadband	$\tau_1 = 1/ 4J_{IS} $	broadband
relative shift $\Delta\nu_{IS} = \nu_I - \nu_S$	$\tau_2 = 1/(2\Delta\nu_{IS})$	$\tau_2 = 1/(2\Delta\nu_{IS})$	$\tau_2 = 1/(2\Delta\nu_{IS})$	broadband	broadband
average shift $(\nu_I + \nu_S)/2$	$\tau_3 = \tau_2/2$	broadband	broadband	broadband	broadband
relative sensitivity (%) (neglecting relaxation)	$\nu_{RF} = (\nu_I + \nu_S)/2$	100	100	25	25

^a If there is no need for any adjustment, the experiment has a broadband character with respect to the corresponding parameters.

of $J_{IS} = J(H_5'H_5'') = -12.5$ Hz. The sample was initially dissolved in deuterated degassed DMSO in view of reducing intermolecular dipolar interactions with the solvent. For both H_5' and H_5'' protons, we determined the same nonselective longitudinal relaxation time constant $T_1(H_5') = T_1(H_5'') = 0.7 \pm 0.03$ s, while the lifetime of the singlet state was found to be $T_S(H_5', H_5'') = 26 \pm 3$ s, which is longer than T_1 by a factor of 37 ± 6 . In nondeuterated degassed DMSO, we have measured a similar value. This shows that the long lifetimes of singlet states can be exploited in protonated solvents. Removal of paramagnetic oxygen by using a few pump–freeze–thaw cycles or, as in our case, by letting a stream of argon bubble through the sample is essential to obtain the maximum value of $T_S(H_5', H_5'')$, which otherwise can be as low as 15 s. It is however difficult to seal degassed samples while using sample tubes with

a limited height to combat convection. For a similar saccharide where none of the protons were substituted by deuterons, we measured $T_1(H_5') = T_1(H_5'') = 0.34 \pm 0.02$ s and $T_S(H_5', H_5'') = 1.5 \pm 0.2$ s. Thus, partial deuteration leads to a 2-fold extension of the longitudinal relaxation time constant T_1 and to a 17-fold extension of the singlet-state lifetime T_S .

Figure 5 shows a two-dimensional singlet-state exchange (SS-EXSY) spectrum of the selectively deuterated monosaccharide, recorded using sequence III of Figure 1. The 2D spectrum was deliberately acquired while placing the carrier 160 Hz away from the average shift of the two proton spins, to illustrate the broadband character of the method. The mixing time during which spins were maintained in the singlet state was $\tau_m = 12$ s. As expected, each spin gives rise to antiphase doublets in both dimensions with respect to the J coupling.

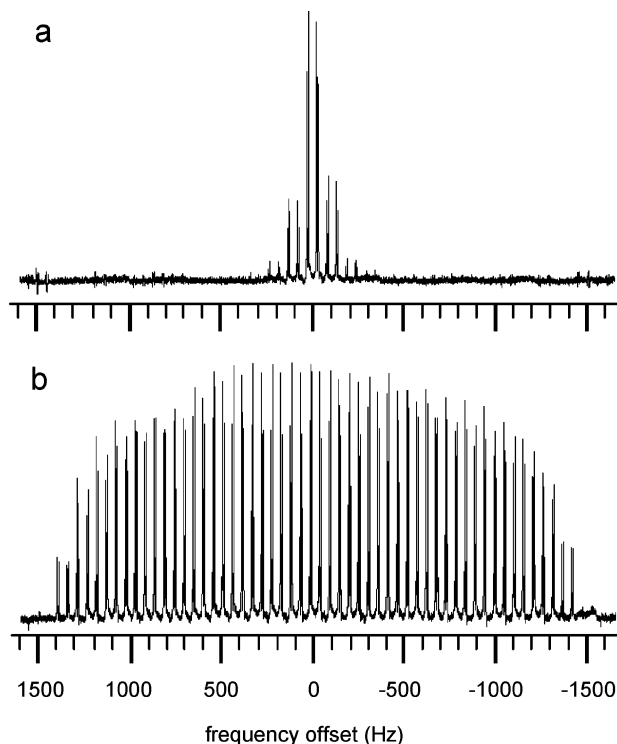


Figure 4. Signal intensities (obtained with sequence II) of the partly deuterated saccharide shown in Figure 2, as a function of the offset $\Delta\nu = \nu_{av} - \nu_{RF}$ between the center of the two resonances $\nu_{av} = (\nu_1 + \nu_S)/2$ and the radio frequency carrier ν_{RF} . (a) CW decoupling; (b) WALTZ16 decoupling. The frequency ν_{RF} was used for all pulses and during the decoupling period, and it was incremented in 33 steps of 100 Hz. The amplitude of the decoupling field was $\nu_1 = \omega_1/2\pi = 976$ Hz, and the duration of the mixing period was $\tau_m = 10$ s. The fixed intervals were the same as those in Figure 2. At each step a signal consisting of two in-phase doublets is detected, as in Figure 2.

This 2D experiment is conceived to excite singlet states in different environments (for example in two distinct environments A and B where the spins I and S have chemical shifts and couplings $\nu_1^A, \nu_S^A, J_{IS}^A$ and $\nu_1^B, \nu_S^B, J_{IS}^B$, respectively). The chemical shifts are first labeled in the evolution interval t_1 , so that the coherences at point b in sequences III and V are modulated at the frequencies $\nu_1^A, \nu_S^A, \nu_1^B$, and ν_S^B . Singlet states SS^A and SS^B are then excited at point d for both environments A and B. During the interval τ_m , exchange may occur between the environments A and B, leading to a partial interconversion of singlet-state populations SS^A and SS^B . At point g the singlet-state populations are transformed back into antiphase single-quantum coherence of spins I and S and resume precession at the four chemical shifts in the t_2 detection interval. Thus, even in the absence of exchange, a magnetization that was initially labeled at a frequency ν_1^A during t_1 is detected at frequencies ν_1^A and ν_S^A in t_2 because the spins I and S are mixed to form a common singlet state during τ_m . If exchange between states A and B occurs, additional signals will be detected at frequencies ν_1^B and ν_S^B in t_2 , as shown schematically in Figure 3. Thus, in the presence of exchange, one diagonal and three cross-peak multiplets can be detected at each ω_1 frequency. In the absence of exchange, only one diagonal and one cross-peak multiplet (which arises from the blending of the magnetization of two the spins in the singlet state) are detected, as it can be seen in Figure 5.

The structure of a 2D spectrum obtained using the sequence in Figure 3 is reminiscent of that of ZZ-EXSY¹⁴ spectra, so, by

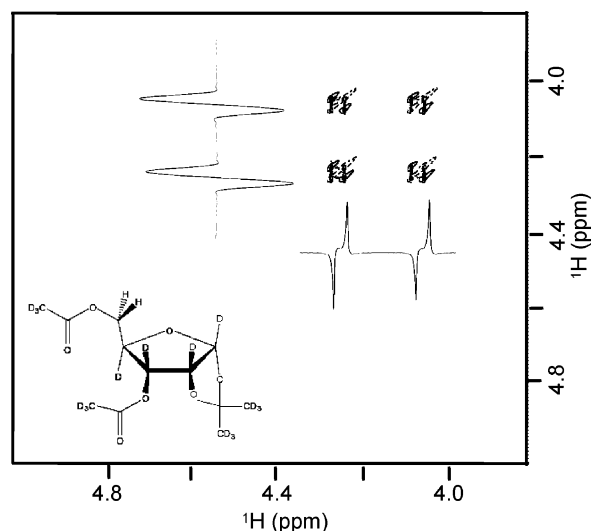


Figure 5. 2D singlet-state exchange spectrum of the $H_{5'}$ and $H_{5''}$ protons in the partly deuterated saccharide again shown in the inset, recorded with sequence III at 300 K and 400 MHz. Positive and negative signals are represented by continuous and dotted contours. A matrix of $32k \times 256$ points was acquired and transformed to $32k \times 1k$ points. The spectral widths in the ω_2 and ω_1 dimensions were 10.2 and 2.6 ppm (ca. 4 and 1 kHz). The carrier frequency ν_{RF} was set at 4.5 ppm, at an offset $\Delta\nu = \nu_{av} - \nu_{RF} = 160$ Hz from the center of the two resonances $\nu_{av} = (\nu_1 + \nu_S)/2$. The duration of the mixing period with WALTZ16 decoupling was $\tau_m = 12$ s, and the amplitude of the decoupling field was 976 Hz. The fixed intervals were the same as those in Figure 2. The peak amplitudes of the g_1 and g_2 pulsed field gradients (PFG) were 73% and 29% of the maximum intensity (50 G/cm), respectively.

analogy, we like to refer to our new singlet-state method as SS-EXSY. The side chain of Tyrosine 35 in basic pancreatic trypsin inhibitor (BPTI) is a good illustration for exchange studies, as the shifts of the four protons in the δ and ϵ positions are well-dispersed.¹⁶ Chemical exchange ($H^{\delta 1} \leftrightarrow H^{\delta 2}$ and $H^{\epsilon 1} \leftrightarrow H^{\epsilon 2}$) appears due to the slow rotation (~ 30 s⁻¹ at 309 K) of the Tyr ring around the $C^\beta-C'$ axis. The singlet state can be created for coupled pairs of protons on both sides of the ring (i.e., the pairs $H^{\delta 1}-H^{\epsilon 1}$ and $H^{\delta 2}-H^{\epsilon 2}$, both with a J -coupling constant of ~ 8 Hz). A 2D SS-EXSY spectrum of the aromatic region of BPTI is shown in Figure 6. As with ZZ exchange spectroscopy¹⁴ four cross-peak multiplets of Tyr35 were observed centered on the chemical shift position of $H^{\delta 2}$ in the ω_1 domain: $H^{\delta 2}$ (diagonal peak, coded in green in Figure 6), $H^{\epsilon 2}$ (due to the mixing of magnetization of the two spins in the singlet state, in blue), and $H^{\epsilon 1}$ and $H^{\delta 1}$ (both of which are due to slow exchange, color-coded in red). For residues Y10, Y21, and Y23 in BPTI, the exchange cross-peaks coincide with the singlet-mixing cross-peaks, due to the fact that the chemical shifts of the $H^{\delta 1}$ and $H^{\delta 2}$ protons, on the one hand, and those of the $H^{\epsilon 1}$ and $H^{\epsilon 2}$ protons, on the other hand, are averaged by fast exchange on the NMR time scale.

The 2D method illustrated by the spectra of Figures 5 and 6 would prove useful if it can be applied to the study of RNA strands that slowly interconvert between different conformations. In a recent study, an RNA 34-mer was shown to exist in three conformations. The rates of the slow exchange processes suggest that refolding involves the simultaneous dissociation of several base pairs. When slowly relaxing longitudinal ¹⁵N-magnetization

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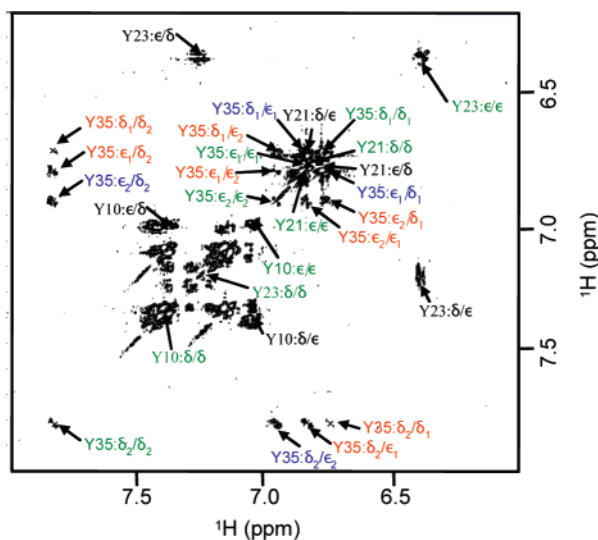


Figure 6. 2D singlet-state exchange spectrum of aromatic protons in BPTI, recorded with sequence V at 310 K and 400 MHz. The sample was not deuterated. A matrix of $4k \times 256$ points was acquired and transformed to $8k \times 512$ points. The spectral widths in the ω_2 and ω_1 dimensions were 5.6 and 2.2 ppm. The carrier frequency ν_{RF} was set at 7.3 ppm. The duration of the mixing period with WALTZ16 decoupling was $\tau_m = 200$ ms, and the RF amplitude of the decoupling field was 1427 Hz. The peak amplitudes of the g_1 and g_2 pulsed field gradients (PFG) were 28.5% and 65% of the maximum intensity (50 G/cm) along the z and y axes, respectively. A Chirp inversion pulse of length $\tau_f = 12$ ms, with a sweep width of 80 kHz and a maximum amplitude of 2348 Hz, was used simultaneously with the gradients for filtering ZQ_x coherences. The assignments of diagonal signals are labeled in green, those of cross-peaks originating from exchange are labeled in red, while those of peaks originating from mixing of magnetization in the singlet state are labeled in blue. Wherever the two types of cross-peaks may be in overlap due to the position of resonances, black labels are used.

was used to store information during the mixing time ($\tau_m = 400$ ms), only two out of three coexisting conformations showed evidence of mutual exchange. Singlet-state exchange spectroscopy, as described in this work, should allow one to extend the mixing time τ_m by at least an order of magnitude and, hence, investigate whether the third fold might be involved in exchange on a slower time scale.

A 2D experiment using long mixing times, as afforded by the lifetime of the singlet state, may prove useful for the purpose of assigning signals belonging to unfolded conformations. The applications are by no means limited to nucleic acids. Proteins have also been shown to feature exchange on slow time scales of hundreds of milliseconds to seconds in global unfolding, allosteric transitions, “breathing” motions, and flips of aromatic rings.^{16,17} Diastereotopic J -coupled pairs of protons in various amino acids can be used for generating singlet states. If a perdeuterated protein is dissolved in H_2O , the δ and ϵ sites in

asparagine and glutamine, respectively, will be protonated, thus providing opportunities to generate isolated singlet states in a deuterated background. Such singlet states would act as probes of exchange processes such as slow rotations around nearby N–C bonds. These types of rotations have been shown to occur in asparagine side chains in the active site of an alpha-neurotoxin, a dynamic event that is believed to play a role in blocking neuromuscular transmissions.¹⁰

Conclusions

This study demonstrates that singlet states with long lifetimes can be excited efficiently over significant spectral ranges. The singlet-state lifetimes are remarkably long in selectively deuterated saccharides that could be incorporated into nucleic acids. Slow exchange processes that occur in such molecules⁷ could hitherto not be characterized by conventional EXSY methods because of short longitudinal relaxation times T_1 but now become experimentally accessible with the SS-EXSY experiment. This method should be useful to monitor dynamic processes such as chemical exchange, refolding of proteins or nucleic acids, and hindered translational diffusion of large molecules in porous media. The time window of such studies can be extended by about an order of magnitude in comparison to conventional EXSY methods employing longitudinal magnetization. In principle, it should also be possible to monitor higher-order cross-relaxation processes, which could convert a singlet state SS^A into another singlet state SS^B through a dual flip-flop process that would lead to an interchange of the spin states of I^A and I^B on the one hand and of S^A and S^B on the other. Although such processes are likely to build up very slowly, they can be expected to do so within the long lifetimes of singlet states.

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Supporting Information Available: Expressions of the relevant product operators in the product base (PB) and singlet–triplet base (STB); description of the pulse sequences in terms of product operators. Supplementary figures containing spectra acquired with sequences II and IV and exponential fits of the signals as a function of the decay time. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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