

Measurement of Slow Diffusion Coefficients of Molecules with Arbitrary Scalar Couplings via Long-Lived Spin States

Riddhiman Sarkar,^[a] Puneet Ahuja,^[a] Paul R. Vasos,^{*[a]} and Geoffrey Bodenhausen^[a, b]

New experiments are described for the determination of very slow diffusion constants by nuclear magnetic resonance (NMR) using long-lived (singlet) states. These experiments are suitable

for molecules or conformations featuring a wide range of J -couplings.

1. Introduction

For nuclear magnetic resonance (NMR) of systems comprising only two scalar-coupled spins I and $S = 1/2$, it has been shown by Carravetta, Johannessen and Levitt^[1–3] that spin populations can be stored in singlet states. These antisymmetric states, $|SS\rangle = N(|\alpha_i\beta_s\rangle - |\beta_i\alpha_s\rangle)$, with the normalisation factor $N = 2^{-1/2}$, have smaller relaxation rates and hence longer lifetimes than any other spin states, because their spin–exchange symmetry makes them immune to the I – S dipolar interaction. Their long lifetimes allow one to monitor various dynamic phenomena that span time scales longer than the longitudinal relaxation times T_1 , notably slow translational diffusion^[4,5] and slow chemical exchange.^[6] Such dynamic processes were hitherto not amenable to NMR studies.

A long-lived state can be excited by generating a difference between the mean population of the three triplet states, $|T_+\rangle = |\alpha_i\alpha_s\rangle$, $|T_0\rangle = N(|\alpha_i\beta_s\rangle + |\beta_i\alpha_s\rangle)$, and $|T_-\rangle = |\beta_i\beta_s\rangle$, and that of the singlet $|SS\rangle$. In Liouville space, this difference can be written as Equation (1):

$$Q_{LLS} = N_{LLS} \left[|SS\rangle\langle SS| - \frac{1}{3} (|T_0\rangle\langle T_0| + |T_+\rangle\langle T_+| + |T_-\rangle\langle T_-|) \right] \quad (1)$$

where $N_{LLS} = \sqrt{3}/2$ is a normalisation factor. The excitation requires the generation of an excess population in selected spin states. Excitation sequences that are tailored to specific coupling constants J_{IS} or chemical shift differences $\Delta\nu_{IS}$ may fail for spin systems with different parameters. The dependence of the excitation efficiency on the chemical shifts can be alleviated considerably by inserting a Thrippleton–Keeler (TK) filter, that is, a combination of strong gradients and frequency-swept pulses designed to suppress zero-quantum coherences.^[7,8] Provided one is willing to resort to two-dimensional (2D) experiments,^[6] it is possible to excite long-lived states over a range of coupling constants J_{IS} , by allowing the J -coupling to act during the evolution time t_1 . In many diffusion studies however, spectral overlap is not a problem, so that two-dimensional techniques are rather wasteful because of their time requirements. None of the one-dimensional (1D) methods known so far can simultaneously excite singlet states in mixtures of molecules featuring a range of J_{IS} coupling constants.

The methods presented herein are intended to fill this gap by demonstrating a new broad-band 1D method that can excite singlet states over a range of scalar couplings and chemical shifts. The new experiments may be used to determine diffusion coefficients in a mixture of slowly-diffusing molecules in solution. They can also be used when only some of the species diffuse slowly, for example, when a mixture of ligands is tested for binding to macromolecules.

2. Methods

The nomenclature of the relative chemical shifts and J -couplings that are pertinent for the excitation of singlet states is shown in Figure 1A. In the singlet-state diffusion-ordered spectroscopy (SS-DOSY) pulse sequence^[4] of Figure 1B, designed for the study of diffusion using singlet states, two requirements must be met, as shown in Equations (2a):

$$\tau_1 = 1/(4J_{IS}) \quad (2a)$$

and (2b):

$$\tau_2 = 1/(2\Delta\nu_{IS}) \quad (2b)$$

We have shown elsewhere^[6,8] how 2D spectroscopy experiments can be made independent of differences in chemical shifts $\Delta\nu_{IS}$ as well as J -couplings. Herein, we demonstrate a way to alleviate these dependencies in 1D experiments as well.

The sequence of Figure 1B relies on the presence of anti-phase single-quantum coherence ($2I_xS_z + 2I_zS_x$) at time point b . The excitation of this coherence must be maximised over a

[a] R. Sarkar, P. Ahuja, Dr. P. R. Vasos, Prof. Dr. G. Bodenhausen
Institut des Sciences et Ingénierie Chimiques
Ecole Polytechnique Fédérale de Lausanne, EPFL
Batochime, 1015 Lausanne (Switzerland)
Fax: (+41) 21 693 9435
E-mail: Paul.Vasos@epfl.ch

[b] Prof. Dr. G. Bodenhausen
Département de Chimie
associé au CNRS, Ecole Normale Supérieure
24 Rue Lhomond, 75231 Paris Cedex 05 (France)

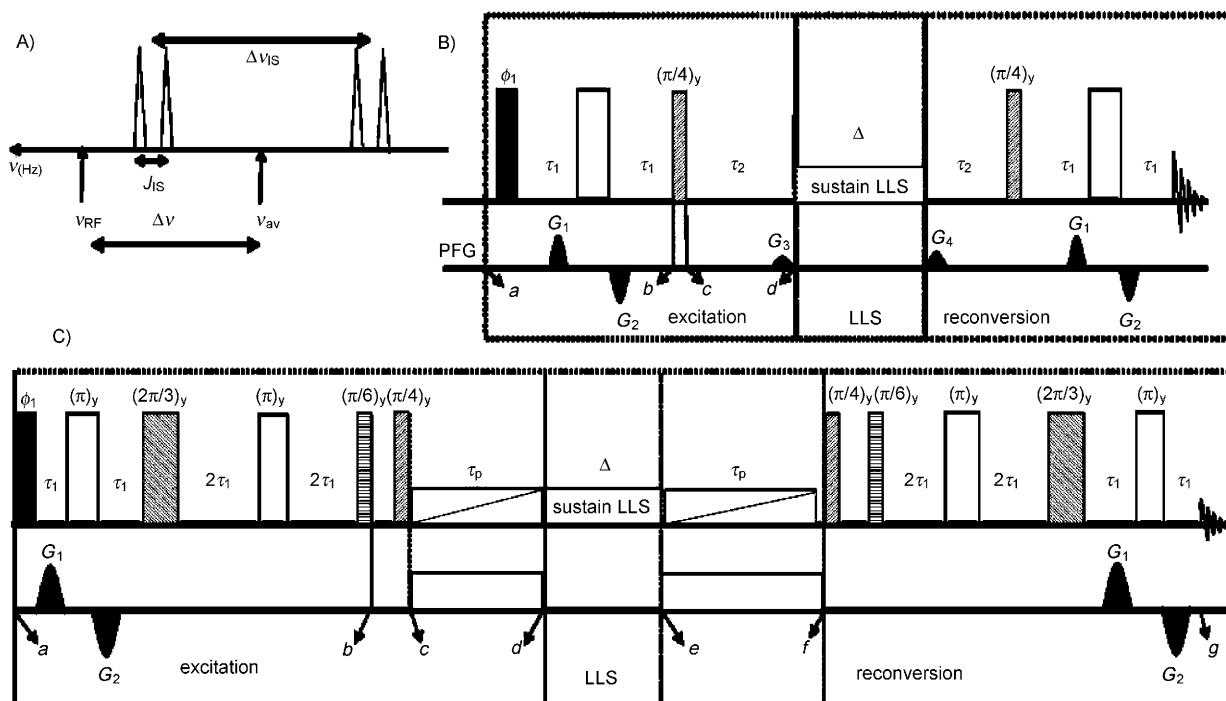


Figure 1. A) Spectral parameters that are relevant for exciting and sustaining singlet-state populations during long intervals Δ in two-spin (sub)systems. B) Typical pulse sequence for diffusion studies using singlet-states. This is a simple variant of singlet-state single-quantum diffusion ordered spectroscopy (SS-SQ-DOSY). The hatched, filled and open rectangles represent $\pi/4$, $\pi/2$, and π pulses, respectively. The pulses are applied along the x axis unless otherwise indicated. The encoding and decoding pulsed field gradients (PFGs) have sine shapes and must be combined in the manner of bipolar pulse pairs (BPPs), with varying amplitudes $G = G_1 = -G_2$. The weak purging gradients G_3 and G_4 are also sine-shaped and have peak amplitudes on the order of 2 G cm^{-1} only. Whenever a triple-gradient probe is available, it is recommended that these latter gradients be applied along a different direction (e.g. along the x axis, assuming that the encoding gradients are applied along z). C) New method for broad-band singlet-state diffusion ordered spectroscopy (BB-SS-DOSY). The hatched rectangles stand for pulses with various flip angles, as indicated. During the intervals $c-d$ and $e-f$, Thrippleton-Keeler (TK) filters consisting of rectangular gradients combined with frequency-swept pulses are inserted. Typical TK filters used in our experiments have durations $\tau_p = 12 \text{ ms}$, sweep-widths of 80 kHz and peak radio-frequency amplitudes of 1.78 kHz ; the amplitudes of the gradients are 14.6 and 17.8 G cm^{-1} for time intervals $c-d$ and $e-f$, respectively. During the interval Δ , where the singlet states are sustained, a sequence of Sinc-shaped pulses, of $400 \mu\text{s}$ duration each and maximum radio-frequency amplitudes of 5.7 kHz , is applied.^[9] The recommended phase cycle in sequences B and C is $\phi_1 = (x, -x)$ and $\phi_{\text{rec}} = (x, -x)$.

broad range of coupling constants J_{IS} in the preparation sequence, that is, between time points a and b .

Broadband (BB) rotation methods drawing inspiration from sequences developed by M. Levitt for inhomogeneous radio-frequency fields^[10] were demonstrated by S. Wimperis^[11] for heteronuclear scalar-coupled systems. In the case of homonuclear scalar-coupled spins, longitudinal magnetisation $I_z + S_z$ can be converted into in-phase transverse $-(I_y + S_y)$ and then further to antiphase coherence $2I_x S_z + 2I_z S_x$ by the coupling J_{IS} via the propagator [Eq. (3)]:

$$U(\beta) = \exp(-i\beta 2I_z S_z) \quad (3)$$

during an interval $2\tau_1$ where the magnetisation rotates under $J_{IS} 2I_z S_z$ through an angle $\beta = 2\pi J_{IS} \tau_1$. This function has a sinusoidal dependence on β ; it can be transformed into a function with a broader maximum in the vicinity of $\beta = \pi/2$ by using a sequence inspired by methods that produce broadband rotations.^[10-12] This sequence concatenates two steps described by Equation (3), as in Equation (4):

$$U^{bb}(\beta) = U^{2\pi/3}(2\beta)U(\beta) \quad (4)$$

These successive evolution periods convert the density operator from $-I_y - S_y$ to $2I_x S_z + 2I_z S_x$ via successive rotations, accomplished within a three-dimensional space subtended by the operators (Figure 2) in Equation (5):

$$\begin{aligned} A_i &= \frac{1}{2}(I_y + S_y) \\ A_f &= I_x S_z + I_z S_x \\ A_e &= I_z S_z - I_x S_x \end{aligned} \quad (5)$$

These three operators satisfy the commutation relationship in Equation (6):

$$[A_f, A_i] = iA_e \quad (6)$$

and cyclic permutations. This space is therefore similar to that defined by the operators I_x , I_y and I_z , for which Malcolm Levitt's original idea for broadband rotations was applied.

In the initial state, the density operator lies along $-A_i$, and at the end of the excitation sequence it is found along A_f . The first rotation $U(\beta)$ takes place around A_e . Indeed, defining A_0 as Equation (7):

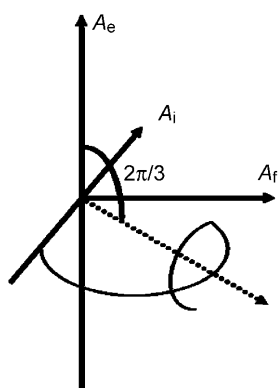


Figure 2. Trajectory of the coherence in the space spanned by operators A_i , A_r and A_e .

$$A_0 = I_z S_z + I_x S_x \quad (7)$$

one can write Equation (8):

$$2I_z S_z = A_e + A_0 \quad (8)$$

The operator A_0 commutes with all terms in the system in Figure 2, and therefore should have no impact on the result.

A system excited from thermal equilibrium by the first $(\pi/2)_x$ pulse, $\sigma_{(1)} = -I_y - S_y$ is transferred towards antiphase coherence by the propagator $U(\beta)$ given in Equation (9):

$$U(\beta) = \exp[-iA_e\beta] \quad (9)$$

with β defined as in Equation (10):

$$\beta = \pi J_{IS} 2\tau_1 \quad (10)$$

For the subsequent rotation, the axis of the effective operator needs to be rotated by 120° in the plane perpendicular to A_i in Figure 2. This is achieved by the insertion of a pair of two $2\pi/3$ pulses that delimits the next 2β period (Figure 3).

These pulses induce a rotation of the effective evolution operator around the $-A_i$ axis, as in Equation (11):

$$\begin{aligned} A_e^a &= \exp[i(I_y + S_y)\alpha] A_e \exp[-i(I_y + S_y)\alpha] \\ &= \exp(iA_i 2\alpha) A_e \exp(-iA_i 2\alpha) \end{aligned} \quad (11)$$

Rotation angles induced by pulses of phase γ around the A_y axis are twice as large as the nominal value of the pulses, α , because $A_i = (I_y + S_y)/2$. Therefore, since a rotation $R_{-y}(2\pi/3) = R_{-A_i}(4\pi/3)$ is applied, the effective evolution operator in the second period is found along (\dots) in Figure 2, at an angle $\pi/6$ from the A_r axis. The evolution during the second

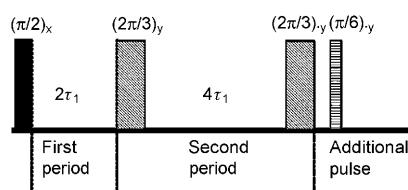


Figure 3. Pulse sequence with two periods and an additional pulse.

period amounts to a rotation through an angle $2\beta \sim \pi$ around this operator. Thus, the final position of the coherence lies in the IVth quadrant of the (A_r, A_e) plane, at an angle $\pi/3$ with respect to the A_r axis and $\pi/6$ with respect to the $-A_e$ axis. To bring it along the A_r axis, an additional pulse $(\pi/6)_{-y} = (\pi/3)_{-A_i}$ must be applied (Figure 3). By combining the two last pulses in Figure 3 into one $(\pi/6)_y$ pulse and introducing $(\pi)_y$ pulses in the middle of each evolution period to refocus chemical shifts, one obtains the a - b excitation segment of Figure 1C.

The efficiency of the new sequence, E^{bb} , in creating antiphase magnetisation was evaluated by calculating the expectation value of $2I_x S_z + 2I_z S_y$ at the end of the sequence in Figure 3 [Eq. (12)]:

$$E^{bb} = \left(1 + \frac{\cos^2 \beta}{2}\right) \sin \beta \quad (12)$$

This may be compared with the $\sin \beta$ profile that is obtained using a simple spin-echo sequence (Figure 4).

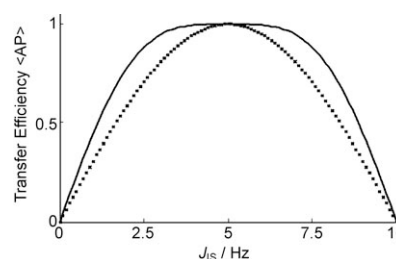


Figure 4. Theoretical plot of the efficiency of the conversion from in-phase to antiphase coherence using the new broadband sequence (—), compared to that of a simple spin-echo sequence (⋯⋯), using an interval $2\tau_1 = 0.1$ s (which results in optimal transfer for $J_{IS} = 5$ Hz).

In the propagator in Equation (4), the first term is obtained by the insertion of two $2\pi/3$ pulses, as explained above. The concatenation leads to a broadband conversion with respect to the angle $\beta = 2\pi J_{IS} \tau_1$, and, consequently, if τ_1 is fixed, with respect to the coupling constant J_{IS} . Thus, we can replace the transfer step a - b in the sequence in Figure 1B by the composite sequence a - b in Figure 1C. As usual, a symmetric sequence f - g is used for reversion before detection. We refer to this new sequence as broad-band singlet-state diffusion-ordered spectroscopy (BB-SS-DOSY).

The interval a - b in the sequence in Figure 1C excites antiphase single-quantum coherence over a reasonably wide range of coupling constants. Under the effect of a $\pi/4$ pulse, the antiphase terms are converted into a superposition of longitudinal two-spin order $2I_z S_z$ and $2I_x S_x$, which is a combination of zero- and double-quantum coherences. Subsequently, a pulsed field gradient combined with a frequency-modulated pulse in the manner of a TK filter^[7] allows one to remove the transverse terms, thus leaving^[8] at time-point d [Eq. (13)]:

$$Q_d = -\frac{1}{2}(|SS\rangle\langle SS| + |T_0\rangle\langle T_0| - |T_+\rangle\langle T_+| - |T_-\rangle\langle T_-|) \quad (13)$$

This generates a difference between the populations of singlet and triplet states, with $\text{Tr}(Q_{\text{LLS}}^\dagger Q_d) = -1/\sqrt{3}$. Step *c-d* is also broadband^[8] with respect to the chemical shift difference $\Delta\nu_{\text{IS}}$, as the state transferred from points *c* to *d* corresponds to longitudinal two-spin order $2I_xS_z$ that does not evolve under the shifts of the spins.

The efficiency of step *a-b* in the BB-SS-DOSY sequence was tested over a range of rotation angles $\beta = 2\pi J_{\text{IS}}\tau_1$ using simulations (Figure 5A) and by varying the interval τ_1 in experiments

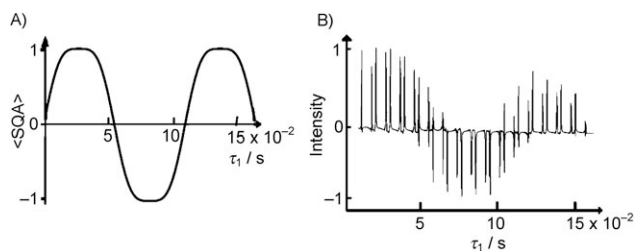


Figure 5. A) Simulations of the expectation values of antiphase single-quantum coherence $2I_xS_z + 2I_yS_x$ in a system with two coupled spins *I* and *S* with $J_{\text{IS}} = 8.8$ Hz, at time point *b* the BB-SS-DOSY sequence of Figure 1C, as a function of the delay τ_1 . B) Experimental signal intensities of the four peaks of 2,3,6-trichlorobenzaldehyde (I), where $J_{\text{IS}} = 8.8$ Hz, using the pulse sequence explained in the text.

on 2,3,6-trichlorobenzaldehyde (I) with $J_{\text{IS}} = J^{\text{II}} = 8.8$ Hz (Figure 5B). In the latter figure, the antiphase coherences are converted into in-phase magnetisation prior to detection by appending a spin-echo sequence with $\tau = 1/(4J_{\text{IS}})$, that is, with $\beta = \pi/2$. Both simulations and experiments confirm that the transformation taking in-phase to antiphase coherence is broadband over a range $0.25\pi < \beta < 0.75\pi$, in the sense that the signal amplitude $S(\beta)$ varies within a small range, $0.8 < S(\beta) < 1$. In larger molecules, losses due to rapid transverse relaxation must be taken into account.

3. Results

To test the ability of the BB-SS-DOSY sequence to determine simultaneously the diffusion coefficients of different molecules in solution using weak encoding/decoding gradients *G* and long diffusion intervals Δ , we have investigated a sample containing 2,3,6-trichlorobenzaldehyde (I) and 2-chloroacrylonitrile (II), each possessing a pair of *J*-coupled protons with coupling constants $J^{\text{I}} = 8.8$ Hz and $J^{\text{II}} = 3.3$ Hz, where long-lived states can readily be excited in separate series of experiments.^[2,4,13] The solvent consisted of 40% D_2O and 60% $[\text{D}_6]\text{DMSO}$ at $B_0 = 14.1$ T (600 MHz) and $T = 286$ K. The concentration of each of the species in solution was ~ 20 mM. The singlet-state lifetimes were found to be $T_{\text{S}}(\text{I}) = 12.5 \pm 0.3$ s and $T_{\text{S}}(\text{II}) = 31.5 \pm 0.4$ s under our experimental conditions (without degassing), while the relaxation time constants of longitudinal magnetisation are $T_1(\text{I}) = 3.97 \pm 0.05$ s and $T_1(\text{II}) = 3.77 \pm 0.01$ s.

The diffusion constants of these two molecules were first measured using the conventional stimulated echo (STE) method.^[14] The signal intensities were fitted with two adjust-

able parameters, the diffusion coefficient *D* and the initial signal intensity S_0 , as a function of the parameter κ , which depends on the amplitude *G* and duration δ of the pulsed field gradients [Eq. (14)]:

$$\kappa = 2\gamma Gps\delta \quad (14)$$

where γ is the gyromagnetic ratio of protons. The factor 2 reflects the fact that bipolar pulse pairs (BPP) are used before and after the diffusion interval Δ , *p* is the coherence order (in the present case, $p = 1$ since single-quantum coherences are encoded), *s* is a shape factor ($s = 2/\pi$ for a PFG with a sinusoidal envelope). The diffusion delay was chosen to be $\Delta = 0.6$ s in our basic STE experiments, and the peak amplitude *G* of the sine-shaped encoding and decoding gradients was varied up to 90% of the upper limit allowed for the probe. This results in spin-echo amplitudes in a convenient range $0.1 < S(\kappa)/S(\kappa=0) < 1$ for $0 < \kappa < 12.4 \times 10^4 \text{ m}^{-1}$ (Figure 6A).

Using the simple SS-DOSY sequence of Figure 1B, the diffusion constants of compounds I and II in the mixture could only be measured in two separate series of experiments because the scalar coupling constants J^{I} and J^{II} are quite different. The intervals of the sequence in Figure 1B were set to $\tau_1 = 1/[4J^{\text{I}}] = 28$ ms and $\tau_1 = 1/[4J^{\text{II}}] = 76$ ms, respectively. The diffusion delay could be set to $\Delta = 4$ s in both experiments, well below the lifetimes of the singlet-state populations in either of the two molecules, that is, $\Delta < T_{\text{S}}(\text{I}) < T_{\text{S}}(\text{II})$. Consequently, the simple SS-DOSY sequence allows us to reduce the

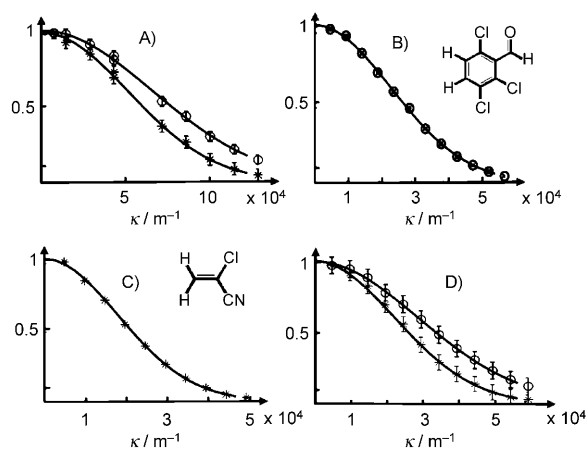


Figure 6. Experimental study of translational diffusion in a mixture of 2,3,6-trichlorobenzaldehyde (I) and 2-chloroacrylonitrile (II) at $T = 286$ K and $B_0 = 14.1$ T (600 MHz for protons). The experimental intensities of resonances of I and II are indicated by (○) and (*), respectively. The diffusion coefficients determined from the curves are given in Table 1. A) Conventional stimulated echo sequence (STE) with a diffusion delay $\Delta = 0.6$ s; the peak amplitudes of the encoding and decoding gradients of duration $\delta = 0.8$ ms are stepped through $2.6 < G < 43 \text{ G cm}^{-1}$. B) Simple singlet-state single-quantum diffusion ordered spectroscopy (SS-SQ-DOSY) with $\Delta = 4$ s and a delay $\tau_1 = 1/[4J^{\text{I}}] = 28$ ms matched for compound I, with gradients of duration $\delta = 0.55$ ms and amplitudes $3 < G < 31 \text{ G cm}^{-1}$. C) Same sequence, with $\tau_1 = 1/[4J^{\text{II}}] = 76$ ms matched for compound II and $3 < G < 26 \text{ G cm}^{-1}$. D) The new method for broadband singlet-state diffusion-ordered spectroscopy (BB-SS-DOSY) with a diffusion delay $\Delta = 3$ s and a compromise interval $\tau_1 = 41 \text{ ms} \approx 1/[2(J^{\text{I}} + J^{\text{II}})]$, with gradients of duration $\delta = 0.55$ ms and amplitudes $3 < G < 31 \text{ G cm}^{-1}$.

maximum gradients by a factor of ~ 2 compared to the basic STE sequence. It is noteworthy that this feature of SS-DOSY experiments allows one to determine diffusion coefficients of large, slowly-diffusing molecules, using regular NMR probes, given that the strain on the required gradient amplitudes is significantly reduced.^[4,5]

The new method for broad-band singlet-state diffusion ordered spectroscopy (BB-SS-DOSY, Figure 1C) was used to determine the diffusion coefficients of the two molecules I and II simultaneously. The interval a – b , as well as the corresponding refocusing interval in the sequence in Figure 1C, were chosen to maximise the function $f = \sin[2\pi J^{(0)}\tau_1] + \sin[2\pi J^{(0)}\tau_1]$ over the interval $0 < \tau_1 < 1/[4J^{(0)}]$. Note that $\tau_1 \sim 1/[2\{J^{(0)} + J^{(0)}\}]$. In practice, we used $\tau_1 = 41$ ms.

The agreement between the diffusion coefficients obtained with and without irradiation during the diffusion delay (Table 1) shows that heating by the radio-frequency field did not lead to any significant convection effects.

The values measured by the SS-SQ-DOSY sequence are higher than those recorded by the new sequence and by STE-DOSY, notably in the case of 2-chloroacrylonitrile. This could be due to interference between purging and encoding gradients (Figure 1).

4. Conclusions

We have shown by simulations and experiments that broad-band sequences can be designed to excite singlet states in samples featuring a distribution of J -coupling constants. This allows the simultaneous determination of slow diffusion coefficients of several slowly-diffusing species, or in mixtures where slowly- and rapidly-diffusing species co-exist. These methods can also be used for the simultaneous determination of relaxation rates $R_5 = 1/T_5$ of singlet states in various environments. It should be kept in mind that a diffusion period during which continuous radio-frequency irradiation is applied to sustain long-lived states, no matter how weak the amplitude of the irradiating field, may lead to heating and hence to undesirable convection currents. A sequence that compensates for these effects can be used^[15] in this case. A partial deuteration of the immediate environment of the two coupled spins can lead to a significant improvement in the lifetimes. Thus, a two-fold extension of the longitudinal relaxation time constant T_1 and a seventeen-fold extension of the singlet-state lifetime T_5 were

obtained for protons 5' and 5'' in a saccharide.^[6] Recent advances in deuteration strategies allow one to label biomolecules so that only a few selected proton pairs remain in a fully deuterated background. For example, it is possible to prepare proteins that only contain aromatic protons in Phe, Tyr and Trp by adding the aromatic amino acid precursor shikimic acid to fully deuterated media.^[16] Singlet states may then be excited on the ring protons of tyrosines, for instance. Long-lived states have also been excited in systems with more than two coupled spins;^[17,18] this should greatly facilitate the task of deuteration strategies.

Provided these considerations are taken into account, the new methods open the way for studying slow exchange and slow diffusion in biomolecules without resorting to two-dimensional spectroscopy. Studies of proteins that exchange between conformations with different hydrodynamic radii could also benefit from the methods presented in this paper, since long diffusion intervals are needed to obtain reliable diffusion coefficients.

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Keywords: diffusion • long-lived spin states • NMR • pulsed field gradients • singlet states

Table 1. Diffusion coefficients D of the two compounds I and II determined from the six curves in Figure 6, corresponding to four different experiments: STE, SS-SQ-DOSY, and the new BB-SS-DOSY. The standard deviations of D for 100 fits of the data in which the experimental intensities are varied within the range of the spectral noise are reported.

Diffusion coefficients D [$10^{-10} \text{ m}^2 \text{ s}^{-1}$] at $T = 286 \text{ K}$	STE	SS-SQ-DOSY $\tau_1 = 1/(4J^{(0)}) = 28 \text{ ms}$	SS-SQ-DOSY $\tau_1 = 1/(4J^{(0)}) = 76 \text{ ms}$	BB-SS-DOSY $\tau_1 = 1/[2(J^{(0)} + J^{(0)})] = 41 \text{ ms}$
Trichlorobenzaldehyde (I) [(○) in Figure 6]	2.0 ± 0.1	2.2 ± 0.1	–	2.0 ± 0.1
Chloroacrylonitrile (II) [(*) in Figure 6]	3.1 ± 0.2	–	3.7 ± 0.2	3.3 ± 0.2

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