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A New Method for Measuring Diffusion Coefficients by 2D NMR using Accordion Spectroscopy

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Translational diffusion measurements are a useful tool for studying supramolecular complexes and for characterizing the association state of molecules that aggregate at NMR concentrations. Pulsed field gradients can be used to measure diffusion coefficients. Spectral overlap problems in complex mixtures can be alleviated by using 2D spectroscopy but the need to record a complete series of 2D spectra with increasing gradient strength makes these experiments extremely time consuming. Concerted incrementation of the gradient strength and the evolution time provides a new version of accordion spectroscopy that allows the measurement of individual diffusion coefficients in complex mixtures in a single experiment.

Key Words: **Accordion spectroscopy; constant time; diffusion; DOSY; pulsed field gradients.**

The widespread availability of shielded gradients has renewed interest in the study of diffusion by NMR using pulsed field gradients (PFG). Diffusion coefficients may be used to prove aggregation of peptides and proteins under the same conditions used for NMR (*1*). In supramolecular chemistry diffusion coefficients may be used to demonstrate the formation of dimers or higher order assemblies of high symmetry and to characterize the inclusion of a low molecular weight guest in a larger host (*2*).

The determination of diffusion coefficients by NMR using PFG is based on the work of Stejskal and Tanner (*3*). In the basic gradient echo experiment (90°–t(PFG)–180°–t(PFG)–Acq) the strength of the PFG pulses is increased in a series of spectra. The intensity of the echo amplitude decreases as a consequence of the change in the spatial position of the molecule during the time interval between the two gradients. The change in signal intensity is related to the translational diffusion coefficient D by the Stejskal–Tanner equation

$$I = I_0 \exp[-(\gamma\delta G)^2(\Delta - \delta/3)D], \qquad [1]$$

where γ is the gyromagnetic ratio, δ the gradient duration, G the gradient strength, and Δ the interval between PFG pulses.

Problems related to the practical implementation of the basic gradient echo experiments include J modulation due to homonuclear coupling and residual eddy currents leading to phase distortions. Additionally, T_2 relaxation in large molecules may be short compared to the interval between PFG pulses (Δ), causing signal decay independent of diffusion (*4*). Finally, spectral overlap poses serious limitations in the extraction of individual diffusion coefficients in complex mixtures. The stimulated echo experiment (STE) (*5*) was designed to circumvent the effect of short T_2 and the longitudinal encode decode (LED) (*6*) sequence minimizes the effect of eddy currents. The problem of signal overlap has been addressed by using advanced fitting procedures (*7–9*) or by using the larger signal spread of 2D NMR experiments. In the 2D approach, the intensity of well-resolved cross-peaks is measured in a series of 2D experiments with increasing gradient strength. The gradient echo has been incorporated in 2D sequences either by adding it as an additional period (*10, 11*) or by directly merging it into the sequence using existing mixing delays or echoes (*12*).

The increased resolution obtained by measuring diffusion coefficients using 2D experiments is obtained at the expense of very long measuring times as the number of individual 2D spectra must be large enough and the signal-to-noise of each of them must be sufficient to allow a correct analysis of the Gaussian decay of the cross-peaks.

The series of 2D spectra recorded with different gradient strengths can be regarded as a 3D data set with diffusion encoded in the third dimension. A reduction of dimensionality, with the corresponding time saving, can be obtained by projecting the 3D experiment in two dimensions by making two of the time variables functionally dependent.

Accordion spectroscopy, introduced by Bodenhausen and Ernst in the early 1980s (*13*) is based on the concerted

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incrementation of the evolution and mixing delays in a 2D experiment:

$$\tau_m = \chi t_1. \qquad [2]$$

In this way the information that could be obtained from a series of 2D experiments with different mixing times (e.g., NOE build-up rates) is obtained in a single 2D experiment and is encoded in the lineshape of the cross-peaks.

In this paper we describe a variant of accordion spectroscopy for the measurement of diffusion coefficients using a single 2D experiment.

The time allowed for diffusion between the gradients forming the echo must be kept constant to avoid interference from relaxation effects and therefore the direct implementation of the accordion strategy using two time variables seems not possible. However, by incrementing the *strength of the gradients* proportionally with the evolution time we obtain a new variant of accordion spectroscopy. We suggest the name GAUDI (gradient accordion used for diffusion) for this type of experiment.

Using a linear relationship between the gradient strength and the evolution time,

$$G = \xi t_1 \qquad [3]$$

signal decay during t_1, neglecting relaxation, is related to the diffusion coefficient by

$$I(t_1) = I_0 \exp[-(\gamma\delta\xi)^2(\Delta - \delta/3)Dt_1^2]. \qquad [4]$$

After 2D Fourier transform both diagonal- and cross-peaks have a Gaussian shape in f_1 from which the diffusion coefficients can be obtained by curve fitting to the Fourier transform of Eq. [4]

$$I(\omega) = (I_0/2)(\pi/AD)^{1/2} \exp[-\omega^2/4AD], \qquad [5]$$

where ω is the frequency difference with respect to the center of the peak and $A = (\gamma\delta\xi)^2(\Delta - \delta/3)$. The GAUDI technique can be implemented in any two-dimensional experiment with a diffusion filter.

The practical implementation of the GAUDI approach has some obvious drawbacks: (i) the need to correct for the intrinsic line width, (ii) the lower sensitivity and resolution resulting from diffusion-broadened lines, and (iii) the interference from scalar coupling.

The method we use to eliminate the intrinsic Lorentzian lineshape is to record a reference spectrum under exactly the same conditions of the GAUDI spectrum but in which the gradients used to measure diffusion are kept inactive. After the first Fourier transform, the oscillatory part of the interferogram at each frequency in the reference spectrum is removed by taking the absolute value. The resulting smoothly decaying function is inverted and used as a weighting function for t_1 in the GAUDI spectrum.

The fact that the diffusion information is encoded in the linewidth reduces the maximum resolution that can be obtained compared to a conventional 2D experiment. The sensitivity is also affected by the broadening and by the possible presence of t_1 noise. These effects can be partially compensated by the resolution enhancement introduced using the reference spectrum and by the fact that the information can be obtained in a single experiment and therefore a longer accumulation can still provide a considerable saving in global time as compared to a complete series of 2D experiments.

Signal modulation due to scalar coupling is a general problem for measuring diffusion coefficients by the standard methods. The presence of poorly resolved multiplets also must be considered during the fitting to Eq. [5] of the data obtained in a GAUDI type experiment. However, this problem can be eliminated by the use of a constant time (CT) version of the experiment (*15*, *16*). Evolution due to scalar

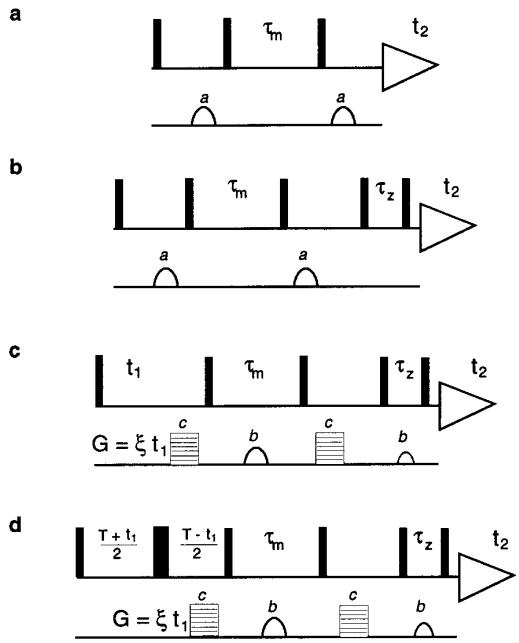
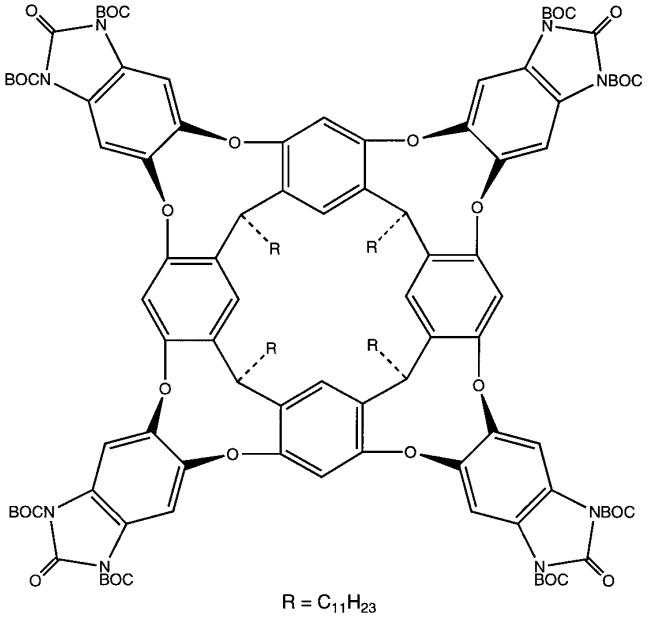


FIG. 1. Sequences of (a) a 1D stimulated echo experiment (STE), (b) a 1D longitudinal encode decode experiment (LED), (c) a 2D GAUDI–NOESY experiment, and (d) a CT-GAUDI–NOESY experiment. Square gradient pulses were used. Gradients labeled *a* are incremented in a series of experiments between 0 and 30 G cm^{-1}. Gradients labeled *b* (13.4 G cm^{-1} and 8 G cm^{-1}) are used for coherence selection. Gradients labeled *c* are accordion gradients changing from 0 to 30 G cm^{-1} within a single 2D experiment. In experiments (c) and (d) the first and the last two pulses were cycled according to a standard NOESY phase cycle. τ_m was 200 ms and τ_z was 300 ms.

coupling, in contrast to that due to chemical shifts, is not modulated between different increments of t_1 and therefore all signals appear as singlets in f_1, although with an intensity that depends on the particular couplings involved. The length of the constant time must be adjusted for the particular peaks of interest.

Figure 1 compares the sequences of standard STE and LED experiments with the diffusion-filtered NOESY and CT-NOESY sequences used for the GAUDI–NOESY and CT-GAUDI–NOESY experiments described below. In the STE experiment the refocusing 180° pulse found in the basic echo sequence is split so that magnetization is stored along the *z* axis during the time between the gradients. The final delay in the LED experiment allows for the decay of eddy currents leading to undistorted signals. By including an evolution delay after the first pulse, the LED experiment becomes identical to a NOESY with a *z* filter. Zero-order coherences are selected either by phase cycling or using additional gradients during τ_m and τ_z. Cross-relaxation can take place during both delays, and therefore the mixing time for NOESY ($\tau_m + \tau_z$) and the delay for diffusion (τ_m) can be optimized independently. The final zeta filter permits the simultaneous selection of both the echo and the antiecho pathways and allows phase-sensitive detection using the standard States *et al.* method (*17*).

In order to test the accuracy of the diffusion coefficients measured in GAUDI experiments we have examined two different samples. The first one, BOC-resorcinarene tetraurea (BOC-RTU),



gives several singlet cross-peaks in NOESY spectra and therefore represents a very favorable case while the second, peptide T (H-ASTTTNYT-NH_2) in d_6-DMSO, represents a more realistic case of a molecule where the NOESY cross-peaks have poorly resolved fine structure due to scalar coupling.

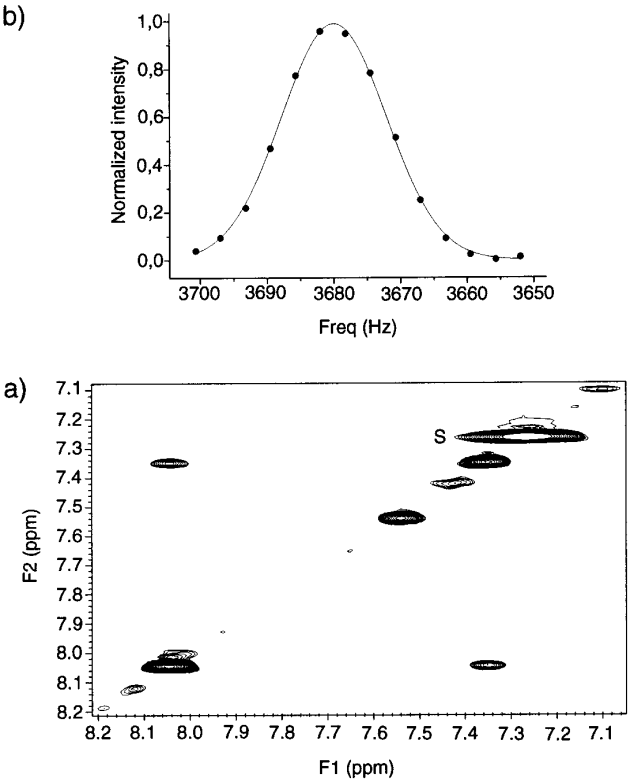


FIG. 2. (a) Expansion of the aromatic region of a CT-GAUDI–NOESY spectrum of BOC-RTU. The solvent signal (marked with S) shows an increased linewidth in f_1 due to fast diffusion. (b) Gaussian curve fitting of a f_1 trace across the same cross-peak shown in (a). The continuous line is the best fit to the experimental points. The number of points is set by the digital resolution which was increased using linear prediction.

Figure 2 shows a portion of the GAUDI–NOESY spectrum of BOC-RTU in d_6-benzene containing a resolved cross-peak and an expansion of a f_1 trace of the same cross-peak fitted to Eq. [5] using a nonlinear fitting program. The diffusion coefficients obtained with different methods are shown in Table 1.

For the sample of peptide T in d_6-DMSO the use of the constant time version of GAUDI–NOESY clearly corrects for the overestimation of the diffusion coefficients that arises from unresolved scalar couplings. Attempts to optimize the fitting of the lineshape of cross-peaks obtained from the non-constant time version of GAUDI-NOESY using multiple Gaussian lines were not completely succesful. Diffusion coefficients measured using CT-GAUDI spectra are identical, within the experimental error, to those measured using the standard STE experiment.

TABLE 1
Comparison of Diffusion Coefficients (m^2 s^{-1}) at 25°C Measured by Different Methods[a]

Molecule/solvent	STE	CT-GAUDI–NOESY	GAUDI–NOESY
BOC-RTU/d_6-benzene	$4.89\ 10^{-10}$	$4.90\ 10^{-10}$	—
Peptide T/d_6-DMSO	$1.81\ 10^{-10}$	$1.78\ 10^{-10}$	$1.96\ 10^{-10}$[b]

[a] Gradient calibration was performed by measuring the diffusion coefficient of the solvent in each experiment.
[b] Partially resolved multiplet. A multiple Gaussian fit was used in this case.

CONCLUSIONS

The GAUDI strategy provides a suitable fast method for measuring diffusion coefficients from the lineshape of 2D diagonal- and cross-peaks and can be implemented in any diffusion-filtered 2D experiment. In particular, the fact that diffusion information is encoded in the evolution period allows the use of constant time experiments to eliminate the effect of scalar couplings. The coefficients measured by this method are in good agreement with those obtained using standard methods.

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