A New Approach in the Use of Gradients for Size-Resolved 2D-NMR Experiments

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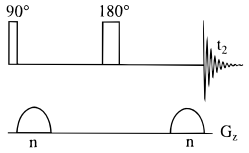
Over the last few years, the use of pulsed-field-gradients (PFG) in NMR has attracted the interest of several research groups. In most experiments, the well-paid static field homogeneity is destroyed, for given periods during the NMR experiments, in order to allow selection of coherence transfer pathways with reduced or no phase cycling, suppression of artifacts, etc.[1,2]

Analysis of translational dynamics through PFG techniques[3] represents another important application offering NMR capabilities similar to other spectroscopic techniques such as light-scattering. In the present work we propose a new, straightforward, and versatile approach to study more complex systems, by extending classical 2D-NMR sequences into 3D-NMR experiments where the third dimension, created by the variation of the gradient strength, provides separation of mixtures of compounds according to their mobility and thus size.

The basic experiment developed so far for the study of translational motion is the gradient echo.[4] Spins, excited by the first 90° pulse, are spatially defocused by the first gradient and refocused by the second one. In this experiment the chemical shift evolution is refocused by the 180° pulse, but not the *J* modulation which causes phase distortion in the resulting spectrum.



But gradient refocusing is also partial because molecules change their position in space, due to Brownian motion. Therefore, variation of the gradient amplitude *G* will result in a change of the signal intensity *I* according to the Stejskal−Tanner equation for a semi-sinusoidal shaped gradient:

$$I = I_o \exp[-(\eta\gamma\delta G)^2(2/\pi)^2(\Delta - \delta/4)D] \qquad (1)$$

where η is the coherence order, γ the gyromagnetic ratio of the nucleus under study, δ the gradient duration, Δ the diffusion time, and *D* the diffusion coefficient for translational motion. A number of improvements of this scheme were recently proposed, including the stimulated echo modification and the longitudinal eddy current delay (LED) pulse sequence,[5] that allow the use of high-power gradients and limit the problems of *J* modulation.

From these experiments the diffusion coefficients can be easily determined through monoexponential fitting, in the simple case of a single-component system. However, when complicated mixtures are encountered signal overlapping poses serious limitations. The extraction of the diffusion coefficients of the individual species from a multiexponential decay becomes quite problematic, without any a priori knowledge of the system. To achieve this goal, methods based on global least-squares analysis[6,7] and advanced fitting procedures[8,9] have been proposed. In the latter approach, known as Diffusion Ordered SpectroscopY (DOSY), the results are displayed in a 2D manner (the diffusion coefficient being along the indirectly observed dimension), facilitating the correlation of protons belonging to the same molecular component of the mixture.

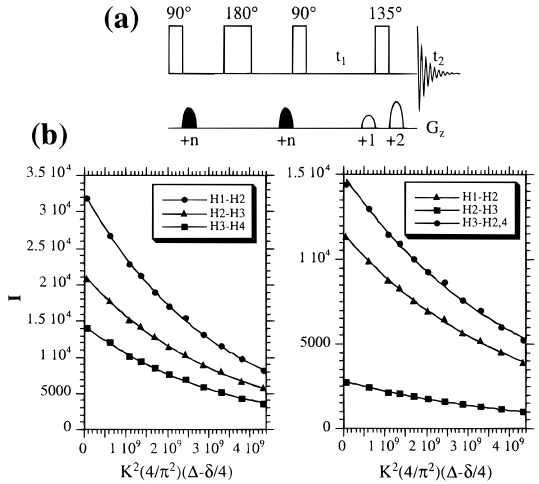


Figure 1. (a) Schematic representation of the PFG-DQS sequence used for the evaluation of diffusion coefficients of ATP and γ-cd. (b) Variation of the cross-peak intensities as a function of gradient parameters is shown (left: ATP cross peaks, right : γ-cd cross peaks). The intensities of γ-cd signals have been halved for clarity.

Searching for NMR solutions to circumvent overlapping, the use of higher magnetic fields was initially proposed.[10] Another alternative is to turn to 2D-NMR experiments where the superposition problem is much less severe. To achieve this goal one possibility is to chain the gradient echo or the DOSY preparation period with a regular 2D-NMR experiment.[11,12] In this case, the elongation of the pulse sequence provokes a reduction of the sensitivity. One possibility devoid of such a limitation is to directly merge diffusion experiments into 2D experiments[13] by taking advantage of NMR pulse sequences comprising either an echo, where a pair of gradients can be easily incorporated, or a gradient pair separated by a mixing period. This situation is now encountered in many experiments where the gradients are used to allow the selection of coherence transfer pathways with reduced or no phase cycling.

A 2D experiment which is perfectly fitted to our purpose is the double quantum correlation,[14] presented in Figure 1a. The preparation module incorporates the two gradients of variable strength and is sufficiently long to be used for the size separation; the last gradient pair is of constant amplitude and serves, optionally, for the coherence selection. Thus no phase cycling is necessary and the spectra can be obtained in the magnitude mode. This experiment was tested on a mixture of ATP/γ-cyclodextrin (50:7 mM).[15] The intensity of the cross

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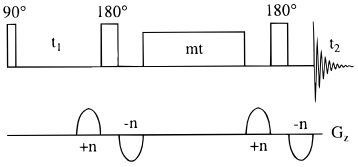
peaks (often more resolved than the signals appearing in a 1D gradient-echo spectrum) was followed as a function of gradient strength on a series of 11 experiments in D_2O at 298 K, recorded in 14 h. Representative cross-peak intensity variations against the area of the gradient pulses are shown in Figure 1b.

Monoexponential fitting of the cross-peak intensities following eq 1 afforded the averaged diffusion coefficients $D_{\gamma-cd} = 2.39\ 10^{-10}$ m^2 s^{-1} and $D_{ATP} = 3.08\ 10^{-10}$ m^2 s^{-1} for ATP and γ-cyclodextrin, respectively, with a dispersion smaller than 7%. The quality of the exponential fitting was $R > 0.997$. This permits size separation of the two compounds and assignment of their respective cross peaks. Ideally this procedure could be automated and inverse Laplace transformation may be used for the reconstruction of the third dimension. This proves the capability of this scheme (also applicable in the case of the DREAM[16] and RELAY experiments) to separate the signals corresponding to the two components of the mixture.

Alternatively, bracketing of the mixing period in 2D sequences with gradient pairs of increasing strength results in a 3D experiment, where the new dimension represents the diffusion coefficient for translational motions. Suitable experiments with a sufficiently long fixed delay include the NOESY/ROESY and TOCSY experiments. They retain all advantages of the gradient experiments and permit for instance total suppression of the phase cycling and phase sensitive spectral representation through echo−antiecho 2D Fourier transformation. Moreover, the use of self-compensating bipolar gradients[17]



ensures minimum recovery delay and fewer phase distortions due to the refocusing of the chemical shift evolution during the gradient pulses (thus avoiding backwards linear prediction).

Of course, this principle implies that the gradient dependence of coherent or incoherent magnetization transfer obeys eq 1. This was verified through selective 1D experiments. Figure 2a shows the variation of both direct (H-1 of γ-cd) and rOe cross peak (H-2 and H-4) intensities as a function of the diffusion parameters, obtained from a series of off-resonance ROESY experiments.[18] Exponential fitting of both curves gave almost identical values (2.06×10^{-10} m^2 s^{-1} and 2.12×10^{-10} m^2 s^{-1} respectively), similar to that obtained from the simple gradient echo experiment. Moreover, it should be noticed that the PFG off-resonance ROESY experiment combines two dynamic filters, particularly valuable for the study of interaction processes: one related to the rotational motion (angle θ of the effective spin-lock and the static magnetic field axis) and the other related to translational motion (gradient strength). This scheme was successfully extended to the 2D version of the off-resonance ROESY experiment and the results will be presented elsewhere.

Finally, this principle was applied to the 2D TOCSY sequence. A series of 10 PFG-TOCSY experiments were recorded over 43 h. Processing of the diagonal and cross peaks as a function of diffusion parameters showed that those

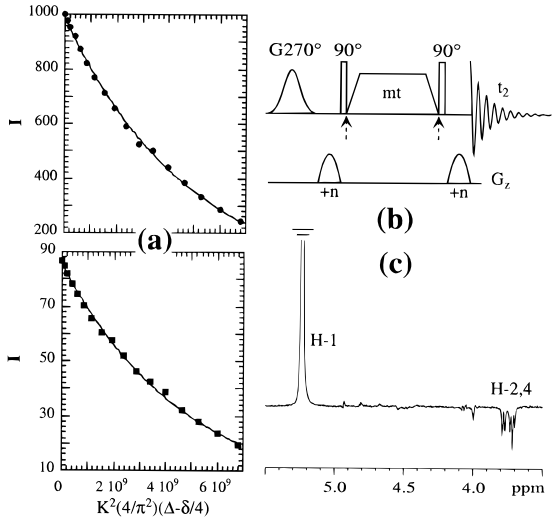


Figure 2. Stejskal−Tanner plots produced from a series of PFG selective 1D off-resonance ROESY experiment performed on a mixture of ATP/γ-cd (a); the continuous line represents the integral of cyclodextrin direct peak (H-1) initially excited by a Gaussian 270° pulse and the dotted line the sum of the cross peaks (H-1 and H-2,4). The pulse sequence is shown in (b) and a representative spectrum in (c).

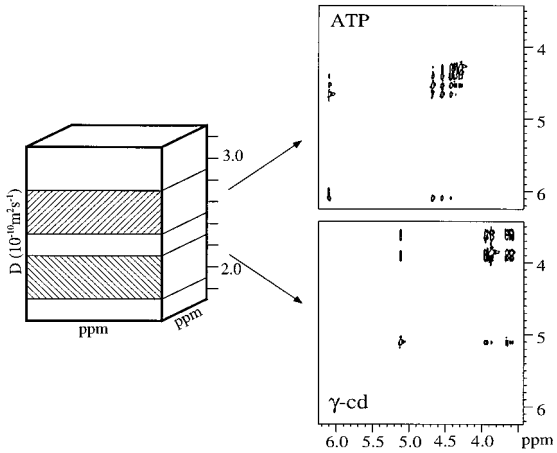


Figure 3. Three-dimensional representation of the results obtained from a series of 10 PFG-TOCSY experiments on a mixture of ATP/γ-cd. A 65 ms MLEV-17 supercycle was used for spin-locking.

corresponding to γ-cd exhibit an average $D_{\gamma-cd} = 2.15 \times 10^{-10}$ m^2 s^{-1} and the rest $D_{ATP} = 2.72 \times 10^{-10}$ m^2 s^{-1}. In both cases the dispersion of the values was found to be less than 10% and the quality of the exponential fitting was $R > 0.992$. The spectra presented in Figure 3 were manually reconstructed following the analysis of each 2D peak, following the general scheme proposed by Morris and Johnson.[7,8] Size separation of the two components is clearly achieved. Better digitization in the third dimension by increasing the number of the recorded experiments should influence the precision of the results and possibly extend the scope and applications of the described experiment.

In conclusion it is demonstrated here that variable strength PFG defocusing−refocusing schemes can be successfully incorporated in many 2D pulse sequences, combining the spectral assignment with the size separation of the components of mixtures. Further technological advances in terms of hardware (linear amplifiers capable of producing stronger gradients with shorter recovery delays) are expected to improve the quality of the resulted spectra and extend the application of this technique to larger molecules and to mixtures of compounds with closer diffusion coefficients.

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(15) ^{1}H-NMR experiments were performed on a Bruker AMX-600 spectrometer upgraded with a bath cooling unit. A triple-resonance HCN probe equipped with self-shielded *z* gradient coils (maximum field strength 0.48 T m^{-1}) was used. Gaussian and trapezoidal pulses were created through the SHAPE program and subsequently were inputted to the selective excitation unit. Spectra were processed via the UXNMR program and the monoexponential fitting was carried out with the Kaleidagraph package (Abelbeck Software).

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