▌**NMR Spectroscopy**

Directly Decoupled Diffusion-Ordered NMR Spectroscopy for the Analysis of Compound Mixtures

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Abstract: For the analysis of compound mixtures by NMR spectroscopy, it is important to assign the different peaks to the individual constituents. Diffusion-ordered spectroscopy (DOSY) is often used for the separation of signals based on their self-diffusion coefficient. However, this method often fails in the case of signal overlap, which is a particular problem for ^{1}H-detected DOSY spectra. Herein, an approach that allows the acquisition of homonuclear broadband-decoupled DOSY spectra without the introduction of an additional decoupling dimension, by instant decoupling during acquisition, is presented. It was demonstrated on a mixture of six alcohols, and the investigation of the binding of a dodecapeptide to membrane mimetics.

Introduction

NMR spectroscopy is one of the most often used techniques for the structure determination in the chemical sciences. The analysis of mixtures constitutes a particular challenge, because signals in regular NMR spectra cannot be assigned to the individual compounds, unless they are scalar coupled. A solution to this problem is the use of diffusion-ordered NMR spectroscopy (short DOSY),[1] which allows the separation of compounds through their respective self-diffusion coefficient. This experiment consists of at least two pulsed field gradients and a delay Δ between them, which allows diffusion to take place. The magnetization of spins, which did not change their positions during Δ, is refocused by the second gradient, whereas the intensities of other signals are reduced according to Equation (1):

$$I_G = I_{G=0} \exp(-(\gamma \delta G)^2 D(\Delta - \delta/3)) \tag{1}$$

This Stejskal–Tanner equation describes the connection between the signal intensity I_G for different gradient strengths G; γ is the gyromagnetic ratio of the observed nuclei; D the self-diffusion coefficient; and δ the length of the gradient pulse. Since many years, regular pulsed field gradient stimulated echo (PFG-STE) experiments were carried out to separate signals based on their molecular sizes.[2] To reduce possible artifacts, these experiments can be enhanced, for example, by using bipolar field gradient pulse pairs and a 180° hard pulse in the middle,[3] instead of one single-field gradient pulse, which gives several advantages: minimization of artifacts, lower signal loss by spin diffusion, reduced eddy currents, and refocusing of the deuterium lock signal.[4] An additional eddy current delay is implemented to reduce effects of magnetic-field inhomogeneity.[5] By using such state-of-the-art DOSY experiments mixtures of a few compounds can be separated routinely. High-resolution (HR) DOSY experiments, in which peaks of all components are separated, provide the easiest way to analyze these spectra. The diffusion constant of each peak can be obtained by exponential fitting of the intensity versus the gradient strength. This approach leads to problems in the case of overlapping signals. They can be partially overcome in some instances by more complicated data processing, such as fitting the decay with two different diffusion coefficients (D).[6,7] Another mathematical solution are multivariate methods, which are based on least-square minimization of the global diffusion coefficient data set, followed by an optimization of the amplitudes of different components.[8–10] But regardless how the data are processed, the diffusion coefficient difference between compounds must be at least 30%,[11] and only a hand full (three to four) different compounds can typically be measured. Due to their high gyromagnetic ratio and widespread occurrence, protons (^{1}H) are the most commonly used nuclei for DOSY NMR measurements. Spectral overlap is a particularly severe problem for proton NMR due to its limited chemical-shift range (ca. 15 ppm) and even more homonuclear scalar coupling. One possibility to overcome this limitation is the use of different nuclei with higher frequency ranges. For this purpose, ^{6}Li,[12] ^{13}C,[11,13,14] ^{19}F,[15,16] ^{29}Si,[17] or ^{31}P[18] DOSY experiments have been described. However, their applicability is limited to molecules containing these nuclei and/or their low natural abundance or sensitivity.

Another possibility to obtain less overlapped DOSY spectra is by spreading the signals into a third dimension. Total correlation spectroscopy (TOCSY),[19–21] heteronuclear multiple-quan-

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tum correlation spectroscopy (HMQC),[22] heteronuclear single-quantum correlation spectroscopy (HSQC),[21] correlation spectroscopy (COSY),[23] and nuclear Overhauser effect spectroscopy (NOESY)[24] experiments have been combined with diffusion-ordered spectra for obtaining better signal dispersion. However, these 3D DOSY experiments require much longer measurement times and more elaborate data processing. An alternative to obtain [1]H-detected DOSY spectra with high resolution is the use of singlet-only spectra. This can be achieved, for example, by the suppression of multiplets by spin-echo editing.[25] Components, which do not contain any singlet signals, are of course completely eliminated by this method. Another approach is to use homonuclear broadband decoupled (pure-shift) ^{1}H spectra to get rid of scalar couplings.[26] Regular 1D pure-shift spectra can be obtained by several methods,[27–31] but only a few of them are applicable to DOSY experiments. The first decoupled DOSY spectra were obtained by adding a spin-echo sequence before[32] or after[33] a regular DOSY experiment. An improvement of these 2D/J-resolved DOSY is 2DJ/IDOSY,[34] in which the experimental time is reduced by a factor of four. The result is a J-resolved DOSY experiment, which of course is essentially also a 3D experiment, because of an additional J-resolved dimension. Another method to obtain decoupled DOSY spectra is the diffusion weighted z-COSY experiment, in which a stimulated echo is combined with an anti z-COSY sequence.[30] More recently, spatially and frequency-selective decoupling (also called Zangger–Sterk or ZS method) was used to obtain pure-shift DOSY spectra,[35] and improved by replacing the PFG sequence by a "one-shot" sequence.[27] For the spatially selective decoupling, the NMR sample tube is split into a series of small slices and selective decoupling of a different narrow frequency range is done in each slice for a different signal. Therefore, the problem of homonuclear decoupling is transformed to a spatially separated pseudo-heteronuclear decoupling situation. Combined slice and frequency-selective excitation is achieved by frequency-selective excitation during a weak pulsed-field gradient.[27,31,36] Decoupling is then achieved by a frequency and spatially selective spin echo. Recording of the FID is started at the end of the spin echo when full decoupling is achieved. However, scalar coupling evolves during the acquisition of the FID. This problem was overcome by acquiring data chunks (ca. 20 ms) of this FID and record the entire dataset in a series of different, incremented scans. In combination with a DOSY sequence, this constitutes a "double-pseudo" 3D NMR experiment, in which the decoupled frequency axis is obtained by concatenation of the individual FID data chunks. Besides this special processing (in addition to the regular DOSY processing), the sensitivity of the resulting spectrum is severely compromised. This results from both the slice-selective excitation and the need to acquire several scans to construct the complete FID. Recently, we have presented a modified method of spatially selective homonuclear broadband decoupling, in which the individual data chunks were recorded within one FID, which is interrupted every approximately 20 ms for repeated decoupling.[29] The big advantages of this approach are its increased sensitivity per instrument time, and even more importantly the absence of any

special data processing. Herein, we present a homonuclear broadband decoupled BPP-LED DOSY experiment, acquired with spatially decoupling during acquisition. The recorded data set can be processed like a regular 2D DOSY. Its applicability is presented on a mixture of alcohols, and an investigation of the potential binding of a protein-derived dodecapeptide to membrane mimetics in a solution containing the peptide and its single amino acids together with 1-myristoyl-2-hydroxy-sn-glycero-3-phosphocholine (MHPC) micelles. It is shown that in complex spectra with many overlapping peaks, this decoupled DOSY sequence is able to separate many more peaks according to their diffusion coefficient, whereas a regular DOSY fails in separating some signals.
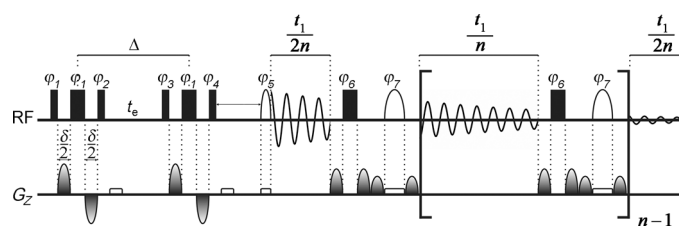

Figure 1. Pulse sequence used for the decoupled DOSY sequence. Δ is the diffusion time (≈ 0.1 s), δ is the gradient pulse length (typically, 2–4 ms), t_e is the eddy current delay (5 ms), and n is the loop counter (depending on the frequency range and desired resolution in the direct dimension 40–80). The following phase cycle was used: $\phi_1 = x$, $\phi_2 = x, x, -x, -x$, $\phi_3 = \phi_5 = x, x, x, x, -x, -x, -x, -x, y, y, y, y, -y, -y, -y, -y$, $\phi_4 = x, -x, x, -x, -x, x, -x, x, y, -y, y, -y, -y, y, -y, y$, $\phi_6 = x, -x$, $\phi_7 = -x, x$, $\phi_{ref} = x, x, -x, -x$, $\phi_{rec} = x, -x, -x, x, -x, x, x, -x, -y, y, y, -y, y, -y, -y, y$.

Theory

The pulse sequence used for the instantly decoupled DOSY (shown in Figure 1) is based on a bipolar pulse pair sequence with eddy current delay (BPP-LED). A selective 90° pulse during a weak-field gradient pulse replaces the last 90° hard pulse. The acquisition is interrupted every approximately 25 ms for continuous decoupling within each scan. The strength of the weak-pulsed field gradient must be strong enough to encompass the whole expected frequency range, but should not be too high to avoid excessive sensitivity loss. To cover a ^{1}H frequency range of 10 ppm (5 kHz) on a 500 MHz spectrometer (field strength ≈ 117 kG, detection volume ≈ 1 cm) by spatially selective excitation, a gradient strength of 1.2 G cm^{-1} (117 kG $\times$ 10 ppm ≈ 1.2 G) must be applied during the soft pulses. For the first 90° selective excitation pulse we used a 60 ms Eburp2 pulse, which excites a frequency range of approximately 80 Hz. This means that compared to non-selective excitation, only 1.6% (of the 5 kHz spectral range) are excited and corresponds to an equally high sensitivity loss. If sensitivity is limiting and/or a smaller chemical shift range is needed, the duration of the spatially selective pulse can be reduced, leading to increased signal intensities. For the 180° soft decoupling pulse, used repeatedly during acquisition, we used a 10 ms Gaussian pulse. The number of decoupling blocks applied during the FID depends on the re-

quired total acquisition time. We recorded typically around 50 loops, resulting in a total acquisition time of 1 s, because each chunk of the FID is approximately 20 ms. It should be noted that decoupling during acquisition leads to stronger artifacts compared with a regular DOSY spectrum and therefore broadening of signals in the diffusion dimension.

Results and Discussion

The first example shows a diffusion-ordered spectrum of a mixture of ethanol (EtOH), 1- and 2-propanol (nPrOH and iPrOH), 1-butanol (nBuOH), N-methylaminoethanol (NMEA), and 2-propenol (allyl-OH). A BPP-LED sequence was applied for the coupled DOSY (Figure 2 A) and the same sequence with decoupling during acquisition for the spectrum in Figure 2 B. In re-

gions, in which signals of different components are overlapped (indicated by circles, 3.5–4 ppm and 0.8–1.5 ppm), the regular DOSY experiment has problems to resolve the individual peaks. This gave inaccurate or wrong diffusion constants extracted from the DOSY. In contrast, the decoupled DOSY spectrum can resolve every peak in the whole 2D spectrum.

DOSY spectra are also often used to investigate molecular interactions. The interaction of peptides and proteins with membrane mimetics constitutes a particularly sophisticated case for DOSY experiments due to extensive signal overlap in basically the whole spectral region. We used the directly decoupled DOSY to study the potential binding of a dodecapeptide (KGGEAAEAEAEK) derived from human metallothionein III to 1-myristoyl-2-hydroxy-sn-glycero-3-phosphocholine (MHPC) micelles. Metallothioneins (MTs) are small, cysteine-rich metal-binding proteins, function(s) of which are not fully understood,[37] although they are believed to play a role in metal homeostasis. In contrast to the isoforms MT-1 and MT-2,[38] mammalian MT-3[39] is located mainly in the brain, and has been found also in the plasma membrane and outer membranes of mitochondria.[40] The main difference between MT-1,2 and MT-3 is a hexapeptide insert in the latter. This peptide is located in an unstructured loop of MT-3.[39] To investigate if this loop is potentially involved in membrane binding, we studied the binding of a 12-residue stretch of MT-3, which contains the hexapeptide insert in the middle, to membrane-mimetic MHPC micelles. A solution containing the dodecapeptide (5.7 mM) KGGEAAEAEAEK), 17.1 mM MHPC, and the free amino acids of the peptide (ca. 5 mM) was used.

The individual components of this mixture (MT3-peptide, MHPC, and individual amino acids) were measured separately by a regular DOSY sequence to get their exact diffusion coefficients. All three spectra were overlaid and are presented in Figure 3 A (MHPC in red, MT3-hp in blue, and amino acids in green). Then the compounds were mixed together in one single sample, and a regular DOSY was recorded (Figure 3 B). What can be easily seen is that the coupled spectrum fails in resolving most peaks due to extensive
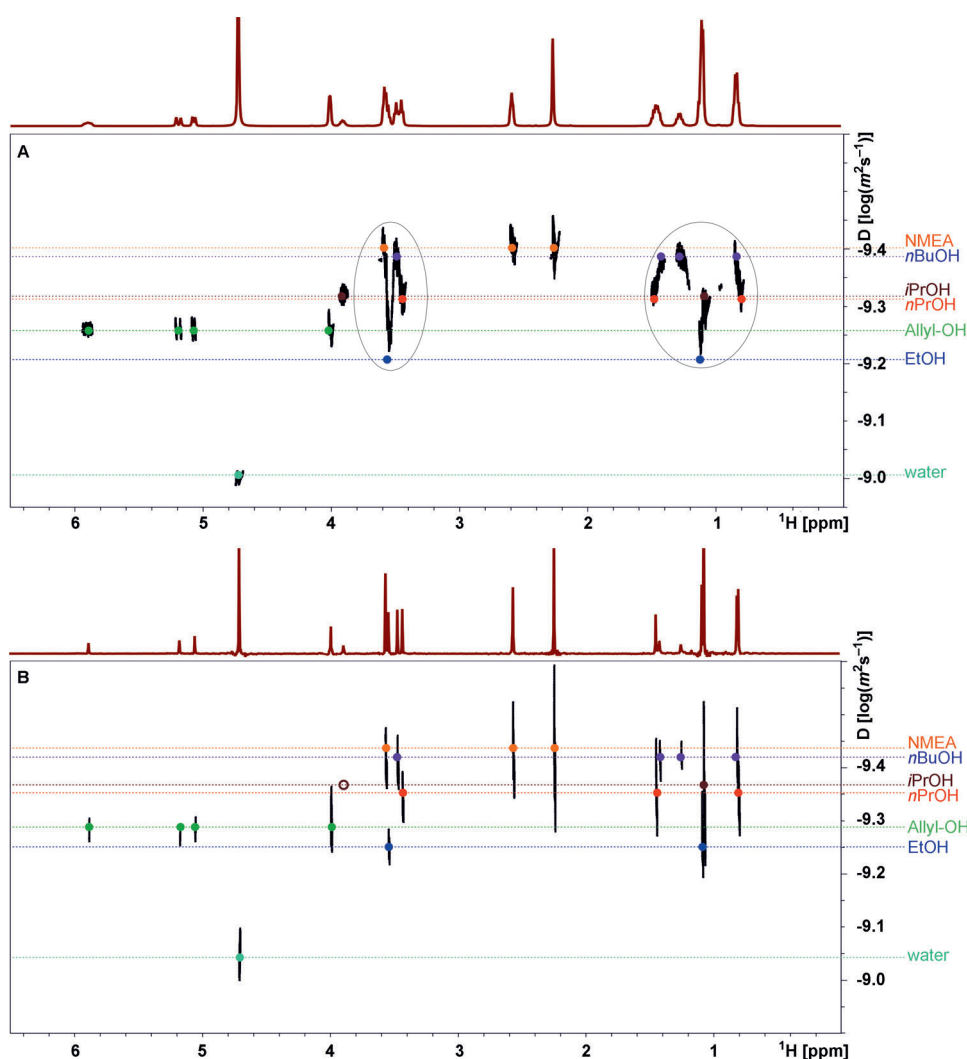

Figure 2. A) Regular DOSY spectrum of a mixture of different alcohols (each at 8% vol.). The colored dots indicate the accurate position (frequency and diffusion constant) of the compound. When peaks of different compounds are partially overlapped, blurred lines with inaccurate diffusion coefficients appear (circled areas). The following parameters were used: 16 scans, 128 gradient increments, 8 ppm spectral width. B) Decoupled DOSY spectrum acquired using the presented method of the same alcohol mixture. All peaks are well resolved in the frequency dimension. With the acquisition parameters of 128 scans, 128 gradient increments and a spectral width of 8 ppm, the duration of the decoupled DOSY experiments was eight times longer than the regular DOSY. Above, both DOSY spectra 1D spectra (regular and decoupled during acquisition) are shown.
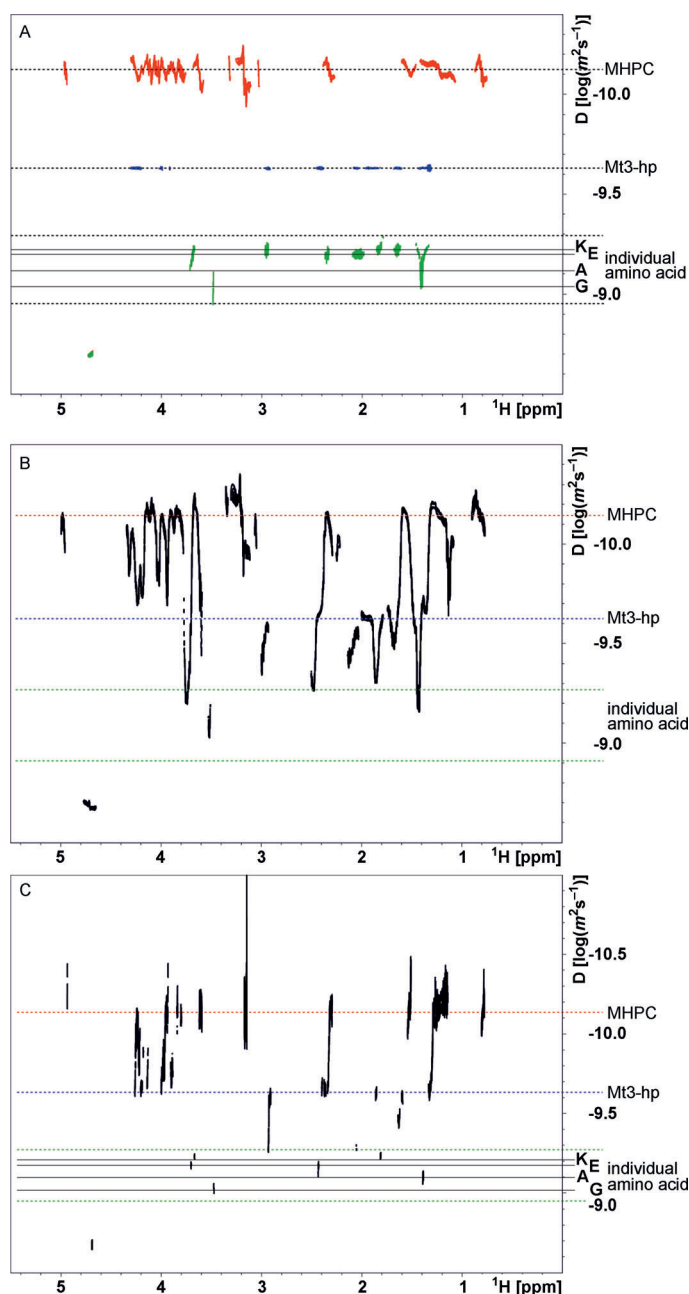
Figure 3. A) Overlay of three DOSY spectra, in which three solutions (containing only MHPC, the peptide, or the amino acid mix), were recorded separately. The acquisition parameters were 16 scans (32 for MT3-hp), 32 gradient strength increments, and a spectral width of 8 ppm. The calibration parameters Δ and δ were optimized separately for all three solutions. B) Regular DOSY spectrum (32 scans, 128 increments) of all three components (MHPC, MT3-hp and amino acid solution) mixed together in one sample. The DOSY experiment failed in separating most of the peaks of different molecules. In the case of the individual amino acids, only the Hα of glycine has the correct diffusion coefficient. C) Homonuclear decoupled DOSY experiment of the mixture. Due to a higher resolution in the ^{1}H dimension, fewer overlapped signals can be observed, leading to less blurred lines and more accurate diffusion coefficients. The parameters for the experiments were 320 scans and 128 increments.

signal overlap. Signals of different components, which are partially or completely overlapped, gave blurred lines in the DOSY from one peak to the other. Especially, for the MT-3 peptide,

the DOSY of the mixture does not allow the extraction of the diffusion coefficient for a single signal. On the other hand, the decoupled DOSY showed several signals of the MT-3 derived peptide at the same diffusion coefficient as the peptide in the absence of any membrane mimetic, showing that there is no interaction between this peptide and MHPC micelles (Figure 3 c).

Furthermore, in this spectrum, it is possible to distinguish between different amino acids. The highest diffusion coefficient was found for glycine (with a signal at 3.50 ppm, Hα), followed by alanine (CH$_3$ group seen at $\delta = 1.45$ ppm). Because spatially selective decoupling causes problems for strongly coupled spin systems, some signals of lysine and glutamate in the area around $\delta = 1.5$–2.1 ppm are missing. However, this just further helps to clean up the spectrum for an easier interpretation of the decoupled DOSY.

Conclusion

We have presented an instantly decoupled 2D DOSY experiment, which gave ω_2 broadband proton-decoupled spectra, which can be processed like regular 2D diffusion-ordered spectra. Applications were shown on a small-molecule mixture and the binding of a dodecapeptide to a membrane mimetic. Although the resolution in the frequency dimension is significantly enhanced, and therefore the number of signals for which diffusion coefficients can be extracted, the resolution in the diffusion dimension is somewhat reduced as a result of increased artifacts due to decoupling during acquisition.

Experimental Section

All experiments were carried out on a Bruker AVANCE III 500 MHz spectrometer by using a 5 mm TCI probe with z-axis gradients at 298 K. The peptide MT3-hp was purchased from PSL (Heidelberg, Germany) in HPLC purified form. 1-Myristoyl-2-hydroxy-sn-glycero-3-phosphocholine (MHPC) was obtained by Affymetrix (Santa Clara, CA, USA). All other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) at > 98 % purity.

Acknowledgements

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