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ARTICLE

Simultaneous convection compensation and solvent suppression in biomolecular NMR diffusion experiments

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Abstract Thermal convection and high intensity solvent resonances can significantly hamper diffusion estimates in pulsed gradient spin-echo nuclear magnetic resonance diffusion experiments on biomolecule samples. To overcome these two problems, a new double functional NMR diffusion sequence, double echo PGSTE-WATERGATE, is presented. The new sequence provides excellent convection compensation and solvent suppression (with a suppression factor in excess of at least 10^5 in a single scan) in biomolecular NMR diffusion experiments. Due to its stimulated echo nature, the new sequence is much less susceptible to spin–spin relaxation than Hahn spin-echo based sequences. Furthermore, the new sequence is not susceptible to spin diffusion due to the application of bipolar pulsed gradients. The new sequence is also much easier to set up compared to previously developed stimulated echo based convection compensation and solvent suppression sequence. The utility of the new sequence is demonstrated on an aqueous lysozyme sample.

Keywords Convection · Diffusion · NMR · PGSE · Protein · Solvent signal suppression

Introduction

Thermal convection can cause erroneous (self-) diffusion coefficient estimates in pulsed gradient spin-echo (PGSE) nuclear magnetic resonance (NMR) diffusion experiments (e.g., Kato et al. 2006; Stilbs 1987). The convection streamlines in an NMR tube are mainly vertical and thus can be modelled as a liquid system with a flow both upward and downward with equal (but opposite) velocity v (e.g., Hedin et al. 2000; Jerschow 2000). For a standard Hahn spin-echo based or stimulated echo (STE) based PGSE sequence (Fig. 1a), the diffusion based spin-echo attenuation in the presence of convection becomes (Hedin et al. 2000; Saarinen and Johnson 1988)

$$E \sim \cos(\gamma \delta g_1 v \Delta) \exp\left(-\gamma^2 \delta^2 g_1^2 D \left(\Delta - \frac{\delta}{3}\right)\right), \quad (1)$$

where γ is the gyromagnetic ratio, δ is the duration of pulsed gradients, g_1 is the strength of pulsed gradients, D is the self-diffusion coefficient, and Δ is the diffusion time. As shown by Eq. 1, in the presence of convection the spin-echo attenuation is modulated by a cosine factor, which can be clearly observed well above ambient temperatures (e.g., ~47°C) in low viscosity samples (e.g., Jerschow and Müller 1998; Mao and Kohlmann 2001). However, when $v^2\Delta \ll D$ and $\delta \ll \Delta$, convection simply accelerates the decay of the spin-echo signal (Hedin et al. 2000). In practice, in freely diffusing samples the existence of convection can be confirmed by the observation of (artifactual) Δ-dependent apparent diffusion coefficients.

The deleterious effects of convection can be partially avoided simply by the use of special sample tubes to restrict sample length (e.g., Hayamizu and Price 2004; Kato et al. 2006) and sample radius (e.g., Goux et al. 1990; Martínez-Viviente and Pregosin 2003). However, these methods sacrifice signal-to-noise (S/N) ratio and therefore are not suitable for low concentration samples which are often encountered in biomolecular NMR. Convection effects can also be partially removed by using specially designed pulse sequences (e.g., He and Wei 2001; Hedin

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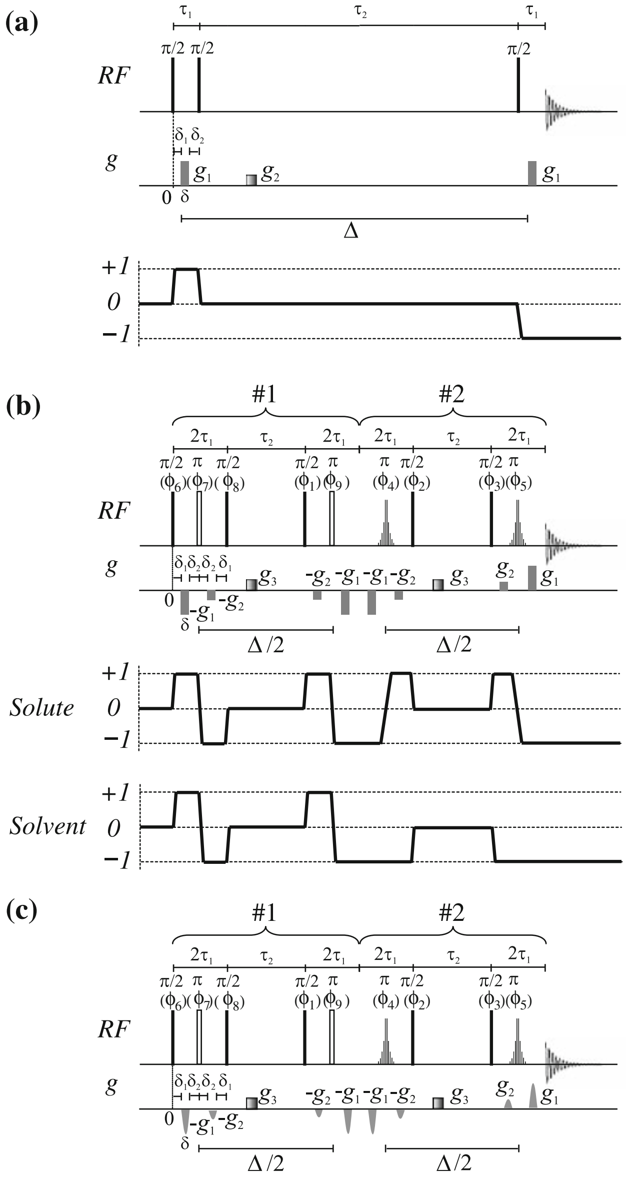


Fig. 1 The standard STE based PGSE sequence (**a**), the double echo PGSTE-WATERGATE sequence with rectangular gradients (**b**), and the double echo PGSTE-WATERGATE sequence with half sine shaped gradients (**c**). The *black bars* and *white rectangles* represent $\pi/2$ and π RF pulses, respectively, g_1, g_2 and g_3 are gradient pulses with different amplitudes, and the bar groupings represent "W5" binomial π pulses (Liu et al. 1998). The desired coherence pathways are also shown. The phase cycle for the double echo PGSTE-WATERGATE sequences is $\phi_1 = (x)_4, (y)_4, (-x)_4, (-y)_4, (-x)_4, (-y)_4, (x)_4, (y)_4$; $\phi_2 = x, y$; $\phi_3 = x, y$; $\phi_4 = x, x, y, y$; $\phi_5 = x$; $\phi_6 = x$; $\phi_7 = y$; $\phi_8 = (x)_4, (y)_4, (-x)_4, (-y)_4$; $\phi_9 = \mathrm{y}$; $\phi_r = (x, x, -x, -x, -x, -x, x, x)_2, (-x, -x, x, x, x, x, -x, -x)_2$. The phases of the pulses of the binomial sequences are given by 0°–0°–0°–0°–0°–180°–180°–180°–180°–180° + ϕ_4–ϕ_4–ϕ_4–ϕ_4–ϕ_4–ϕ_4–ϕ_4–ϕ_4–ϕ_4–ϕ_4 (or ϕ_5–ϕ_5–ϕ_5–ϕ_5–ϕ_5–ϕ_5–ϕ_5–ϕ_5–ϕ_5–ϕ_5)

et al. 2000; Jerschow and Müller 1997, 1998; Loening and Keeler 1999; Nilsson and Morris 2005; Sørland et al. 2000; Zhang et al. 2001), special temperature controlling systems (e.g., Hedin and Furó 1998; Holz et al. 1996; Holz and Weingärtner 1991), sample rotation (e.g., Esturau et al. 2001; Lounila et al. 1996), and replacing variable temperature air flow with liquid (e.g., Boden et al. 1992). Among the suppression methods mentioned above, the pulse sequence methods are the most generally applicable.

Solvent signal suppression is also an important issue in numerous PGSE experiments, especially in biomolecular NMR. It is often necessary to combine convection compensation with solvent suppression. To achieve this double function, Momot and Kuchel developed two Hahn spin-echo based double functional PGSE sequences [i.e., *CONV*ection compensation/*EX*citation sculpting (CONVEX) (Momot and Kuchel 2004) and *D*ouble-*Q*uantum *Diff*usion (DQDiff) (Momot and Kuchel 2005)]. However, these two sequences are more applicable for measuring self-diffusion of small- or medium-sized solutes due to their greater susceptibility to spin-spin relaxation. Simorellis and Flynn (2004) proposed a standard double-stimulated-echo (D-STE) PGSE sequence (Jerschow and Müller 1997) preceded by a WET (Ogg et al. 1994; Smallcombe et al. 1995) sequence and it can be applied to solutes with short spin–spin relaxation times. Although convection compensation and solvent suppression can be successfully achieved by the use of the WET D-STE sequence, it only provides a solvent suppression factor $\geq 10^4$ in a single scan (Smallcombe et al. 1995) and also necessitates the calibration of shaped RF pulses. With only uni-polar gradients, the WET D-STE sequence may suffer serious spin diffusion (i.e., NOE spin exchange) effects on samples containing large proteins and aggregates (e.g., Chou et al. 2004). After each encoding period of the WET D-STE sequence, the spin magnetizations within one molecule are stored along the $+z$ or $-z$ axis depending on their chemical shift offsets and therefore the spin magnetizations will experience significant attenuation through spin diffusion during the subsequent diffusion time; however, if bipolar pulsed gradients are used, the chemical shift effects can be effectively compensated by the application of π pulses and therefore the spin diffusion effects can be greatly suppressed (e.g., Chou et al. 2004; Dvinskikh and Furó 2000). Recently, Ortner et al. proposed the combination of presaturation solvent suppression and D-STE sequence (Ortner et al. 2007). Although the D-STE or double echo scheme provides effective convection compensation, it causes a non-negligible signal loss (i.e., 3/4 of the signal obtained by a Hahn spin-echo based sequence with other attenuation factors such as relaxation and spin diffusion ignored). Therefore, in practice there is always a trade-off between the signal loss due to the use of D-STE scheme and the signal loss due to relaxation.

In this study, a double echo PGSTE-WATERGATE (Fig. 1b, c) sequence was developed for simultaneous convection compensation and solvent signal suppression in PGSE NMR diffusion experiments on solutes with short spin–spin relaxation times (e.g., proteins, protein aggregates, large polymers, and supramolecular assemblies) by modifying the PGSTE-WATERGATE solvent suppression unit (Zheng et al. 2008a) by using the general concept of the D-STE PGSE sequence (Jerschow and Müller 1997). The utility of the new sequence was demonstrated on a 2 mM lysozyme aqueous sample.

Materials and methods

A sample containing 2 mM lysozyme in water (10:90 $^2H_2O/^1H_2O$) was provided in a sealed NMR tube by Bruker (Karlsruhe, Germany) as the standard water-suppression sample.

^1H PGSE NMR spectra were acquired on a Bruker Avance 400 spectrometer (Karlsruhe, Germany) at 400 MHz using a BBO high resolution probe equipped with a gradient coil and a Bruker Avance 500 spectrometer (Karlsruhe, Germany) at 500 MHz using a TXI high resolution probe equipped with a gradient coil at 22 and 37°C. Typical acquisition parameters were: spectral width 8 kHz; digitized into 7–8 K data points; $\pi/2$ pulse length 8–15 μs; number-of-scans = 64 or 128. Diffusion measurements were performed by linearly incrementing the gradient strength (g_1) from ~ 0.2 to ~ 0.5 T m^{-1} [for the standard STE based PGSE experiments (Fig. 1a)] or from ~ 0.1 to ~ 0.5 T m^{-1} [for the double echo PGSTE-WATERGATE experiments (Fig. 1c)] with diffusion times (Δ) of 0.1 and 0.3 s. Please refer Zheng et al. (2008a, b) for detailed information on setting up PGSTE-WATERGATE experiments. The double echo PGSTE-WATERGATE sequence with half sine shaped gradients was used for diffusion measurements in order to avoid possible eddy current effects and improve gradient pulse reproducibility. Integration over the 0.5–1.5 ppm region of each spectrum was used for diffusion data analysis.

Maple 13 (Maplesoft, Waterloo) was used for the Stejskal and Tanner analysis (Stejskal and Tanner 1965) of the new sequence. Origin 8 (OriginLab, Northampton, MA) was used for all diffusion data analysis.

Results and discussion

Convection compensation can be demonstrated either by evaluating the Stejskal and Tanner equation (Stejskal and Tanner 1965) in the presence of convection (i.e., macroscopically) (e.g., Jerschow and Müller 1997) or by looking at the phase shift of each spin (i.e., microscopically) (e.g., Zhang et al. 2001) and the latter is used here. At the end of #1 period (see Fig. 1b, c), the net phase shift of solute spin i introduced by convection is given by

$$\begin{aligned}\Delta\phi_{i,\#1} &= \gamma[1\times(-g_1)+(-1)\times(-g_2)]\delta z_{0,i}\\ &\quad+\gamma[1\times(-g_2)+(-1)\times(-g_1)]\delta\left(z_{0,i}+v_i\Delta\right)\\ &=\gamma(g_1-g_2)\delta v_i\Delta,\end{aligned}\tag{2}$$

where "1" and "-1" stand for the selected coherence levels, $z_{0,i}$ stands for the initial position of spin i, v_i stands for the velocity of convection, and the other parameters are defined in Fig. 1. Similarly, the net phase shift obtained from #2 period is

$$\begin{aligned}\Delta\phi_{i,\#2} &= \gamma[(-1)\times(-g_1)+1\times(-g_2)]\\ &\quad\times\delta\left(z_{0,i}+v_i\Delta\right)+\gamma[1\times g_2+(-1)\times g_1]\\ &\quad\times\delta\left(z_{0,i}+2v_i\Delta\right)\\ &=-\gamma(g_1-g_2)\delta v_i\Delta.\end{aligned}\tag{3}$$

Ideally, the (total) convection based phase shift at the end the sequence is

$$\Delta\phi_{i,\#1}+\Delta\phi_{i,\#2}=0,\tag{4}$$

signifying the complete suppression of convection effects.

For the solvent, we have

$$\begin{aligned}\Delta\phi_{i,\#1} &= \gamma[1\times(-g_1)+(-1)\times(-g_2)]\delta z_{0,i}\\ &\quad+\gamma[1\times(-g_2)+(-1)\times(-g_1)]\delta\left(z_{0,i}+v_i\Delta\right)\\ &=\gamma(g_1-g_2)\delta v_i\Delta,\end{aligned}\tag{5}$$

$$\begin{aligned}\Delta\phi_{i,\#2} &= \gamma[(-1)\times(-g_1)+(-1)\times(-g_2)]\delta\left(z_{0,i}+v_i\Delta\right)\\ &\quad+\gamma[(-1)\times g_2+(-1)\times g_1]\delta\left(z_{0,i}+2v_i\Delta\right)\\ &=-\gamma(g_1+g_2)\delta v_i\Delta,\end{aligned}\tag{6}$$

and

$$\Delta\phi_{i,\#1}+\Delta\phi_{i,\#2}=-2\gamma g_2\delta v_i\Delta\neq 0.\tag{7}$$

Therefore, the diffusion-based solvent signal attenuation will experience a constant convection-based scale factor (≤ 1) during each diffusion measurement in which g_2, δ, and Δ are all kept constant and therefore the efficiency of solvent suppression is not affected.

By using Stejskal–Tanner analysis (Stejskal and Tanner 1965), the diffusion-based spin-echo attenuation for the double echo PGSTE-WATERGATE sequence with rectangular gradients can be determined to be

$$\begin{aligned}E=\exp\Bigg\{-\gamma^2D\delta^2\Bigg[&\left(\Delta-\frac{8}{3}\delta-4\delta_2\right)g^2\\ &+\frac{4}{3}\delta gg_1+\left(8\delta_2+\frac{8}{3}\delta\right)g_1^2\Bigg]\Bigg\},\end{aligned}\tag{8}$$

where $g = g_1 - g_2$ is the effective pulsed gradient. According to Eq. 8, in the limit of $\Delta \gg \delta$ and δ_2, the "gg_1" and "g_1^2" terms can be ignored in diffusion data analysis.

Table 1 Diffusion measurements on lysozyme at different temperature using the standard STE based PGSE and double echo PGSTE-WATERGATE sequences

Sequence	Apparent diffusion coefficient ($\times 10^{-10}$ m^2 s^{-1})			
	22°C		37°C	
	$\Delta = 0.1$ s	$\Delta = 0.3$ s	$\Delta = 0.1$ s	$\Delta = 0.3$ s
STE based PGSE	0.98 ± 0.01	1.00 ± 0.01	2.96 ± 0.02	–[a]
Double echo PGSTE-WATERGATE[b]	0.98 ± 0.01	0.96 ± 0.01	1.42 ± 0.01	1.44 ± 0.01

[a] No acceptable data fitting was obtained due to significant convection effects (Fig. 2b)

[b] Sine-shaped gradients were used

Similarly, the diffusion-based spin-echo attenuation for the double echo PGSTE-WATERGATE sequence with half sine shaped gradients can be determined to be

$$E = \exp\left\{-\frac{4}{\pi^2}\gamma^2 D\delta^2\left[\left(\Delta - 4\delta_2 - \frac{5}{2}\delta\right)g^2 + \delta g g_1 + (3\delta + 8\delta_2)g_1^2\right\}, \qquad (9)$$

To test the convection compensation ability of the new sequence, the diffusion measurements on lysozyme were performed at different temperatures (Table 1). At 22°C little convection took place and therefore both STE based PGSE and double echo PGSTE-WATERGATE sequences provided similar diffusion measurements at different diffusion times. Nevertheless, at 37°C the STE based PGSE sequence provided a notably higher apparent diffusion coefficients with $\Delta = 0.1$ s compared to the double echo PGSTE-WATERGATE sequence and failed to measure diffusion coefficients at relatively long diffusion times (i.e., $\Delta = 0.3$ s) due to serious convection effects (Fig. 2b) while the double echo PGSTE-WATERGATE sequence provided constant diffusion measurements through all different diffusion times (Fig. 2c, d) owing to its convection compensation.

As shown in Fig. 3, the new sequence also affords effective solvent signal suppression due to the inclusion of the PGSTE-WATERGATE (Zheng et al. 2008a) unit which is based on enhanced diffusion differentiation between solute and solvent. With a solvent suppression factor in excess of 10^5 in a single scan, the new sequence outperforms the WET D-STE sequence.

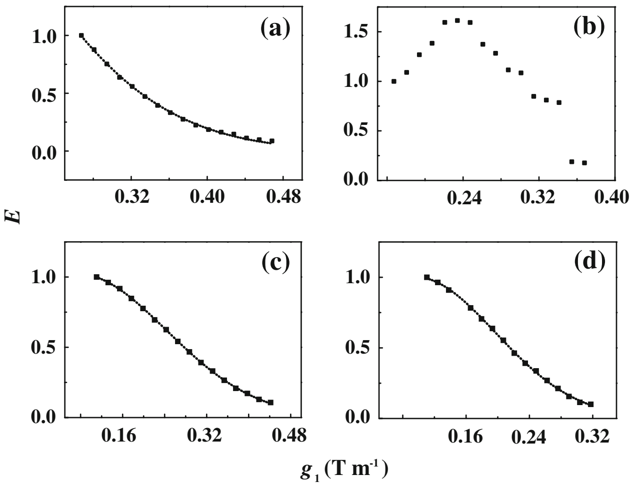
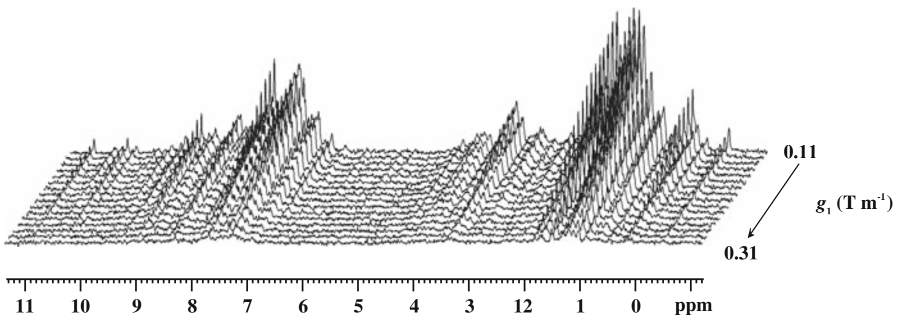


Fig. 2 Representative diffusion attenuation plots of the lysozyme resonance using the standard STE based PGSE sequence with $\Delta = 0.1$ s (**a**) and 0.3 s (**b**) and the double echo PGSTE-WATERGATE sequence (sine gradients) with $\Delta = 0.1$ s (**c**) and 0.3 s (**d**) at 37°C. The *dotted lines* represent non-linear least squares regression of the relevant attenuation equations onto the data

Conclusions

The PGSTE-WATERGATE (Zheng et al. 2008a) solvent suppression unit was successfully modified into a double echo version based on the general concept of the D-STE PGSE sequence (Jerschow and Müller 1997) without adding any pre- or post- suppression unit. The new sequence

Fig. 3 The ^{1}H 400 MHz double echo PGSTE-WATERGATE spectra at 37°C with $g_2 = 0.10$ T m^{-1}, $\delta = 6$ ms, $\Delta = 0.3$ s, number-of-scans = 128, and different g_1 values. The water resonance at ~4.8 ppm was completely suppressed



provides excellent convection compensation and a high solvent suppression factor in excess of 10^5 in a single scan, which is ~ 10 times higher than that of the previously developed WET D-STE sequence. It can be applied to diffusion measurements on a variety of solutes ranging from small molecules like amino acid to large protein aggregates.

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