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A PFG NMR experiment for translational diffusion measurements in low-viscosity solvents containing multiple resonances

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Abstract

Pulsed gradient simulated-echo (PGSE) NMR diffusion measurements provide a facile and accurate means for determining the self-diffusion coefficients for molecules over a wide range of sizes and conditions. The measurement of diffusion in solvents of low intrinsic viscosity is particularly challenging, due to the persistent presence of convection. Although convection can occur in most solvent systems at elevated temperatures, in lower viscosity solvents (e.g., short chain alkanes), convection may manifest itself even at ambient laboratory temperatures. In most circumstances, solvent suppression will also be required, and for solvents that have multiple resonances, effective suppression can likewise represent a substantial challenge. In this article, we report an NMR experiment that combines a double-stimulated echo PFG approach with a WET-based solvent suppression scheme that effectively and simultaneously address the issues of dynamic range and the deleterious effects of convection. The experiment described will be of general benefit to studies aimed at the characterization of diffusion of single molecules directly dissolved in low-viscosity solvents, and should also be of substantial utility in studies of supramolecular assemblies such as reverse-micelles dissolved in apolar solvents.

Keywords: PFG diffusion; Protein encapsulation; Membrane protein; Micelle; Inverted micelle; Reverse micelle; Low-viscosity solvents

1. Introduction

The introduction of multidimensional and multinuclear methods together with the refinement of recombinant technologies that support the incorporation of magnetically active isotopes (^{2}H, ^{13}C, and ^{15}N) into proteins and nucleic acids, has been the driving force of progress in solution NMR. These enhancements have made NMR a central tool in studies of the structure and function of biologically active macromolecules of significant size and complexity. Larger molecules ($M_w > 20$ kDa) represent a challenge to solution NMR-based approaches [1], since increasing size leads to slower reorientational motions, which in turn generate correspondingly increased rates of transverse relaxation rates ($R_2 = 1/T_2$), which compromise the otherwise very powerful potential of solution NMR methods as an analytical tool.

Recently, a method that directly reduces the effective rotational correlation time has been developed that involves encapsulation of a water-soluble macromolecule in a shell of surfactant (usually dioctyl sulfosuccinate, AOT), forming a *reverse micelle* particle that is subsequently dissolved in a low-viscosity solvent, e.g., the short-chain *n*-alkanes [2–8]. Bax and co-workers [9] have investigated a similar strategy that involves liquefied CO_2 (solvent) and the ammonium salt of perfluoroheptanoic acid (surfactant). The results of these studies confirm that macromolecules of significant size and complexity can be encapsulated at concentrations compatible with multidimensional, multinuclear NMR experiments [6] and that encapsulated molecules retain both native structure [10] and native-like biological

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activity [11–14]. Importantly, the tumbling rates of properly prepared encapsulated macromolecules dissolved in appropriately low-viscosity solvents are more rapid than for molecules in aqueous solution, and such encapsulated molecules exhibit spectroscopic properties that are superior to those of the free molecules in aqueous solution [2]. Encapsulation thus promises to provide a fundamental enhancement of solution NMR methods applied to larger molecules.

As a prelude to detailed NMR studies of encapsulated macromolecules, the samples must be characterized to insure that the desired hydrodynamic behavior has been established. The key feature of the characterization is the determination that the desired particle radius has been achieved; which itself depends on the appropriate level of hydration of the encapsulated molecule. In addition, it must be established that the preparation is monodisperse and that the sample is stable—a key consideration for samples that must be maintained under pressure, e.g., butane, propane, ethane, etc. NMR relaxation experiments would in principle provide the most direct and relevant assay of the effective tumbling rate of the particles, however, for macromolecules such experiments are relatively time-consuming and require substantial detailed data analysis. Alternatively, PFG diffusion experiments provide essentially equivalent information, require only a few minutes to record a complete data set and the data are very readily analyzed.

The translational diffusion coefficient is commonly used to determine the effective (solvated) radius of a molecule or complex aggregate under study. The relationship between bulk viscosity, temperature, and particle radius is concisely expressed in the Stokes–Einstein equation:

$$D_t = \frac{k_B T}{6\pi\eta r}, \tag{1}$$

wherein D_t is the translational diffusion constant for a sphere, η is the absolute viscosity, k_B is the Boltzmann constant, r is the solvated particle radius, and T is the absolute temperature (K). The relative ease with which PFG NMR diffusion experiments can accurately measure the translational diffusion coefficient make such experiments the method of first choice for hydrodynamic measurements of complex polymers, perhaps the most important subspecies of which are biopolymers, e.g., proteins, nucleic acid oligomers, and their complexes. Given complete knowledge of the solution components, the oligomeric state of a molecule (e.g., protein) can be directly determined by examining its effective hydrodynamic radius [15].

Although PFG NMR diffusion experiments may be of several distinct types, certain general features are found in all experiments. These common features include formation of a gradient-associated echo, a diffusion interval; the length of which depends on the relative magnitude of the translational diffusion coefficient, and a mechanism for applying a spatially dependent pulsed magnetic field gradient. The latter component, of course, lies at the heart of the approach. Stejskal and Tanner [16] modified the spin-echo (SE) experiment to include pulsed magnetic field gradients, producing a PFG SE diffusion experiment with significantly improved resolution and sensitivity. In the PFG SE experiment, the resonance intensities are monitored as a function of the strength of the field gradient pulses, and the results are fitted to a Gaussian function that depends on the length of the diffusion interval, the PFG strength and the translational diffusion coefficient (see Section 4). Although the experiment produces acceptable results for samples that possess long transverse relaxation times, the approach is not appropriate for application to systems in which T_2 is comparable to the length of the diffusion interval (typically 10–200 ms), due to substantial losses in signal intensity.

A major improvement in the sensitivity of PFG STE experiments was realized in a modified version of the experiment due to Tanner [17]. In this experiment, the transverse magnetization is converted into longitudinal magnetization following generation of the gradient-induced z-coil, and the magnetization is stored along the z-axis for the bulk of the diffusion interval. Since T_1 is significantly longer than T_2 for molecules that tumble relatively slowly, the experiment produces a substantial increase in sensitivity. The echo is produced by conversion of the longitudinal magnetization back into transverse magnetization and subsequent rephasing of the transverse magnetization by a second gradient pulse that is identical to the initial gradient pulse. This variant is therefore referred to as the stimulated-echo diffusion experiment, PFG STE. Gibbs and Johnson [18] further modified the PFG STE to include an additional delay interval, T_e, that would allow PFG-induced eddy currents in the surrounding metal probe components to dissipate, and this variant has been named the longitudinal-encode-decode sequence (PFG STE LED). Modern PFG coils have relatively short eddy-current recovery times (in some cases less than 1 ms), so that this additional delay included in the LED experiment is not always strictly necessary. In practice however, the interval remains a common element of current PFG diffusion pulse sequence designs, wherein the interval supports more effective phase-cycling (artifact rejection) and solvent suppression modules.

A fundamental assumption of the experimental design of all of the PFG diffusion experiments discussed thus far is that the translation of the molecules is stochastic, as is expected for translation driven by Brownian motion. Convection currents, or vector flow generally, cause artifacts that interfere with diffusion measurements by generating additional dephasing of

the vectors, leading to unpredictable loss of signal intensity, e.g., dephasing due to the nonrandom transport is superposed on the dephasing arising from the random Brownian motion. Assuming that the sample is confined in a standard NMR tube, the nonrandom motion will be entirely due to convection, which is defined as flow arising from a density gradient, e.g., flow driven by gravitational forces in an otherwise static sample volume.

The temperature at which onset of convection is established in a fluid confined within an infinitely long cylinder may be expressed using a dimensionless parameter, γ, defined in the following expression:

$$\gamma = \left(\frac{g\alpha}{\nu\kappa}\right) * r^4 T', \tag{2a}$$

$$T' = \frac{\Delta T}{d}, \tag{2b}$$

in which g is the acceleration due to gravity, α is the coefficient of thermal volume expansion, ν is the kinematic viscosity (η/ρ), κ is the thermal diffusivity; which is equal to $k/\rho c_p$, where k is the thermal conductivity and c_p is the heat capacity at constant pressure, r is the inner radius of the cylinder (tube) and $T'(\Delta T/d)$ is the temperature change across the height of the sample, $d \gg r$ [19,20]. The parameter γ adopts a limiting value, γ_c, that is equal to 67.4 for the case of a perfectly insulating wall and 215.8 [20] for the case of a perfectly conducting walls [19]. Convection arises within the sample volume if γ exceeds γ_c, and thus a critical temperature, T'_c, may be identified, above which convection will be expected to be present:

$$T'_c = \frac{\Delta T}{d} \propto \left(\frac{\nu\kappa}{g\alpha}\right)\left(\frac{\gamma}{r^4}\right). \tag{3}$$

A sample calculation by Lounila et al. [20] indicates that for a sample of neat benzene at 20 °C in a glass (essentially a conducting wall) 10 mm OD NMR tube ($r = 4.5$ mm) the transition to convective flow occurs if the temperature gradient exceeds $3.5\,\mathrm{K\,m^{-1}}$.

Jershow and Muller [21] observed that if the nonrandom flow components are highly regular, it should be possible to record and sum a pair of diffusion experiments, one which stores magnetization during the diffusion interval along the +z direction, the other which stores magnetization along the −z direction. The components of the apparent diffusion due to net flow should compensate one another, effectively negating the effects of convection. The experiment developed based on these considerations makes use of tandem-stimulated echoes, and is known as the PFG double stimulated echo pulse sequence, PFG DSTE.

The experiment described here is based on the PFG DSTE experiment, and we have shown the effectiveness of the approach in application to a challenging system consisting of a sample of water-containing reverse micelles in *n*-pentane. The onset of convective flow is directly proportional to the kinematic viscosity, and comparison of the kinematic viscosities of water and *n*-pentane, 1 St and ~0.36 St, respectively, indicates that convection will be significantly more prevalent in the lower viscosity solvent (refer to Eq. (3)). Furthermore, the effectiveness of encapsulation as an enhancement to standard approaches depends on access to solvents with kinematic viscosities that are even lower than *n*-pentane, for example for propane, ν is ~0.19 St. Clearly, a diffusion experiment that is robust in terms of its tolerance to the influence of net flow is essential. The use of hydrocarbon solvents likewise generates a substantial solvent suppression challenge. Such solvents produce multiple resonances, all of which are relatively intense. Effective solvent suppression schemes must therefore be capable of simultaneously scaling the intensity of at least two relatively broad resonances (methyl and methylene).

2. Results

The effects of convection on the results of an uncompensated PFG STE LED diffusion experiment [15] modified to include WET solvent suppression [22,23] are shown in Fig. 1. The data shown was recorded at 30 °C on a sample consisting of 75 mM AOT in *n*-pentane with a water-loading ($[H_2O]/[AOT]$) of 30. The irregular intensity oscillations are a hallmark feature of flow effects. Depending on the viscosity of the solvent used, the transition to convective flow can be remark-

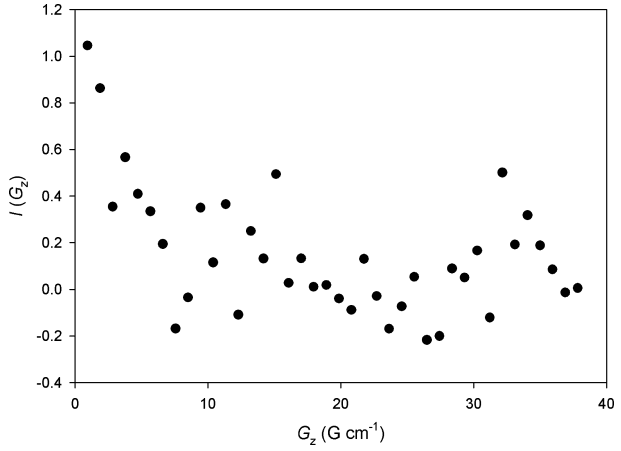

Fig. 1. Plot of resonance intensity, $I(G_z)$, versus applied gradient field strength for data recorded using a conventional, uncompensated, longitudinal-encode-decode, WET PFG STE LED pulse sequence. A WET-based solvent suppress scheme was employed to suppression the proton resonances of the solvent. The sample was 75 mM AOT in *n*-pentane with a w_0 of 30. The data were recorded at 30 °C with a spectral width of 4000 Hz into 8192 complex points. The diffusion interval was 100 ms and the length of the gradient employed to prepare and refocus the *z*-coil was 3 ms.

ably sharp, e.g., experimentally, about 1 °C in pentane. The phenomenon of convection within the NMR cell appears to be intrinsic to the design, e.g., a long thin cylindrical geometry tends to promote the onset of convection. Indeed, a recent article by Hayamizu and Price [24] describes a specialized NMR cell that defeats convection by employing a sample chamber geometry wherein the length is small relative to the width. Although undoubtedly effective, the small volume of the design together with the requirement for custom fabrication limits the applicability of the approach.

A representative spectrum recorded on the AOT/water/pentane sample using the PFG WET DSTE experiment is shown in Fig. 2 (PFG strength equal to $\sim 7.5\,\mathrm{G\,cm^{-1}}$). The spectrum reveals that the effective intensity of the *n*-pentane ^{1}H resonances has been scaled to a level similar to that of the surfactant, 75 mM in this instance. The solvent resonance suppression provided by the WET sequence is exquisitely selective, e.g., no distortion in the intensity of resonances of either water or the surfactant occurs as a result, nor are there any baseline distortions or other spectral artifacts. Furthermore, as the spectrum shown represents a member of the diffusion array, it is clear that high-resolution spectral lineshapes are obtained using the method.

A complete data set obtained using the WET PFG DSTE experiment is shown in Fig. 3. The influence of convective flow has been completely compensated, as is made clear by the absence of any hint of artifacts in the plot of resonance intensity versus applied gradient strength, e.g., the irregular oscillations have been completely eliminated. The fitted value of the diffusion coefficient, $2.1 \times 10^{-6}\,\mathrm{cm^2\,s^{-1}}$, agrees with the extrapolated value of the diffusion coefficients measured at lower temperatures (wherein convection does not manifest itself) using a simple LED experiment [8]. We have generally

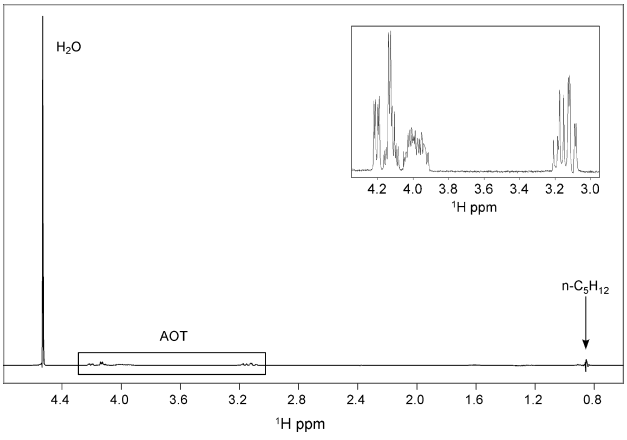

Fig. 2. One-dimensional ^{1}H spectrum of a sample of 75 mM AOT, w_0 of 30, in *n*-pentane recorded using the WET PFG DSTE pulse sequence (this particular spectrum was recorded with a PFG strength of $\sim 7.5\,\mathrm{G\,cm^{-1}}$). Spectral parameters and temperature were identical to those specified in Fig. 1.

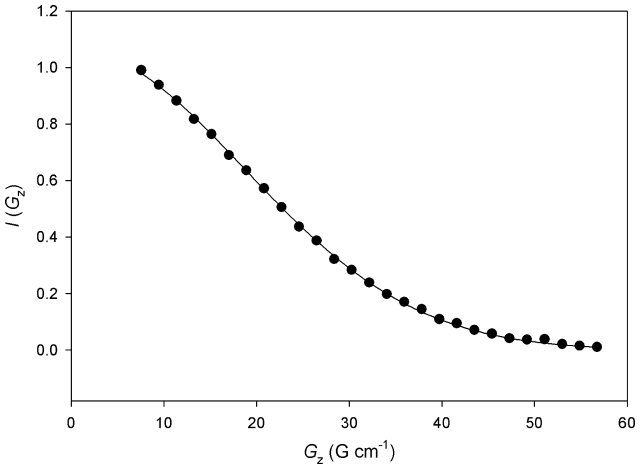

Fig. 3. Plot of resonance intensity versus applied gradient field strength for data recorded using the WET DSTE LED pulse sequence. The sample and conditions employed were identical to those described in the legend of Fig. 1, except that the gradient field strength array was extended to higher maximal values.

observed that the intensity of field gradient pulses departs from a linear dependence on the current to the PFG coil at low current values, and that this effect is more prevalent with shorter gradient pulse lengths (<5 ms). The WET PFG DSTE experiment described here appears to be somewhat more sensitive to such effects compared to simple LED experiments. This condition is easily remedied by restricting employed gradient strength values to those that fall in the linear response regime, in the authors' experience, between 2 and $10\,\mathrm{G\,cm^{-1}}$ on the low field side and between 60 and $70\,\mathrm{G\,cm^{-1}}$ on the high field side for modern triple-resonance triple-axis gradient probes. There are no significant consequences of this condition, and the only requirement is the need to monitor the linearity of the PFG coil, which is a standard procedure for quantitative studies of diffusion.

3. Discussion and conclusions

Measurement of translational diffusion is among the most convenient methods for characterizing effective particle radii for both single-molecules and complexes across a wide range of molecular weights and conditions. The pulsed-gradient double stimulated echo method (DSTE) provides an accurate and relatively straightforward approach to such diffusion measurements under virtually all conditions.

A significant general challenge to efficient NMR characterization of molecules dissolved in hydrocarbon solvents involves suppression of the solvent resonances. ^{1}H NMR spectra of molecules dissolved in alkane solvents will be dominated by the methyl resonance and methylene resonances of the solvent. The concentration

of the neat hydrocarbon solvents ranges from ~6 M for *n*-octane, ($\rho = 0.703\,\mathrm{g\,mol^{-1}}$) to ~13 M for propane, ($\rho = 0.585\,\mathrm{g\,mol^{-1}}$). The concentration of dissolved molecules will naturally be much lower, e.g., surfactant concentrations for reverse-micelle preparations will be in the range of 50 to 200 mM. The density of *n*-pentane is $0.626\,\mathrm{g\,mol^{-1}}$, which corresponds to ~8.7 M for the neat liquid, and the relative concentration of methyl protons in neat *n*-pentane is thus 52.2 M (2 equivalent methyl resonances, each from three protons). Taking the concentration of the dissolved molecules to be 100 mM, the dynamic range between the solvent and protein resonances will be greater than 500:1.

One way in which the dynamic range may be reduced will be to employ deuterated solvents. Deuterated versions of most *n*-alkanes are readily available, although their cost is extremely high. Furthermore, commercially available deuterated *n*-alkane solvents are generally less than 98% enriched in the deuterium isotope, which given their high relative concentration means that suppression of the residual protonated resonance may be necessary in cases wherein lower solute concentrations are used.

We have found that that effective suppression of solvent resonances that are both complex and intense may be effectively suppressed using a WET (Water suppression Enhanced through T_1 effects) pulse sequence [22,23]. The WET sequence consists of a series of alternating long, low-power shaped RF-pulses and field-gradient pulses that provide superior suppression of solvent resonances. An important practical element of the effectiveness of the WET sequence is the ability to incorporate tailored excitation that allows suppression of multiple resonances. Effective solvent suppression, especially for multiline solvents such as the *n*-alkanes, requires RF be applied simultaneously at several frequencies (i.e., the methyl and methylene resonances). This is accomplished by comodulating a standard waveform (e.g., sinc, SEDUCE, even a rectangular waveform) at multiple frequencies [25,26]. The required complex RF waveforms modulated to produce simultaneous multiple excitations are readily generated using standard pulse generation algorithms available on most commercial spectrometer systems. The WET solvent suppression component of the experiment renders the requirement for deuterated solvent completely unnecessary, even when the solute concentration is as low as a few millimolar.

The data presented confirm that WET PFG DSTE LED experiment described here provides a facile and accurate method for measuring the translational diffusion coefficients for complex molecular aggregates dissolved in low-viscosity solvents. Furthermore, we have also applied the experiment to measurement diffusion coefficients to relatively simple aqueous solutions and obtained results with accuracy and precision identical to that recorded using standard PFG STE LED experiments (data not shown).

4. Experimental

Dioctyl sulfosuccinate (AOT) was obtained from Sigma, and used without additional purification. AOT is hygroscopic and must be stored in a dessicator. *n*-Pentane was obtained from J.T. Baker and used without further purification. The sample used in this study consisted of a 75 mM solution of AOT in *n*-pentane with a water loading, w_0 ([H_2O]/[AOT]), of 30. The sample volume was 1 mL and the sample tube employed was a 5 mm OD screw-cap tube purchased from Wilmad Glass. Convection was established in the sample by raising the temperature to 30 °C. The presence of convection was confirmed by recording a WET STE LED experiment, which is sensitive to the effects of convection (see Fig. 1).

All data were recorded on a Varian INOVA NMR spectrometer operating at 500 MHz ^{1}H. The probe employed in this study was a Varian indirect single-axis (*z*-coil) PFG probe. Maximum gradient strength of the PFG amplifier and probe combination was $62\,\mathrm{G\,cm^{-1}}$. Spectral parameters, as well as phases of the RF pulses are given in the legend to Fig. 4. The pulse sequence and an associated parameter file are available upon request or may be obtained from the NMRFAM website.

An extremely versatile and effective WET-based solvent suppression scheme allows the experiment to be readily applied to systems that involve complex solvents with multiple resonances. The WET sequence consists of four shaped RF pulses alternating with magnetic field gradient pulses. The WET-associated RF pulses were 6 ms in duration and of the SEDUCE type [27,28]. The analytical form for a single SEDUCE pulse is defined in Eqs. (4a) and (4b) below:

$$v_2(t) = v_0 \sin^2\pi\left(t + \tfrac{1}{2}\right) \operatorname{sec}\mathrm{h}(500\mathrm{tc}(t)), \tag{4a}$$

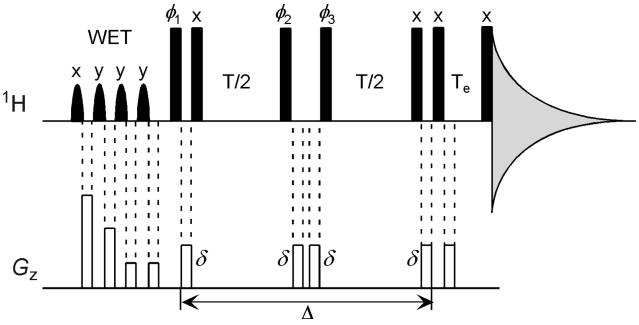

Fig. 4. WET PFG DSTE pulse sequence. The WET component of the sequence is discussed in detail in the text. All remaining RF pulses were 90° (π/2), with phases designated directly above each pulse. Pulses labeled with an explicit phase were not cycled; the remaining pulses were cycled according to the following schedule: ϕ_1: *x*, *y*, −*x*, −*y*; ϕ_2: −*x*, −*y*; ϕ_3: 4(−*x*), 4(*x*). The received was cycled according to the following schedule: ϕ_{rec}: *x*, *x*, 4(−*x*), *x*, *x*. The length of the gradient pulses (labeled δ in the figure) was 3 ms and the gradient pulse applied during the interval labeled T_e was 0.5 ms.

$$c(t) = 10\tanh^2(0.08|t|); \quad -\frac{1}{2} \leqslant t \leqslant \frac{1}{2}. \tag{4b}$$

SEDUCE pulses were delivered as laminar waveforms [25,26], with phase-encoded extrema at 1114 Hz (2.2 ppm) and 1318 Hz (2.6 ppm) upfield of the carrier frequency aimed at the methylene and methyl resonances respectively. WET-associated PFG pulses used were 1 ms in duration and the intensities of the individual sequential PFG pulses was approximately 28, 14, 7, and 3.5 G cm^{-1}. The total interval between RF pulses in the WET module was ~7 ms, e.g., the sum of the RF and PFG pulses. In principle, solvent suppression could be optimized by varying the duration between RF pulses, e.g., optimized for differences in solvent and solute T_1s, however in practice such adjustments translated into small changes in the suppression and the overall suppression of solvent resonances is extremely effective (see Fig. 2).

The PFG NMR diffusion experiment described in this article employs a tandem-stimulated echo approach. The experiment makes use of constant-time diffusion with variation of the strength of the applied gradient pulses to produce an array of one-dimensional experiments wherein the intensity is scaled both by the diffusion interval and the magnitude of the gradient pulses. The diffusion interval and the gradient strength used to install the z-coil must be empirically balanced depending on the maximal gradient strength available and the absolute value of the diffusion coefficient. In the current study, the total diffusion interval was 100 ms. To further improve sensitivity, the transverse magnetization is converted into longitudinal magnetization following generation of the z-coil. For macromolecules, wherein the product of the rotational correlation time and the Larmor frequency are much larger than unity, this element makes a substantial contribution to the overall sensitivity of the experiment.

The diffusion experiment is conducted using an array of PFG strengths, between 7.5 and 57 G cm^{-1} in the present example. The translational diffusion coefficient, D_t, is fit to the following Gaussian expression:

$$I(G_z) = I_0 \exp[-(\gamma\delta G_z)^2(\Delta)D_t], \tag{5a}$$

$$\Delta = T + 4\delta/3 + 2\tau, \tag{5b}$$

wherein $I(G_z)$ is gradient-dependent intensity, I_0 is the initial intensity, G_z is the strength of the applied field gradient pulse (which is arrayed in this experiment), δ is the duration of the field-gradient pulse and T is a delay that together with the length of the gradient pulses and associated delays, τ, determines the effective diffusion interval [21]. The sequence also minimizes the influence of eddy currents by including an additional delay time period before the signal acquisition, which also serves to accommodate additional phase cycling and could in principle support an additional solvent suppression component (not necessary in the current implementation).

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