

Slow Diffusion of Macromolecular Assemblies by a New Pulsed Field Gradient NMR Method[†]

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Abstract: The translational diffusion coefficient of an integral membrane protein/surfactant complex has been measured using a novel pulsed field gradient NMR method. In this new approach, the information about the localization of the molecules is temporarily stored in the form of longitudinal magnetization of isotopes with long spin–lattice relaxation times. This allows one to increase the duration of the diffusion interval by about 1 order of magnitude. Unlike standard proton NMR methods using pulsed field gradients and stimulated echoes, the new method can be applied to macromolecular assemblies with diffusion coefficients well below $10^{-10} \text{ m}^2 \text{ s}^{-1}$, corresponding to masses in excess of 25 kDa in aqueous solution at room temperature. The method was illustrated by application to a water-soluble complex of tOmpA, the hydrophobic transmembrane domain of bacterial outer membrane protein A, with the detergent octyl-tetraoxyethylene (C_8E_4 ; overall mass of complex ~ 45 kDa). The diffusion coefficient was found to be $D = (4.99 \pm 0.07) \times 10^{-11} \text{ m}^2 \text{ s}^{-1}$, consistent with measurements by size exclusion chromatography and by ultracentrifugation. The method has also been applied to a solution of recombinant human tRNA₃^{Lys}, which has a molecular mass of 24 kDa, and the diffusion coefficient $D = (1.05 \pm 0.015) \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$.

I. Introduction

Pulsed field gradient (PFG) methods used in nuclear magnetic resonance (NMR) provide one of the most versatile means of studying transport phenomena such as flow and diffusion in fluids.^{1,2} By applying gradients in three orthogonal dimensions, it is possible to determine the direction of flow or the anisotropy of translational diffusion. PFG-NMR methods can provide a measurement of the translational diffusion coefficient D of a complex, which is directly related to its Stokes radius R_S and correlation time τ_c . Other approaches to determining the size of a macromolecule or a supramolecular assembly include ultracentrifugation, molecular sieving, quasi-elastic light scattering, and small-angle X-ray or neutron scattering. One advantage of PFG-NMR methods, however, is that measurements can be carried out under experimental conditions (protein and detergent concentration, temperature, viscosity, etc.) identical with those used for collecting structural NMR data. Unfortunately, the small diffusion coefficients ($D < 10^{-10} \text{ m}^2$

s^{-1}) associated with biological macromolecules or supramolecular assemblies with masses in excess of 50 kDa are difficult to measure by standard PFG-NMR methods using stimulated echoes.^{2–4} This limitation results from the rapid longitudinal relaxation of the nuclei (usually protons) that carry the information about the localization of the molecules during the diffusion interval. It is shown in this paper that the duration of this interval can be increased by about 1 order of magnitude by storing the information in the form of longitudinal magnetization of heteronuclei, such as nitrogen-15, that have much longer spin–lattice relaxation times than protons. This novel method, called “heteronuclear stimulated echoes” (X-STE), allows one to gain about 1 order of magnitude in the measurement of D , so that diffusion coefficients of molecules with molecular masses in excess of 100 kDa should be readily measurable.

One of the principal hurdles that must be overcome prior to determining the structure of integral membrane proteins by solution-state NMR is the optimization of their solubilization. Ideally, the hydrophobic transmembrane surface of the protein should be covered with the thinnest possible layer of surfactant, so that the overall dimensions of the resulting assembly remain as small as possible. The concentration of free surfactant micelles must be minimized so as to reduce the viscosity of the solvent. Large particle size or a highly viscous medium entail slow rotational diffusion, long tumbling correlation times τ_c ,

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[†] Abbreviations: NMR, nuclear magnetic resonance; PFG, pulsed field gradient; STE, stimulated echo; X-STE, heteronuclear stimulated echo; tOmpA, transmembrane domain of *Escherichia coli* outer membrane protein A; C_8E_4 , octyl-tetraoxyethylene; sds-page, sodium dodecylsulfate polyacrylamide gel electrophoresis.

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broad NMR lines, and hence poor resolution and sensitivity. The new method is illustrated here by application to tOmpA, the transmembrane domain of the bacterial outer membrane protein OmpA, complexed by the detergent octyl-tetraoxyethylene (C₈E₄; overall mass of complex ~45 kDa). tOmpA is one of the largest membrane proteins whose 3D structure has been studied by solution NMR.^{5,6} The diffusion coefficient of tOmpA with C₈E₄ obtained by X-STE NMR was found to be $D = (4.99 \pm 0.07) \times 10^{-11} \text{ m}^2 \text{ s}^{-1}$ at 23 °C, in good agreement with measurements obtained by size exclusion chromatography and by ultracentrifugation. The method was also applied to a solution of tRNA₃^{Lys}, which has a molecular mass of 24 kDa and the diffusion coefficient $D = (1.05 \pm 0.015) \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$.

II. Pulsed Field Gradient NMR

The success of the original pulsed field gradient spin-echo NMR method introduced by Stejskal and Tanner¹ has led to the development of many variants, particularly methods that employ so-called stimulated echoes, where the information about the localization of the molecules is temporarily stored in the form of longitudinal magnetization.³ Such experiments are usually carried out using the magnetization of protons, because of the favorable gyromagnetic ratio that allows one to “encode” the initial spatial position with good accuracy without resorting to very intense gradients. Other nuclei, such as carbon-13, have been employed to achieve a better dispersion in the chemical shift dimension.^{7,8} Byrd and co-workers⁹ have proposed a method that includes water-selective pulses to suppress the intense solvent signal, combined with an additional interval (longitudinal eddy current delay) that allows transient perturbations to die out. Undesirable effects of eddy currents can also be circumvented by so-called “bipolar gradients”.^{10–12} It has also been shown that diffusion can be distinguished from flow or convection.⁴ The development of novel experimental methods has been accompanied by substantial efforts to extract reliable information from the attenuation of the signals as a function of gradient amplitude, which ideally should obey a Gaussian function. Diffusion coefficients can be deduced from these curves by various approaches that use inverse Laplace transformations. This has led to the so-called DOSY (Diffusion Ordered Spectroscopy) representation:² i.e., two-dimensional plots where diffusion coefficients appear along the ordinates and chemical shifts along the abscissae, allowing the separation of individual components in a complex mixture according to their diffusion coefficients.

The storage of information in the form of longitudinal proton magnetization is of course subject to spin-lattice relaxation $T_1(^1\text{H})$. In macromolecules such as proteins and nucleic acids, this limits the useful duration of the diffusion interval Δ . In samples such as those used here, with masses on the order of

50 kDa, $T_1(^1\text{H})$ typically is ~100 ms. The diffusion interval therefore must be limited to $\Delta < 100 \text{ ms}$ (if $\Delta = T_1(^1\text{H})$, the signal intensity decreases to $\exp\{-\Delta/T_1(^1\text{H})\}$, i.e., to 37%). This limitation means that the very small diffusion coefficients typical of larger molecules, which require longer diffusion intervals, are difficult to measure. Special probes that are equipped with heavy-duty coils capable of generating very intense gradients in principle allow one to determine small diffusion coefficients in short intervals, but it is difficult to carry out modern high-resolution triple-resonance triple-gradient experiments with such special probes. The conventional proton STE methods, using a 600 MHz spectrometer equipped with standard gradients for high-resolution NMR, allow one to determine the diffusion coefficient in aqueous solution of a small protein such as ubiquitin⁹ (8.6 kDa; $D = 1.49 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$). However, it is difficult to determine D values below $\sim 10^{-10} \text{ m}^2 \text{ s}^{-1}$, as are expected for complexes larger than ~25 kDa. When a polyhistidine tag is attached to the protein, it may be possible to observe the proton signals due to this mobile tag because of their relatively narrow lines and slow proton spin-lattice relaxation.¹³ However, histidine tags are not always available and, even if they are, interpreting their signals may be problematic because of baseline problems and exchange of amide protons with the solvent. Although the tOmpA construct used in the present work does carry a polyhistidine tag, we have found it difficult to determine its diffusion coefficient using the proton STE method.

Since nitrogen-15 enrichment is a prerequisite for most modern biomolecular NMR methods, the optimization of solubilization conditions that must precede structural studies can usually be carried out with isotopically enriched proteins or nucleic acids. It is then possible to transfer magnetization between amide proton $^1\text{H}^{\text{N}}$ and ^{15}N nuclei through the scalar couplings $^1J(^1\text{H}, ^{15}\text{N}) = -92 \text{ Hz}$. Most heteronuclear correlation methods exploit the favorable dispersion of the ^{15}N chemical shifts in so-called HSQC (Heteronuclear Single Quantum Correlation) spectra. In the present work, we do not use ^{15}N chemical shifts, but we take advantage of another favorable feature of ^{15}N spins in biomolecules: namely, the fact that their longitudinal relaxation times $T_1(^{15}\text{N})$ are usually longer than the $T_1(^1\text{H})$ values of the protons. Typically, for the tOmpA/detergent complexes used in this study, $T_1(^{15}\text{N}) = 1 \text{ s}$ whereas $T_1(^1\text{H}) = 100 \text{ ms}$. This makes it attractive to store the information in the form of longitudinal *nitrogen* magnetization. A similar advantage can be obtained with other heteronuclei. We shall therefore refer to the new method as “X-STE”, for “heteronuclear stimulated echoes”.

The pulse sequence is shown in Figure 1. The initial 90° nitrogen-15 pulse at point **a** and the subsequent gradient G_1 serve to saturate the equilibrium nitrogen magnetization. The G_z gradients of duration δ and variable amplitude G_{encode} and G_{decode} are used for “bipolar encoding” in the first two τ delays and for “bipolar decoding” in the last two τ delays. Their effect can be summed up by the factor $\kappa = \gamma s G_{\text{max}} \delta$, where γ is the proton gyromagnetic ratio, s the shape of the (usually not rectangular) encoding and decoding gradient pulses, G_{max} their peak amplitude, and δ their duration. The phase accumulated by the magnetization during each gradient pair is proportional to the z

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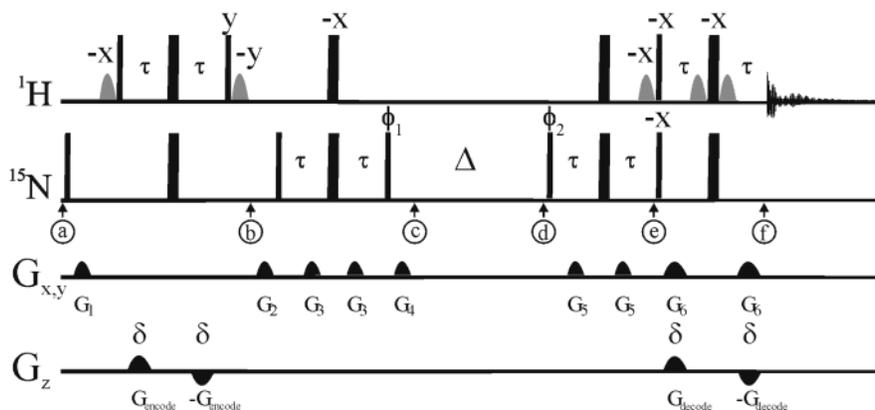


Figure 1. Pulse sequence designed for the measurement of diffusion coefficients using longitudinal magnetization of a heteronucleus such as nitrogen-15 with stimulated echoes (X-STE). Narrow and wide pulses represent 90 and 180° pulses, respectively. Unless otherwise stated, all pulses are applied along the x axis of the doubly rotating frame. The encoding and decoding gradients $G_{\text{encode}} = G_{\text{decode}}$ have variable amplitudes and typical durations $\delta = 1.3$ ms each; the total diffusion delay ($\Delta + 6\tau$) was 1 s. The INEPT delays were $\tau = |4J_{\text{HN}}|^{-1} = 2.72$ ms ($J_{\text{HN}} \approx -92$ Hz). The phase cycling employed is $\Phi_1 = y, -y, y, -y$ and $\Phi_2 = y, y, -y, -y$, with receiver phase $\Phi_{\text{rec}} = x, -x, -x, x$.

coordinate of the initial position of the molecule along the vertical z axis of the sample, $\phi = 2\kappa z$. The gradients G_{encode} and G_{decode} are used to dephase (and later rephase) the transverse proton magnetization during the τ intervals. At point **b**, after an INEPT-type sequence with $\tau = |4J_{\text{HN}}|^{-1} = 2.72$ ms, the longitudinal two-spin order $2H_z N_z \cos(2\kappa z)$ is spatially modulated along the z axis of the sample tube because of the two bipolar gradient pulses, slightly attenuated by the factor $\exp\{-D\kappa^2\tau\}$ due to the onset of diffusion between encoding and point **b** (for simplicity, we shall assume that the two encoding gradients are close to each other and that they are both separated by an interval τ from point **b**). The sequence between points **b** and **c** converts longitudinal two-spin order $2H_z N_z \cos(2\kappa z)$ into nitrogen-15 Zeeman order $N_z \cos(2\kappa z)$, now attenuated by the diffusion factor $\exp\{-D\kappa^2 3\tau\}$ at point **c**. The reconversion of N_z into observable proton magnetization H_y at point **f** follows a roughly symmetrical path. Assuming again that the two decoding gradients are close to each other and separated by an interval τ from point **e**, the resultant signal is attenuated by translational diffusion during six short τ intervals and during the much longer Δ interval. The sine-shaped proton pulses are water flip-back pulses,¹⁴ typically Gaussian 90° pulses of 1.2 ms duration. Selective pulses are used between points **e** and **f** to suppress the solvent signal with the “Watergate” method.¹⁵ The gradients G_x and G_y are used to purge unwanted magnetization components. A four-step phase cycle is used to eliminate signals of protons that are not coupled to ^{15}N (isotope filtration) and to ensure that longitudinal relaxation causes the signals to converge asymptotically to zero rather than toward their equilibrium value.

It has been shown by Jerschow¹⁶ that external heating or cooling of ordinary NMR sample tubes may lead to convection effects in the vertical direction, with the solution rising on one side of the tube and falling on the opposite side. Velocities on the order of 0.1 mm/s have been measured for aqueous solutions. Although convection effects can be distinguished from diffusion by cleverly designed variants of stimulated echo sequences,⁴ they cannot easily be separated from diffusion effects with our X-STE method. It is therefore important to minimize convection

effects, by using Shigemi tubes where the solution is constrained to a small volume of limited height (as for both of our samples), by slowly spinning the sample around the vertical axis, or by avoiding asymmetric heating or cooling. When using ordinary tubes, the diffusion coefficient should be measured in two orthogonal directions, by using encoding gradients either along the z axis or along a transverse axis. As expected from flow velocity profiles,¹⁶ transverse gradients give a slightly smaller apparent diffusion coefficient, which we consider to be the best measure.

If convection can be ruled out, and if a triple-gradient probe is not available, all gradients can be applied along the z axis, provided suitable precautions are taken to avoid accidental refocusing. The Watergate sequence must then be performed after decoding. This procedure allows one to employ slightly longer decoding gradients, since their duration is no longer limited by the length of the selective pulses applied to the water resonance.

The ratio of the signal S attenuated by diffusion (recorded with $G_{\text{encode}} = G_{\text{decode}} \neq 0$) to the reference signal S_0 (recorded with very weak gradient amplitudes) obeys the equation

$$S/S_0 = \exp\{-D\kappa^2(\Delta + 6\tau)\} \quad (1)$$

Both the reference signal S_0 and the attenuated signal S are damped by transverse proton and nitrogen relaxation and by longitudinal nitrogen-15 relaxation, which may be expressed by a common factor f :

$$f = \exp\{-4\tau/T_2(^1\text{H})\} \exp\{-4\tau/T_2(^{15}\text{N})\} \exp\{-\Delta/T_1(^{15}\text{N})\} \quad (2)$$

This may be compared to the attenuation factor in the homonuclear proton stimulated spin-echo (STE) method as used by Byrd and co-workers:⁹

$$f' = \exp\{-4\delta/T_2(^1\text{H})\} \exp\{-\Delta/T_1(^1\text{H})\} \quad (3)$$

It should be emphasized, however, that in eq 2 the delay τ is constrained to $\tau = |4J_{\text{HN}}|^{-1}$ (2.7 ms), while in eq 3 δ is equal to the duration of a single bipolar gradient. In eqs 2 and 3, the effective transverse relaxation rates are averages of the trans-

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verse relaxation rates of in-phase and anti-phase coherences:

$$1/T_2(^1\text{H}) = \{R_2(\text{H}_x) + R_2(2\text{H}_x\text{N}_z)\}/2 \quad (4)$$

$$1/T_2(^{15}\text{N}) = \{R_2(\text{N}_x) + R_2(2\text{H}_z\text{N}_x)\}/2 \quad (5)$$

If we neglect for simplicity the effects of transverse relaxation in the τ intervals, the main difference between eqs 2 and 3 lies in the factors $\exp\{-\Delta/T_1(^{15}\text{N})\}$ and $\exp\{-\Delta/T_1(^1\text{H})\}$. If one accepts an attenuation of the signal intensity by the factor $e^{-1} = 0.37$ in both experiments, the delay Δ can be extended by about a factor of 10, from ca. 100 ms in the homonuclear STE method to ca. 1 s in the X-STE experiment, which opens the way to measuring much smaller diffusion coefficients. The key advantage of the new method is that relaxation during the diffusion delay is no longer a limiting factor. For very large molecules, however, signal losses due to transverse relaxation during the fixed τ intervals will take a heavy toll in sensitivity. These effects can be attenuated by deuteration¹⁷ and by exploiting the so-called TROSY (transverse relaxation-optimized spectroscopy) effect.¹⁸

III. Applications

The X-STE method was applied to the complex of the transmembrane domain of *Escherichia coli* outer membrane protein A (tOmpA) with octyl-tetraoxyethylene (C_8E_4). The original tOmpA plasmid was kindly provided by G. Schulz (Freiburg University). It encodes residues 1–171 and includes, as compared to the wild-type sequence, three mutations (F23L, Q34K, L107Y) that have proven helpful in solving the structure by X-ray crystallography.¹⁹ An eight-residue polyhistidine tag was added to the C-terminus of tOmpA.²⁰ The protein was overexpressed in *E. coli* grown on minimum medium enriched in nitrogen-15, purified as inclusion bodies, and refolded as described.²¹ The solutions used for our diffusion studies contained 1 mM tOmpA/ C_8E_4 complex in 20 mM Tris buffer, pH 8, $\text{H}_2\text{O}:\text{D}_2\text{O}$ ratio 9:1, and ~ 300 mM free C_8E_4 (critical micellar concentration ca. 8.5 mM). tOmpA/ C_8E_4 complexes appeared to be monodisperse upon size exclusion gel chromatography, with the apparent Stokes radius $R_S = 3.3 \pm 0.3$ nm. Contrast variation small-angle neutron scattering indicates that tOmpA/ C_8E_4 complexes comprise ~ 25 kDa of detergent (~ 80 molecules) per 20 kDa of protein.²²

Figure 2 shows proton signals of tOmpA in C_8E_4 recorded at 23 °C on a Bruker DRX 600 MHz spectrometer using a standard triple-resonance and triple-gradient TBI probe. The signals stem from proton magnetization that has been transferred back and forth to nitrogen-15 nuclei. The overall pulse sequence lasted about 1 s, and the experiment required 11 min for each of the 16 increments of the gradient amplitudes. Not surprisingly, given that solubilization conditions have not been optimized, the proton spectra of tOmpA are not well resolved. However, sds-page results and the dispersion of ^1H and ^{15}N spectra (data not shown) indicate that the protein is folded. The breadth of

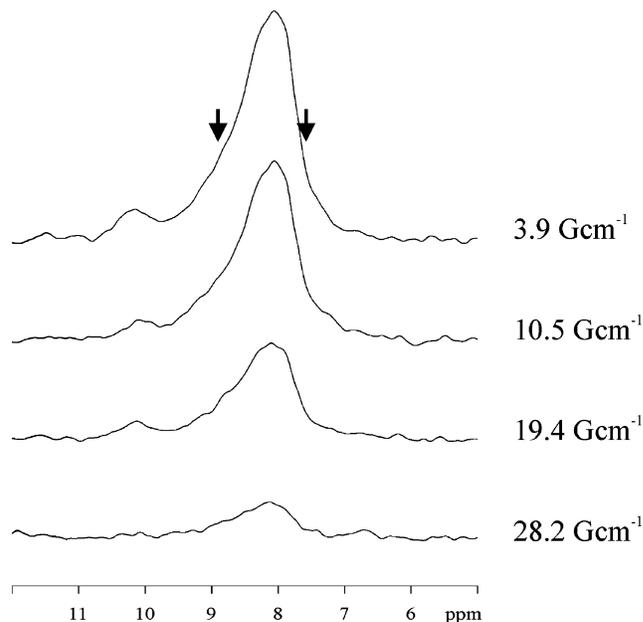


Figure 2. Nitrogen-15-filtered proton spectra of ^{15}N -tOmpA in C_8E_4 , pH 8, $T = 23$ °C. Arrows indicate the limits of the region (between 7.6 and 8.9 ppm) over which signals were integrated to obtain the plot shown in Figure 3. The diffusion delay was $\Delta + 6\tau = 1$ s; each sine-shaped encoding gradient lasted $\delta = 1.3$ ms. The free induction decays were multiplied by exponentially decaying functions corresponding to 100 Hz line broadening. Each spectrum resulted from 256 transients obtained in 11 min.

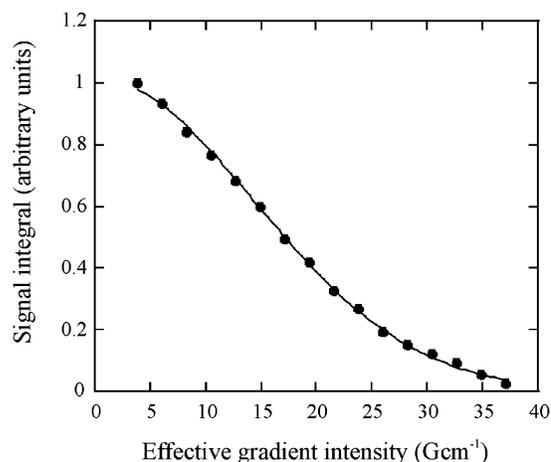


Figure 3. Decay of the integrated signal of the tOmpA/ C_8E_4 complex as a function of the amplitude of the encoding and decoding gradients $G_{\text{encode}} = G_{\text{decode}}$. Proton signals were integrated over the amide region (see Figure 2). The diffusion coefficient, determined by fitting to eq 1, is $D = (4.99 \pm 0.07) \times 10^{-11} \text{ m}^2 \text{ s}^{-1}$.

the resonances may be due to several factors, including viscosity, enhancement of proton relaxation by high pH, and heterogeneity. Nevertheless, the experiment is quite effective, which should make it a useful tool for optimizing solubilization conditions.

Figure 3 shows the decay of the signal intensity of the tOmpA-detergent complex at 23 °C as a function of the amplitude of the gradients $G_{\text{encode}} = G_{\text{decode}}$, which were incremented in 16 steps. The Gaussian decay is in agreement with eq 1. The diffusion coefficient determined by fitting this decay to eq 1 is $D = (4.99 \pm 0.07) \times 10^{-11} \text{ m}^2 \text{ s}^{-1}$. Random errors are small, but systematic errors of up to 4% may arise from the calibration of the gradients. The value of D resulting from X-STE measurements is in reasonable agreement with independent estimates obtained by size exclusion chromatog-

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raphy, $D = (6.4 \pm 0.6) \times 10^{-11} \text{ m}^2 \text{ s}^{-1}$ at room temperature and 19 mM C_8E_4 , and by sedimentation velocity analytical ultracentrifugation, $D = (5.7 \pm 0.3) \times 10^{-11} \text{ m}^2 \text{ s}^{-1}$ determined at 20 °C under similar conditions.^{23,24} The diffusion coefficient determined by NMR is slightly smaller, probably because the higher concentrations in both complex and detergent increase the viscosity of the medium. For the sake of comparison, the diffusion coefficient $D = 7.76 \times 10^{-11} \text{ m}^2 \text{ s}^{-1}$ has been reported for ovalbumin (45 kDa) at 20 °C and infinite dilution in water.²⁵

In the case of the tOmpA/ C_8E_4 complex studied here, which has an overall mass of ca. 45 kDa and an expected rotational diffusion correlation time τ_c of 20 ns, the gain of X-STE over homonuclear proton STE is approximately a factor of 10 in the duration Δ of the diffusion interval, since $T_1(^{15}\text{N}) = 1 \text{ s}$ and $T_1(^1\text{H}) = 100 \text{ ms}$. The larger the complex, the slower the diffusion coefficient, and the more important the gain in terms of Δ will be, which makes this method particularly adapted to the study of large molecules.

The X-STE method is not limited to proteins, nor indeed to biomolecules. In nitrogen-15-enriched nucleic acids (DNA or RNA), one may use the one-bond couplings $J(\text{NH})$ between the “donor” nitrogen nuclei of guanosines and uridines and the imino protons that are responsible for holding the Watson–Crick base pairs together. We have applied the X-STE method to a solution of recombinant human tRNA₃^{Lys} (kindly provided by F. Dardel), which has a molecular mass of 24 kDa. The diffusion coefficient was found to be $D = (1.05 \pm 0.015) \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$.

Conclusions

It has been shown that slow translational diffusion constants can be determined accurately for nitrogen-15-enriched molecules by using a variant of stimulated echo pulsed field gradient NMR, where the information is stored in the form of longitudinal Zeeman magnetization of nitrogen-15 nuclei. This approach may be applied to many other heteronuclei such as carbon-13 or

phosphorus-31. The method allows one to focus on the diffusion coefficient of a selected component in a complex mixture, even if the NMR spectra are poorly resolved, the medium is very viscous, and the molecules are tumbling slowly. This method should simplify the optimization of experimental conditions for NMR studies of macromolecules in solution. It can be readily applied to measuring diffusion coefficients in three orthogonal spatial directions, so that the anisotropy of translational diffusion can be investigated. The X-STE approach can also be adapted to measuring flow, in vivo as well as in vitro.

Note Added in Proof

The pertinent parameter to describe the memory of proton magnetization in homonuclear stimulated echo experiments depends on the details of the sequence. If the proton chemical shifts are *not* refocused in the interval during which the encoding occurs,⁹ the longitudinal proton magnetization is modulated not only as a function of the spatial position but also as a function of the chemical shifts. The modulation of proton magnetization within a molecule will be attenuated in the Δ interval by intramolecular cross-relaxation (with rates of about 10 s^{-1} for $\tau_c = 20 \text{ ns}$). On the other hand, if the chemical shifts are refocused by a 180° pulse (a condition for the use of bipolar gradients for encoding), all protons within the same molecule have the same longitudinal magnetization and the memory will be determined by a combination of intrinsic $R_1(^1\text{H})$ and chemical exchange with the solvent.

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(23) The detergent employed for this measurement was octyl-polyoxyethylene ($\text{C}_8\text{-POE}$) at a 240 mM concentration. $\text{C}_8\text{-POE}$ and C_8E_4 are virtually identical detergents, except for the $(\text{CH}_2\text{O})_n$ polyoxyethylene polar head, which is polydisperse in $\text{C}_8\text{-POE}$, with $\langle n \rangle = 5$, yielding an average molecular mass $\langle M \rangle = 350 \text{ Da}$, while it is monodisperse in C_8E_4 , with $n = 4$ and $M = 306 \text{ Da}$.

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