Measuring Molecular Motion

Using NMR Spectroscopy to Study Translational Diffusion

Jennifer N. Maki and Nikolaus M. Loening*

Department of Chemistry, Lewis & Clark College, Portland, OR 97219

This chapter demonstrates how to use NMR spectroscopy to measure the rate of translational diffusion. Once measured, this rate can provide insight into the size and shape of the molecules in a sample and can even be used to match peaks in the NMR spectrum with the different components of a sample mixture. Of the possible applications of diffusion, the experiment presented focuses on using measurements of the rate of translational diffusion to reveal how the viscosity of a solution varies with the concentration of the solutes.

The term *diffusion* describes a variety of physical processes that involve the random motion of particles or the dispersal of energy. In the context of NMR spectroscopy, diffusion can refer to translational, rotational, and spin diffusion. Both translational and rotational diffusion have to do with the physical motion of particles. Translational diffusion refers to the seemingly-random motion of a particle due to its kinetic energy and its interactions with other particles as shown on the left in Figure 1. Rotational diffusion, which also arises due to kinetic energy, refers to the reorientation of a particle relative to the rest of the system as shown on the right in Figure 1. The third process, spin diffusion, refers to the spread of spin polarization via dipolar couplings or, more rarely, scalar couplings.

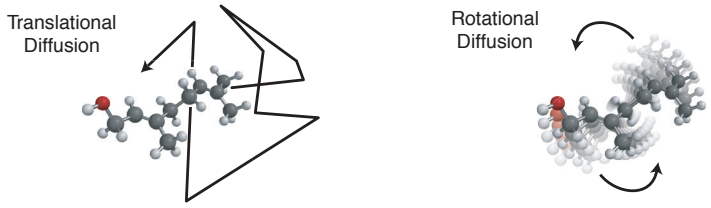

Figure 1. Translational diffusion refers to the seemingly random motion of molecules through space, whereas rotational diffusion describes the reorientation of a molecule relative to the rest of the sample.

The rate at which these processes occur can be measured using NMR spectroscopy. The rate of spin diffusion can be measured using an NOE build-up experiment and the rate of rotational diffusion is usually inferred from measurements of the transverse and longitudinal relaxation rates. The procedure described in this chapter demonstrates how NMR spectroscopy can be used to measure the rate of translational diffusion as well as how this rate can provide insight into the effect of the solute molecules on the bulk properties of the solution.

Background

In a gas or liquid, the individual molecules have enough kinetic energy to overcome the intermolecular forces binding them to other molecules in the sample. Consequently, the molecules move about at a speed determined by their kinetic energy. As a molecule moves through the sample, it changes direction and speed due to collisions with other molecules. Over a period of time, the movement of the molecule forms a zigzag path known as a *random walk* as shown on the left side of Figure 1. This movement occurs even in the absence of external effects (such as concentration gradients or electric fields) and is referred to as *translational diffusion*.

Consider the movement of a molecule along one of the spatial dimensions (the *z*-axis). In a specified period of time, the molecule will move a certain distance resulting in a displacement Z_1 as shown on the left in Figure 2. For a single experiment on a single molecule, it is hard to specify the value of Z_1 because the random walk of a molecule is (as the name implies) random. In a second experiment, the molecule moves another distance Z_2, which will usually be somewhat different than Z_1. The center part of Figure 2 illustrates the result of repeating such an experiment several times.

Solely based on physical intuition it is possible to deduce several properties of this motion. For example, small values for the displacement should

be more likely than large values since the individual steps of the molecule's random walk are more likely to cancel out than to add constructively. Another property is that, on average, the displacement of the molecules will be zero. This is because the molecules are just as likely to move in one direction along the *z*-axis as the other. Physically, this has to be the case for a stationary sample…if the average displacement of the molecules was non-zero, than the sample would be moving rather than stationary. The results of a large number of experiments (or a large number of molecules in a single experiment) follow a well-defined trend as shown on the right in Figure 2.

Although it is difficult (if not impossible) to predict the distance that a single molecule will move in an experiment, it is possible to statistically define the *probability* that this molecule will move a certain distance. The statistics of the random walks of individual particles on the molecular level result in the bulk property of translational diffusion. The average rate of translational diffusion is described quantitatively by the diffusion coefficient (*D*). The value of the diffusion coefficient depends not only on the identity of the sample molecules, but also on the solvent and the temperature (and, to a very minor degree, the pressure). Statistically, the probability for a molecule to be displaced a distance *Z* in time *t* is described by a Gaussian distribution function (*1*):

$$P(Z) = \frac{1}{\sqrt{4\pi Dt}} \exp\left(-\frac{Z^2}{4Dt}\right) \tag{1}$$

Larger diffusion coefficients correspond to more rapid movement of the molecules; therefore larger displacements are more likely and the distribution function will be broader. Likewise, larger values of *t* also result in a broader distribution function as the molecules have a longer period of time to diffuse.

As expected, the average displacement calculated using eq 1 is zero:

$$\overline{Z} = \int_{-\infty}^{\infty} Z \times P(Z)\, dZ = \int_{-\infty}^{\infty} (\text{odd function}) \times (\text{even function}) = 0$$

The mean-square displacement, on the other hand, is

$$\overline{Z^2} = \int_{-\infty}^{\infty} Z^2 P(Z) dZ = 2Dt$$

Therefore, the root-mean-square displacement for a molecule is

$$Z_{\text{rms}} = \sqrt{\overline{Z^2}} = \sqrt{2Dt}$$

The diffusion coefficient is on the order of 1×10^{-5} cm^2 s^{-1} for a typical small molecule in solution, and a typical NMR diffusion measurement experiment is 0.1 s long. These values correspond to a root-mean-square displacement along a single axis of 14 μm.

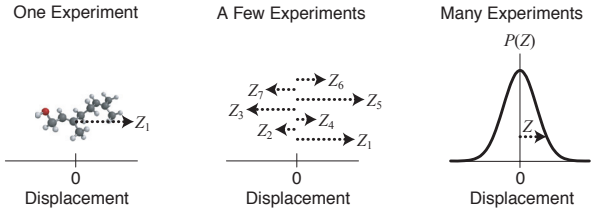

Figure 2. The random walk motion of a single particle during a specific time period will lead to a displacement Z_1, as shown on the left. The center image illustrates the distribution of displacements that result from repeating the experiment several times. After a large number of experiments, the Gaussian distribution shown on the right appears.

Predicting the Diffusion Coefficient. Ideally, a molecule in a gas or liquid will move about at a speed determined by its kinetic energy. In a liquid, however, the distance that the molecule travels is restricted by the "cage" of solvent molecules surrounding it. Only after repeated collisions with the solvent cage will the molecule squeeze into the next cage. This restriction can be modeled as a frictional force, F_f, that is negatively proportional to the velocity, v:

$$F_f = -fv$$

where f is a proportionality constant known as the friction coefficient. For a spherical object moving through a liquid with viscosity η, Stokes' law predicts that the frictional force on the object is

$$F_f = -6\pi\eta r v$$

where r is the radius of the object. Consequently, the frictional coefficient is

$$f = 6\pi\eta r$$

Consideration of the relationship between the kinetic energy of a molecule and the friction due to the surrounding cage of solvent molecules leads to the Stokes-Einstein equation (*2*):

$$D = \frac{k_B T}{f} = \frac{k_B T}{6\pi\eta r} \tag{2}$$

where k_B is the Boltzmann constant and T is the temperature. This equation was originally derived for spherical colloidal particles and provides an accurate explanation of their motion; it is only an approximation for the behavior of smaller (and/or non-spherical) particles.

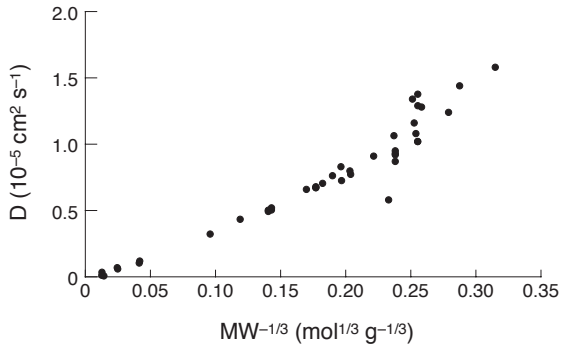

Figure 3. The diffusion coefficient (D) versus the inverse cube root of the molecular weight for a number of dilute solutes in water at 25°C. The lightest molecule included on the graph is methanol (MW = 32 g mol^{-1}) and the heaviest is myosin (MW = 493 kg mol^{-1}). Data are from references 3–4.

Applications of the Diffusion Coefficient. Knowing the value of the diffusion coefficient can be useful for a number of reasons. First, the diffusion coefficient can be used to predict the size of an unknown compound and therefore can be used to estimate the molecule weight. This is possible because eq 2 relates the radius (and therefore the volume) of a spherical molecule to the diffusion coefficient ($V \propto r^3 \propto D^{-3}$). By using this calculated volume and knowing (or assuming) the density, it is possible to estimate the molecular weight. Figure 3 displays the diffusion coefficient versus the inverse cube root of the molecular weight for a number of organic and biological molecules; the trend is apparent although a few outliers exist. As eq 2 is more applicable to large, spherical molecules, it is understandable that the trend shown in Figure 3 is more consistent for large molecules (which appear toward the left side of the graph) than for small ones.

Knowledge of the diffusion coefficient can aid in the determination of the oligomeric state of a compound (*i.e.*, whether a compound is present free in solution or whether it has aggregated to form a dimer, trimer, etc.). The oligomeric state is particularly important in protein NMR where it must be known prior to structure calculations. Although it is possible to determine the oligomeric state using techniques such as dynamic light scattering, these methods are not always applicable at the concentrations used for NMR experiments (*5*). To determine the oligomeric state of a molecule, a comparison is made between the experimentally measured diffusion coefficient and a calculated diffusion coefficient based on the molecule's size and shape (*6*). If the values match, the compound is probably a monomer. If the calculated value is significantly greater than the experimentally measured value, the compound has probably aggregated. This comparison of experimental and calculated values

is most useful for detecting dimerization, as the molecular weights of a monomer and a dimer differ by a factor of two. Differentiating higher oligomeric states, such as whether a molecule is a trimer or a tetramer, becomes progressively more difficult as the ratio of the molecular weights (and therefore the variations in the diffusion coefficients) will be smaller. This method is most applicable to large molecules, such as proteins, but also has been used for smaller systems such as non-covalent assemblies of calix[4]arene units (*6*) and ions in solution (*7*).

Similarly, diffusion coefficients can be used to estimate binding affinities between substrates and proteins. For example, one screening method used in drug discovery is to observe how the apparent diffusion coefficient for a small molecule changes in the presence of a protein. A decline in the diffusion coefficient of the small molecule in the presence of a protein is indicative of binding, in which case the small molecule is a good candidate for a drug. As will be seen later in this chapter, it is important in such experiments to control for changes in the viscosity upon addition of further solute or other solutes as the solution viscosity, and consequently the diffusion coefficients, is very sensitive to the composition of the sample.

Measurements of the diffusion coefficients can also be used to separate signals from different molecules. In NMR spectroscopy, the spectrum of a mixture is equivalent to the superposition of the spectra of the individual components. Using the experiment described later in this chapter, it is possible to measure a diffusion coefficient for each peak in the spectrum. As the peaks from the non-exchangeable nuclei of a compound will share the same diffusion coefficient, it is possible to use the diffusion coefficient to distinguish signals from different components of the mixture (provided they have appreciably different diffusion coefficients). This method of separating signals is known as diffusion-ordered spectroscopy, or DOSY (*8*). DOSY is particularly useful in situations where it is not convenient or possible to physically separate a mixture; DOSY is capable of separating the signals from different components without the need to resort to chromatography. Another use for DOSY is in cases where it is the *mixture* of components and their interactions that is of interest.

Magnetic Field Gradients and NMR Spectroscopy

Before running an NMR experiment, it is typical to spend some time "shimming" the magnet. The shimming procedure is carried out in order to make the magnetic field as homogeneous as possible; this is necessary because spatial inhomogeneities in the main magnetic field interfere with the resolution of the NMR experiment. However, observing translational diffusion by NMR spectroscopy requires a spatially-dependent magnetic field. In other words, it is

necessary to reintroduce inhomogeneities to the main magnetic field to measure the diffusion coefficient.

Experimentally, these inhomogeneities are generated using gradients. Gradients are coils inside the NMR probe that generate well-defined spatial variations of the magnetic field. Most gradients are designed to generate a linear variation in the magnetic field along the axis of the main magnetic field (B_0); this axis is usually defined as the z-axis and therefore such a gradient is called a z-axis gradient. The symbol G_z represents the strength of such a gradient [$G_z = \partial B(z)/\partial z$]. By changing the magnitude and sign of the electrical current running through the coil of the gradient it is possible to vary the magnitude and the sign of G_z. Likewise, the gradient can be switched off ($G_z = 0$) by switching off the electrical current.

Although z-axis gradients are the most common type of magnetic field gradients, it is possible to generate gradients along other axes, such as the x-axis, the y-axis, or even along the rotor axis of a magic-angle spinning probe. In a typical NMR probe, each gradient coil can generate a magnetic field gradient of several tens of Gauss cm^{-1} for a period of several milliseconds (a "gradient pulse"); weaker gradient pulses can be generated for much longer periods. In all cases, the direction of the magnetic field generated by the gradient is parallel to B_0; the gradient axis is the axis along which the magnitude, not the direction, of the field varies.

Magnetization and Gradients. The Larmor frequency (ω) for the nuclei in a sample depends on the magnetic field and the magnetogyric ratio (γ):

$$\omega = -\gamma B_0$$

When a gradient is applied, the magnetic field varies linearly along the gradient axis and, consequently, the Larmor frequency varies in the same way. As a result, nuclei at different parts of the sample will precess at different frequencies. On a bulk scale, the result is a spatially-dependent phase for the transverse sample magnetization. As shown in Figure 4, the magnetization will begin to vary in phase across the sample after the gradient is switched on, resulting in a helical profile for the transverse magnetization along the gradient axis. As the signal observed in the NMR experiment arises from the sum of all the transverse magnetization in the sample, the spatially-dependent phase results in a diminished signal because the transverse (x and y) components of the magnetization will tend to cancel one another. The degree to which the signal is diminished depends on how tightly the transverse magnetization is "coiled" along the gradient axis. This signal attenuation due to a gradient pulse is referred to as gradient *dephasing*.

Consider an experiment in which two gradient pulses are applied. The first gradient pulse, often referred to as the *phase-labeling* gradient, dephases any transverse magnetization. A second gradient pulse can be used to reverse

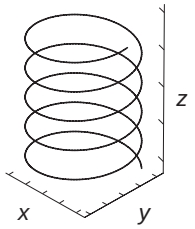

Figure 4. A gradient pulse along the z-axis winds the transverse magnetization into a "helix" (the helix traces out the tips of the magnetization vectors). The pitch of this helix (i.e., how tightly it is wound) depends on the gradient strength and length, and the magnetogyric ratio of the nuclei.

this process if the appropriate combination of pulse length and strength are used. Such a gradient is called a *refocusing* gradient because it uncoils the magnetization helix and restores the transverse magnetization to its initial state. For the magnetization to be properly refocused, the gradient values must be chosen such that the spatially dependent phase sums to zero at the end of the experiment. By carefully choosing the strengths of different gradients in an experiment, it is possible to use gradients in place of phase cycling for selecting different components of the NMR signal. However, this is not the focus of this chapter; instead, we will concentrate on how gradients are used to measure translational diffusion.

Gradients and Motion. The measurement of motion by NMR requires at least two gradient pulses in an experiment: the first to dephase the magnetization and the second to refocus it at some later point in the experiment. To completely refocus the magnetization (and completely recover the NMR signal), the molecules of the sample must not move during the time period in between the gradients. If the molecules *do* move, the observed signal will change because the refocusing gradient will not be able to properly restore the transverse magnetization to its initial phase.

In order to visualize how movement affects the result of a gradient-selected experiment it is useful to consider a discrete version of the magnetization helix shown in Figure 4. In the discrete model (shown in Figure 5), the sample is split into thin slices referred to as *isochromats* in which the magnetic field is approximately homogeneous. Before the first gradient pulse the components of the magnetization in the various isochromats are aligned as shown on the left in Figure 5. During the gradient, the nuclei precess at different frequencies in each isochromat. This variation in precession results in the situation shown on the right of Figure 5. In the absence of motion, a suitable refocusing gradient will realign the magnetization. However, if the nuclei move in between the phase-labeling and refocusing gradients, the signal

at the end of the experiment will change in phase, amplitude, or both, depending on the nature of the movement.

Coherent Motion. Coherent motions are ones that persist in direction and magnitude over the timescale of an experiment. Examples of coherent motions are laminar flow, plug flow, and convection. The simplest type of coherent motion to deal with is uniform flow of the sample in one direction. As shown in Figure 6, uniform downward motion of the sample in between the phase-labeling and refocusing gradients results in the realignment of the transverse magnetization at the end of the experiment. However, the overall phase of the magnetization is not the same as the initial phase; the magnetization has acquired a phase that depends on the sample velocity. Since the phase of the transverse magnetization is uniform across the sample at the end of the experiment, the spectrum will not change in intensity, but will change in phase depending on the flow velocity. Consequently, the velocity of a sample can be estimated by measuring the change in the phase of the spectrum relative to the spectrum of a stationary sample. Variations of this technique have been used, for example, to measure the flow of blood, the rate of water moving through vascular plants, and the non-Newtonian flow of polymers (*1*).

Incoherent Motion. The effects of incoherent motions, such as diffusion and turbulent flow, on the NMR signal are somewhat different than those from coherent motions. Whereas coherent motions can change the phase and/or the intensity of the signal, incoherent motions only affect the signal intensity. The reason for this is that incoherent motions results in a symmetrical distribution of phases for the individual spins in the sample. The intensity of the NMR signal (S) is related to the ensemble average of the phases (ϕ) of the individual spins according to:

$$S \propto \overline{\exp(i\phi)}$$

In the case of a symmetrical distribution of phases about some value, the resulting signal will not change in phase. However, the cancellation of signal components from individual spins will reduce the intensity of the signal.

A mechanism of motion is considered incoherent if the correlation time for movements is short in comparison to the timescale of the experiment. The correlation time refers to the time-scale over which a molecule maintains the same velocity. In molecular diffusion, the velocities of individual molecules change frequently due to collisions, whereas in turbulence the velocities of small regions of the sample change frequently. In NMR spectroscopy, the timescale of the experiment is always much longer than the correlation time for small molecules undergoing unrestricted diffusion, so translational diffusion behaves as an incoherent process.

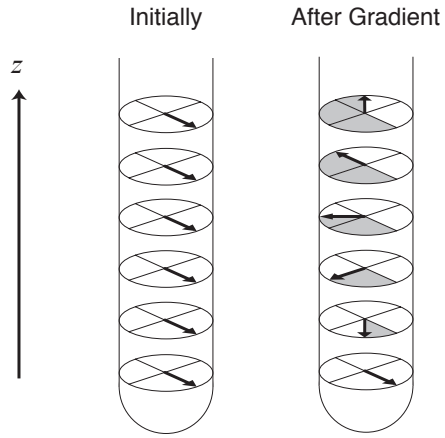

Figure 5. As shown on the left, the components of the sample magnetization in the isochromats are aligned at the start of a gradient pulse. After the gradient pulse, these components will have precessed by different amounts according to their location in the sample as shown on the right. Due to the gradient, the magnetization has a phase (indicated by the shading) that varies with position in the sample.

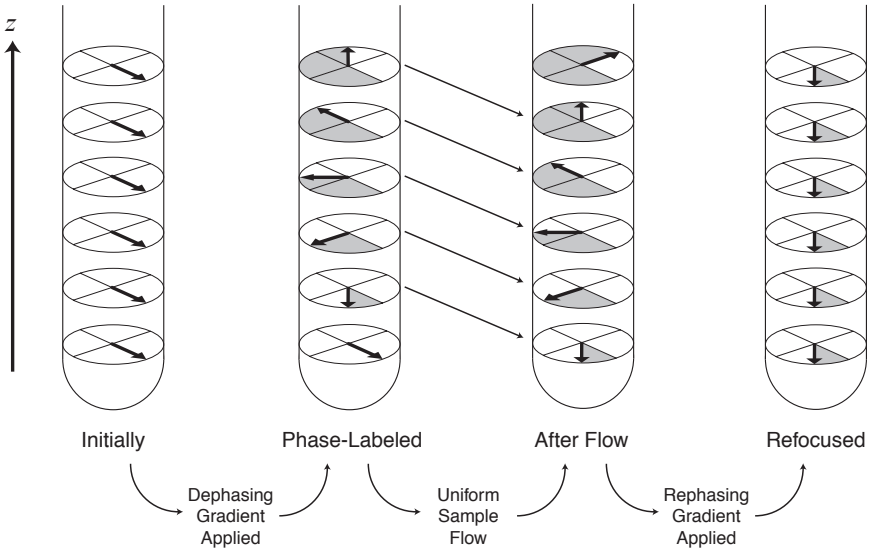

Figure 6. Uniform movement of the sample along the gradient axis between the phase-labeling and refocusing gradient will result in a velocity-dependent phase as shown by the shading.

Measuring Diffusion Coefficients

A Gaussian distribution function (eq 1) describes the distribution of molecular displacements due to translational diffusion. Therefore, in an NMR experiment with dephasing and refocusing gradients there will be a Gaussian distribution of phases due to translational diffusion between these two gradients pulses. As this Gaussian distribution of phases is symmetric the effect of molecular diffusion in the NMR experiment is *only* to attenuate the signal; diffusion does not cause the signal to change in phase. The degree to which diffusion attenuates the signal can be calculated based on a modified version of the Bloch equations (which are a set of differential equations that provide a semi-classical description of the NMR experiment). The result of this derivation (*9*) is that the attenuation of the signal (S) relative to the signal in the absence of diffusion (S_0) is

$$S = S_0 \exp\left[-D\gamma^2 G^2 \delta^2 s^2 \left(\Delta - \tfrac{1}{3}\delta\right)\right] \tag{3}$$

where γ is the magnetogyric ratio, G is the gradient strength, δ is the length of the gradient pulses, Δ is the time between the gradients, and s is a shape factor which accounts for how the gradient pulses are ramped on and off. Typically, s will be either 1 (for rectangular-shaped gradient pulses) or $2/\pi$ (for half-sine bell shaped gradient pulses) (*10*). If we take the natural log of this equation, we find

$$\ln S = \ln S_0 - D\gamma^2 G^2 \delta^2 s^2 \left(\Delta - \tfrac{1}{3}\delta\right) \tag{4}$$

Consequently, a plot of $-\ln S$ versus $\gamma^2 G^2 \delta^2 s^2 (\Delta - {}^1/_3\,\delta)$ should result in a straight line with slope D and y-intercept $-\ln S_0$. One subtle point for using eq 4 is that, when linear regression is used to fit the data, all the points are weighted equally. This can yield inaccurate results, as noise will disproportionately affect some of the data. This problem is not severe if the signal-to-noise ratio for all the data is good; if this is not the case then directly fitting the data to eq 3 using a non-linear fit yields more accurate results (*11*).

Figure 7 illustrates the diffusion-dependent signal attenuation for a sample of camphene in deuterated methanol in a series of experiments with increasing gradient strength. The spectra were integrated between 1.1 and 0.95 ppm and, as shown on the right of Figure 7, the integrals were plotted versus $\gamma^2 G^2 \delta^2 s^2 (\Delta - {}^1/_3\,\delta)$ using a semi-log plot. The slope of this plot corresponds to the diffusion coefficient. For this sample, linear least-squares was used to fit the data, resulting in an estimated diffusion coefficient of $1.339\ (\pm 0.002) \times 10^{-5}$ cm^2 s^{-1}. The quoted error is the standard deviation of the slope resulting from the linear regression analysis, which usually underestimates the experimental error inherent in the technique. This value for the diffusion coefficient was deduced using the integrals of the methyl peaks; other camphene peaks in the spectrum should provide identical results.

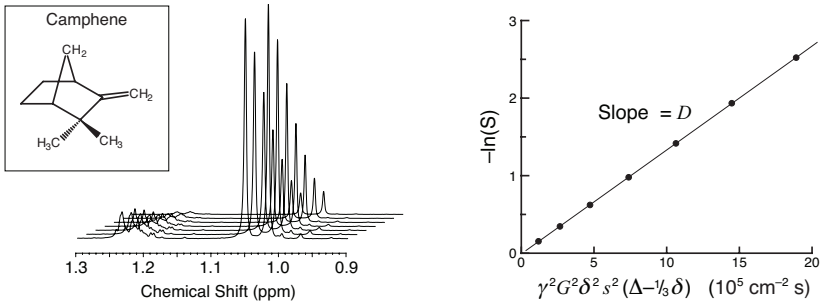

Figure 7. On the left is a stacked plot for a small portion of the camphene ^1H NMR spectrum. The spectra differ in the amount of diffusion-dependent signal attenuation; the degree of attenuation was controlled by varying the gradient strength. The semi-logarithmic plot on the right is based on the integrals for the region between 0.95 and 1.1 ppm. The linear least-squares fit to the data is indicated by the solid line. The sample temperature was 300.0 K (26.9 ºC).

The Accuracy of Measured Diffusion Coefficients. The value of the diffusion coefficient depends primarily on three physical factors: the identity of the molecule of interest, the solvent, and the temperature. There are also several experimental factors that affect the accuracy of the measurement of the diffusion coefficient, including the uniformity of the gradient (usually only a very minor effect), other sources of motion, distortions of the baseline, and the uniformity of the temperature of the sample. The experiment for measuring diffusion coefficients that is presented later in this chapter was designed to minimize some of these sources of interference.

Solvent Viscosity. According to the Stokes-Einstein equation (eq 2), the diffusion coefficient is inversely proportional to the solvent viscosity. The viscosity depends not only on the temperature of the sample, but also on the *composition* of the sample. For example, changing the concentration of the solute, or adding other solutes to the sample, will alter the solvent viscosity. Whether a solute increases or decreases the viscosity depends on the nature of both the solute and the solvent. For example, geraniol and quinine both increase the viscosity of chloroform, whereas surfactants will often decrease the viscosity of aqueous solutions.

The effect of solvent viscosity is particularly relevant in NMR spectroscopy as, due to the intrinsically low sensitivity of the method, samples with relatively high concentrations are used; concentrations between 0.1 M and 1 M are not unusual. Consequently, it is necessary to know the exact composition of a sample when determining the diffusion coefficient; otherwise the results may not be reproducible.

Restricted Diffusion. The theory used to derive eqs 1 and 3 assumes that the movement of the sample molecules is unbounded. If this is not the case, then the molecules are subject to *restricted diffusion* and the signal attenuation due to diffusion will be less than what eq 3 predicts. Fortunately, a typical solution-state NMR sample tube has a diameter of several millimeters whereas the sample molecules move only a few tens of micrometers during an experiment. Therefore, the effect due to restricted diffusion can be safely ignored and eqs 1, 3, and 4 can be used without difficulty.

Motion. NMR experiments involving gradient pulses are sensitive to both coherent and incoherent motions. This means that any attempt to measure the diffusion coefficient in the presence of another source of movement produces results that reflect *both* movements. Consequently, diffusion measurements should be performed *without* sampling spinning.

In addition, convection can arise due to small temperature gradients in the sample; this is particularly common for low viscosity solvents such as chloroform or acetone (*12*). In diffusion-measurement experiments, sample convection introduces an additional component to the signal decay that results in overestimates of the diffusion coefficient. Fortunately, convection is, at least to a first approximation, a coherent motion and therefore the signal variation due to convection can be removed by using the double-stimulated echo experiment described later in this chapter (*13*).

Gradient Calibration. To make accurate measurements of the diffusion coefficient, an accurate value for the strength of the gradient needs to be known. Typically, this value will be determined when the NMR system is installed and is unlikely to need to be recalibrated. If the value is unknown, it can be determined in two ways. The first method involves making a diffusion measurement using a sample with a known diffusion coefficient. By working backwards from the known diffusion coefficient, it is possible to determine the strength of the gradient.

The second method involves using the spectrometer to acquire an image of a sample with a known geometry; the strength of the gradient can then be deduced by comparing the dimensions of the sample with the frequency separation of the features in the image. For example, calibration of a z-axis gradient can be performed using a vortex plug that had been inserted into an NMR sample tube that is filled with a 1:9 solution of water in deuterated water as shown at the top of Figure 8. The length of the sample (Δz) is related to the frequency separation of the features in the image ($\Delta \nu$) according to

$$\Delta \nu = \frac{1}{2\pi} \gamma G \Delta z$$

where, once again, γ is the magnetogyric ratio, G is the gradient strength.

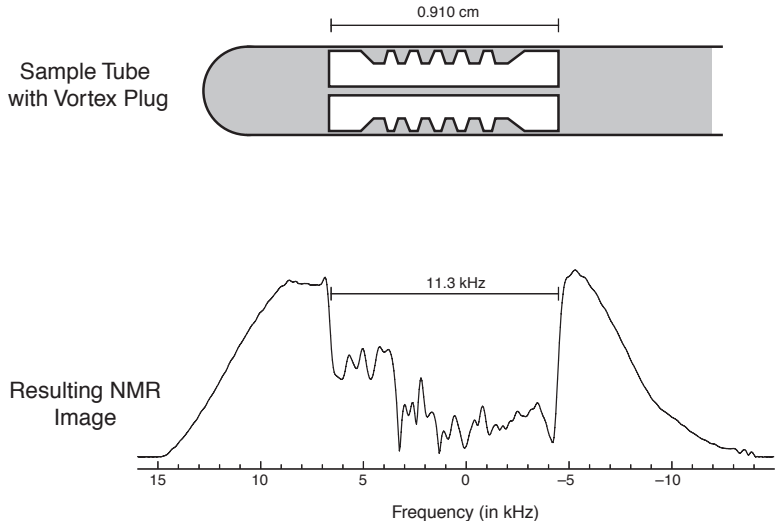

Figure 8. The strength of the gradient can be calibrated by imaging a sample with known geometry. In this case, the sample consisted of a 5 mm NMR sample tube with a 0.910 cm vortex plug inserted into the active region. The solvent surrounding the plug (indicated by the shading in the figure) was 10% H_2O/90% D_2O (by volume). The bottom part of the figure shows the NMR image of this sample acquired at 300 MHz for ^1H. This image reveals an 11.3 kHz separation when the imaging gradient is set to 5% of its maximum value; this corresponds to a maximum gradient strength of 58.0 G cm^{-1}. The signal goes to zero at the edges of the image due to the limited length of the radiofrequency coil.

The vortex plug shown in Figure 8 is 0.910 cm in length and the resulting image has a frequency separation of 11.3 kHz when the gradient is at 5% of its maximum strength. Therefore, the gradient strength is

$$G = \frac{2\pi\,\Delta\nu}{\gamma\,\Delta z} = \frac{2\pi\left(11300\text{ Hz}\right)}{\left(26750\text{ rad G}^{-1}\text{ cm}^{-1}\right)\left(0.910\text{ cm}\right)} = 2.92\text{ G cm}^{-1}$$

and the maximum gradient strength is (2.92 G cm^{-1} / 5%) = 58.0 G cm^{-1}.

Pulse Sequences. Measuring the diffusion coefficient requires the use of an NMR experiment which includes both dephasing and rephasing gradients. The simplest experiment used for measuring diffusion is the gradient spin echo (SE) experiment shown at the top of Figure 9. To achieve a significant amount of diffusion-dependent signal attenuation, the delay time (Δ) must usually be between 0.1 and 0.5 s. This delay is long enough that effects due to transverse

relaxation and homonuclear scalar couplings interfere with the diffusion measurement.

Compared to the SE experiment, the stimulated echo (STE) experiment shown in the middle of Figure 9 is much better for measuring diffusion coefficients as the sample magnetization is "stored" as longitudinal magnetization in the middle part of the experiment. During this storage period, the molecules continue to diffuse but the magnetization does not evolve due to couplings and is subject to longitudinal relaxation instead of transverse relaxation.

The preferred pulse sequence for measuring diffusion coefficients is the double stimulated echo (DSTE) experiment shown at the bottom of Figure 9. The advantage of this sequence is that it is only sensitive to incoherent motion (*i.e.*, diffusion). Effects due to coherent motions are refocused by the second stimulated echo in the sequence, which means that effects due to sample convection are compensated for (at least to first-order) in the resulting spectra (*14*). Consequently, the DSTE experiment provides more accurate values for the diffusion coefficient, particularly for samples in low-viscosity solvents such as chloroform or acetone.

Experimental

Sample Preparation. First, prepare a series of five samples of geraniol in deuterated chloroform; the samples should range in concentration from around 0.02 M to 1.00 M. It is useful to make sure that the samples are filled to the same height as this will greatly reduce the amount of shimming necessary when changing samples. Next, prepare a similar series of quinine samples. These samples should cover a similar range of concentrations as the geraniol samples. Studying concentrations above 1 M is unfortunately not possible as the solution approaches saturation at this concentration. Note that quinine is usually only 90% pure; the remainder is usually hydroquinine, which differs from quinine only in that the terminal alkene is saturated.

Data Acquisition. Run a simple one-dimensional (1D) experiment for the first sample to make sure that the sample is shimmed adequately, that the 90º pulse time is calibrated properly, and that the spectral width includes all the peaks in the spectrum. Next, setup and run a DSTE experiment. On Bruker systems, the standard DSTE sequence is called "dstegp3s". This sequence should be used with an eight-step phase cycle. Usually, eight values for the gradient strength are sufficient to observe the attenuation of the signal due to diffusion.

Repeat the 1D and DSTE experiments for each of the geraniol samples and then for each of the quinine samples. For the more concentrated quinine samples the linewidths will be noticeably wider. This effect is due to the

increased viscosity of for the concentrated samples. Due to this increase, the quinine molecules tumble more slowly, which leads to faster transverse relaxation rates and, in turn, broader lines in the spectrum.

The variable temperature (VT) control system should be on during the experiments. This is necessary because the diffusion coefficients depend strongly on the sample temperature. For consistent results, it is best to wait a few minutes after inserting each sample to ensure that the sample has had time to equilibrate to the system temperature.

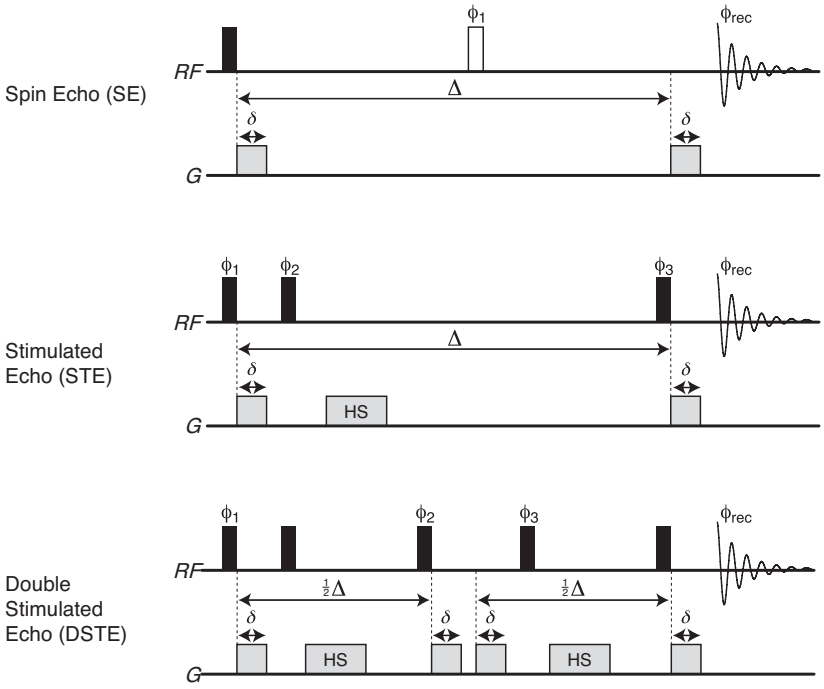

Figure 9. Pulse sequences for the spin echo (SE), stimulated echo (STE) and double stimulated echo (DSTE) experiments. The top line for each experiment indicates the radiofrequency (RF) pulses; filled and open rectangles represent 90° and 180° RF pulses, respectively. The bottom line for each experiment indicates the gradient pulses (G). The gradient pulses labeled "HS" are homospoils; to ensure that the signal is properly refocused all other gradient pulses in a sequence should have the same length, magnitude, sign, and shape. The phase cycles for the experiments are as follows. SE: $\phi_1 = y, -y;\ \phi_{rec} = x$. *STE:* $\phi_1 = x, x, x, x, -x, -x, -x, -x;\ \phi_2 = \phi_3 = y, -y, x, -x;\ \phi_{rec} = x, x, -x, -x, -x, -x, x, x$. *DSTE:* $\phi_1 = x, y, -x, -y;\ \phi_2 = -x, -y;\ \phi_3 = -x, -x, -x, -x, x, x, x, x;\ \phi_{rec} = x, x, -x, -x, -x, -x, x, x$. *All other RF pulse phases have a phase of x.*

Data Processing. Once acquired, each DSTE experiment should be phased and Fourier transformed in the directly-detected dimension. It is usually a good idea to baseline correct the spectra as baseline errors generate systematic errors in the calculated diffusion coefficients. Next, select a region and integrate each of the rows in the data set. Usually, the best region to use is one that has a strong signal and that is well removed from solvent and impurity peaks. For geraniol, the methyl region (1.8 – 1.5 ppm) works well. For quinine, one of the peaks in the aromatic region (8.5 –7 ppm) or the methyl peak at ~3.8 ppm are suggested.

On Bruker spectrometers running XWIN-NMR the analysis of the data can be performed using the relaxation (t1/t2) environment. However, the interface for this function is somewhat Byzantine, so we use a program developed by one of the authors to integrate each row (*14*) and then analyze the data using a separate program.

Once measured, the integrals should be fit to eq 4 using linear regression (or to eq 3 using a non-linear fitting routine) to determine the diffusion coefficients. For our data, we fit the data to eq 3 using the solver macro in Excel.

Results and Discussion

The results from measuring the diffusion coefficient for a series of geraniol and quinine samples are shown in Figure 10. In both cases, the diffusion coefficients decrease as the concentrations of the solutes increase, but the variation is much more drastic for the quinine solutions than for the geraniol solutions. The observed variation is primarily due to changes in viscosity. For example, geraniol is a liquid at room temperature and has a higher viscosity than chloroform. Therefore, combinations of the two liquids should have intermediate viscosities. According to the Stokes-Einstein equation (eq 2), increases in viscosity reduce the diffusion coefficient, a conclusion that is in agreement with the observed trend.

The quinine samples show a similar, but much more pronounced, variation, as indicated by the percentage scale on the right side of each graph in Figure 10. This difference can be attributed to the larger size of the quinine molecules as well as the presence of stronger intermolecular forces than those found in the geraniol solutions.

Using the diffusion coefficient for the least concentrated samples and assuming that the viscosity of the solution is the same as the viscosity of chloroform, it is possible to use eq 2 to estimate the molecular radius. Based on our results, the estimated radii for geraniol and quinine are 0.3 nm and 0.5 nm, respectively. These results agree well with average radii determined using molecular modeling programs.

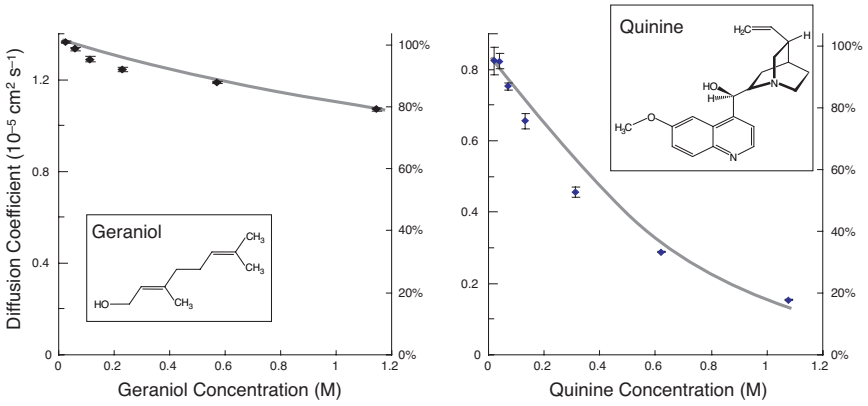

Figure 10. Experimental results for the measurement of the diffusion coefficients for a series of geraniol samples (on left) and a series of quinine samples (on right). The right axis on each graph shows the percent change (100% corresponds to the diffusion coefficient for the least concentrated sample). The data were acquired on a Bruker NMR spectrometer operating at 300 MHz for ^{1}H. At each concentration, seven (geraniol) or nine (quinine) distinct peaks were integrated and fit to determine the diffusion coefficient. The data represent the average value for each concentration along with the standard deviation. The gray lines indicate "theoretical" values of the diffusion coefficients predicted using eq 2 as explained in the text. The sample temperature was maintained at 300.0 K (26.9 ºC) throughout the diffusion measurement experiments.

We measured the viscosity of solutions of geraniol and quinine in chloroform at concentrations ranging from 0 to 1 M using an Ostwald viscometer. Based on these results and our estimated molecular radii, we used eq 2 to calculate the variation of the diffusion coefficient with concentration. These "theoretical" diffusion coefficients are shown as gray lines in Figure 10. The close correspondence between the experimental diffusion coefficients and the calculated values indicate that the sample viscosity is the primary reason for the observed variation in the diffusion coefficients.

Conclusion

NMR spectroscopy is a powerful technique for measuring molecular motion since the technique is non-invasive and does not require physically "tagging" the sample. The rate of translational diffusion can be easily measured in a relatively short NMR experiment, and this information can be used to make inferences about the size of the sample molecules as well as to observe the effect of the

solutes on the bulk properties of the solution. Additionally, measurements of the diffusion coefficients can provide insights into the oligomeric state of molecules or be used to monitor protein-ligand binding. However, as the results of such measurements are highly dependent on the sample composition, temperature, and solvent viscosity, these influences need to be included in any interpretation of the diffusion coefficients.

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14. The integration routine (which works from within XWIN-NMR or TOPSPIN) can be downloaded from the author's website at: `http://www.lclark.edu/~loening/software/introws`