NMR Diffusion Spectroscopy as a Measure of Host−Guest Complex Association Constants and as a Probe of Complex Size

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The complexes of cyclohexylacetic acid and cholic acid with β-cyclodextrin were studied by NMR diffusion coefficient measurements. The diffusion coefficient of the 1:1 cyclohexylacetic acid/β-cyclodextrin complex, $K_a = 1800 \pm 100$ M^{-1}, is slightly slower ($3.23 \pm 0.07 \times 10^{-6}$ cm^2 s^{-1}) than that of β-cyclodextrin ($3.29 \pm 0.07 \times 10^{-6}$ cm^2 s^{-1}). The diffusion coefficient of the 1:1 cholic acid/β-cyclodextrin complex, $K_a = 5900 \pm 800$ M^{-1}, is significantly slower ($2.93 \pm 0.07 \times 10^{-6}$ cm^2 s^{-1}) than that of β-cyclodextrin. The results indicate that caution should be exercised when studying host−guest complexation by the so-called 'single point' technique. A novel data treatment is introduced which takes into account the diffusion behavior of all of the species when determining K_a. Experimentally determined diffusion coefficients of complexes are also a useful probe of the size of host−guest complexes.

Introduction

The NMR titration method is widely used to determine association constants of intermolecular complexes, especially in the field of supramolecular chemistry. The most frequently seen application of the technique is for the determination of K_a for a host−guest complex and using ^{1}H chemical shift information.[1] Recently there has been growing interest in the use of pulsed field gradient methods that give information on K_a via the molecular self-diffusion coefficient.[2−7]

The basis of the chemical shift method is that for a complex in fast exchange on the NMR time scale, the observed chemical shift of a proton is the weighted average of the chemical shifts in the native (nonexchanging) environments.[8] Equations 1 and 2 describe the formation of a host−guest complex of stoichiometry 1:1, and the NMR result when the observed proton is located on the host molecule.

$$K_a = [HG]/[H][G] \quad (1)$$

$$\delta_{obs} = X_H\delta_H + X_{HG}\delta_{HG} \quad (2)$$

where [HG], [H], and [G] are the equilibrium concentrations of host−guest complex, host, and guest, respectively, δ_{obs} is the observed ^{1}H chemical shift, X_H and X_{HG} are the mole fractions of noncomplexed host and complex, respectively, and δ_H and δ_{HG} are the chemical shifts of the host and host−guest complex, respectively. Note that the equilibrium concentrations required to calculate K_a cannot be determined from the single experiment suggested by eq 2 because δ_{HG} cannot be directly observed. In other words there is no unique solution for K_a from a single experiment data point. In practice, an NMR determination of K_a involves measuring over a range of host−guest concentrations to define an NMR binding curve. It is then a straightforward matter to compute a calculated curve and iterate to minimize the difference between the calculated and observed data to find K_a and δ_{HG}.

Another NMR based, but quite different approach, for determination of K_a involves the measurement of diffusion coefficients (D).[9] Procedures for measuring D by NMR are well established and most modern instruments are already equipped with the hardware required to implement the experiments. The method is based on size discrimination between the small guest and the usually much larger host. Like any other NMR method, the experiment observable is a mole fraction weighted average of contributions from species in fast exchange. Hence, we can write an expression that describes the observed diffusion behavior of the guest molecule in a solution containing an appropriate host.

$$D_{obs} = X_G D_G + X_{HG} D_{HG} \quad (3)$$

which can be rearranged to give an expression that describes the equilibrium concentration of host−guest complex

$$X_{HG} = (D_G - D_{obs})/(D_G - D_{HG}) \quad (4)$$

An advantage of this technique is that it suggests the possibility of removing one of the unknowns from the

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binding equation. Because the host molecule is usually very much larger than the guest, it seems reasonable to assume that the diffusion coefficient of the host−guest complex is the same as that of the host molecule (a measurable quantity). So, combining eq 4 with eq 1 and accounting for mass balance gives

$$K_a = X_{HG}/((1 - X_{HG})([H]_0 - X_{HG}[G]_0)) \qquad (5)$$

where $[H]_0$ and $[G]_0$ are the total concentrations of host and guest, respectively.

This approximation removes the need to perform titrations to describe binding curves, and in principle K_a can be derived from single experiments. The procedure is directly analogous to the chemical shift single point procedure.[10]

For studies of small molecules binding to macro (biological) molecules, the assumption that the diffusion coefficient of the large molecule is unperturbed by binding of the small molecule should be sound. However, for the smaller molecules typical of contemporary host−guest chemistry, the relationship $D_{HG} = D_H$ may not necessarily be true.

In view of the increasing popularity of the diffusion method to measure K_a we wanted to test the assumption that $D_{HG} = D_H$ for a typical medium-sized host molecule. In the first part of this paper we present some calculated curves showing the effects of fast exchange on observed diffusion coefficients in a three-component system. These curves model the behavior of a hypothetical host−guest system where the size of the host molecule is altered by complexation of a guest molecule. In the second part of the study we present some experimental data for two model systems. They are the β-cyclodextrin (**1**) complexes of cyclohexylacetic acid (**2**) and cholic acid (**3**). We show that binding of guests by cyclodextrins does have an effect on D_H and that careful NMR diffusion measurements can be used to probe the size of the complex as well as its thermodynamic stability.

Experimental Section

β-Cyclodextrin [68168-23-0] was purchased from Aldrich and dried for 12 h at 50 °C before use. Cyclohexylacetic acid [5292-21-7] was purchased from Aldrich and used as received. Cholic acid [81-25-4] was purchased from Lancaster and used as received. The NMR experiments were performed on a standard Bruker DRX400 spectrometer operating under XWINNMR version 2.5, using a 5 mm inverse geometry probe fitted with an actively shielded *z* field gradient coil, and fitted with a BGPA 10 gradients generator and BGU II control unit.

A series of eight solutions were prepared such that they were all 0.5 mM in **1** and covered a range of concentrations from 0.22 to 2.5 mM in either **2** or **3**. All of the solutions also contained 0.05 M phosphate buffer (pH 7.5) and a trace of MeOH to act as a chemical shift reference. Diffusion coefficients were measured using the BPPLED pulse sequence.[11,12] Data were acquired with a 60 ms diffusion delay, 1.8 ms bipolar gradient pulses, 6 ms spoil gradient pulse (30% full power), and a 100 ms eddy current delay. The bipolar pulse gradient strength was varied from 10 to 80% over seven increments. Data were collected at 298 K. Signal averaging ranged from 40 scans to 1024 scans as required for adequate signal-to-noise. The experimentally observed diffusion coefficients were then determined from plots of ln *I* versus g^2 where the *I* is the integrated intensity of a specified region of the NMR spectrum. The slope of the line of this curve is $D/(\Delta - \delta/3 - \tau/2)\gamma^2\delta^2$. Correlation coefficients for all lines were >0.995. Each species was measured at two different chemical shifts, and the experiments were performed in duplicate. Hence, the results and error ranges are the mean and mean deviations of four data points.

Curve fitting was accomplished with an Excel spreadsheet. The spreadsheet was configured to solve the general speciation equation for formation of a 1:1 complex. It accepts as input a table of the initial concentrations of the various species present and the observed variable (e.g., diffusion coefficients). The input page also requires a list of the various parameters (K_a, D_H, D_G, D_{HG}) required to define the system. If any of these parameters are unknown, then an estimate is entered. The program then calculates a predicted data set and goes on to find the magnitude of the difference between the observed data and the predicted data. The embedded Solver tool is then used to minimize this difference for any one or more of the input page parameters that were unknown at the start. Significantly better fits were obtained when all three *D* values were allowed to be changed during the iteration.[13] This approach was used to produce all of the graphical figures for this communication. The spreadsheet is available from the authors.

Results and Discussion

Effects of Chemical Exchange on the Observed Diffusion Coefficients of Equilibrating Host−Guest Complexes. Figure 1 illustrates the anticipated diffusion behavior of a pair of molecules (e.g., a host and a guest) forming a 1:1 complex. The systems are in fast exchange on the NMR time scale. Each graph shows the experiment observable (the observed diffusion coefficient of the guest and the host) as a function of the solution composition. The example is intended to be representative of a typical host−guest system where the small guest molecule diffuses several times faster than the host molecule. The host−guest complex has a measurably slower diffusion coefficient than the host; ($D_G = 5 \times 10^{-6}$ cm^2 s^{-1}, $D_H = 3 \times 10^{-6}$ cm^2 s^{-1}, $D_{HG} = 2 \times 10^{-6}$ cm^2 s^{-1}). The virtual system is 2 mM in $[G]_0$ and ranges from 0.1 to 10 mM in $[H]_0$. It is conventional to present graphs such as these with the *x*-axis normalized to a simple ratio $[H]_0/[G]_0$.

The case for tight binding ($K_a = 10^5$ M^{-1}) is shown in Figure 1a. The observed diffusion behavior of the guest molecule (a fast-diffusing species exchanging with a slow diffusing species) resembles two straight lines intersecting at the stoichiometry of the complex (1:1). The binding isotherm starts ($[H]_0 = 0$) at the diffusion coefficient of the pure guest molecule (5×10^{-6} cm^2 s^{-1}). Complex is formed essentially quantitatively as host is added, and until a stoichiometry of 1:1 is reached, the observed diffusion coefficient is the mole fraction weighted average between free guest (5×10^{-6} cm^2 s^{-1}) and bound guest (2×10^{-6} cm^2 s^{-1}). Eventually, after the 1:1 mole ratio is reached, all of the guest is fully bound and the diffusion coefficient becomes independent of further increases in $[H]_0$.

In the same graph the observed diffusion behavior of the host molecule traces out a flatter binding curve with the steepest inflection after the stoichiometry 1:1. In this system we are observing a slowly moving host molecule

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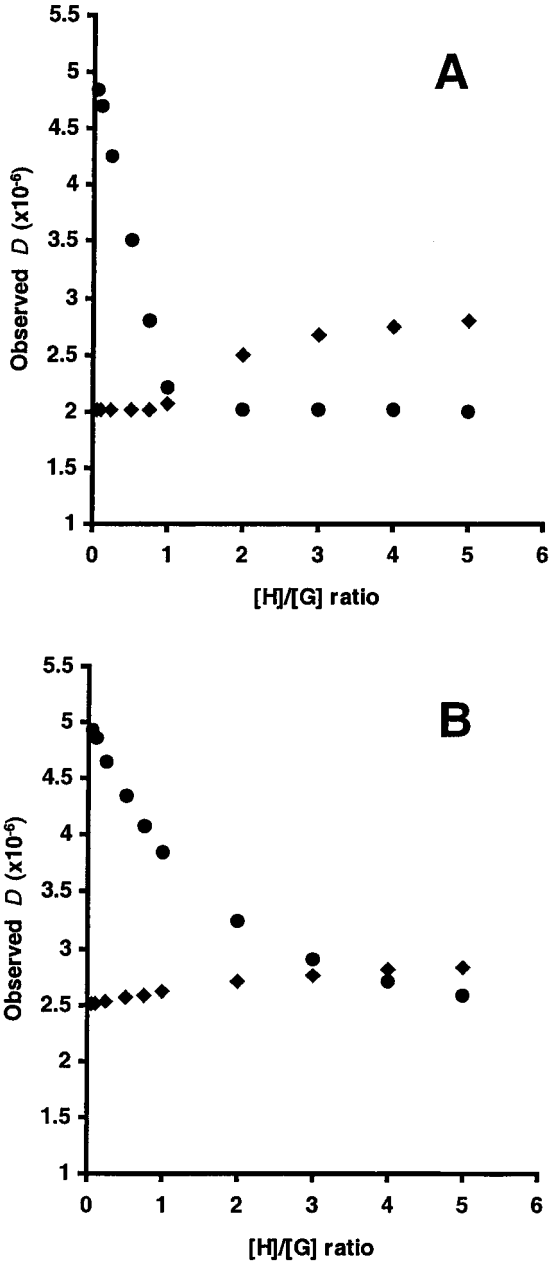

Figure 1. Predicted diffusion behavior of a model host−guest system, (A), $K_a = 10^5$ M^{-1}; (B), $K_a = 5 \times 10^2$ M^{-1}; (● guest or small molecule species; ◆ host or larger molecule).

(3×10^{-6} cm^2 s^{-1}) exchanging with a slower moving complex (2×10^{-6} cm^2 s^{-1}). At stoichiometries less than 1:1 the amount of complex present is limited by $[H]_0$ and the observed diffusion coefficient is approximately the true value of the complex. The line starts to rises slightly with increasing $[H]_0$ because of exchange with a small amount of uncomplexed host (3×10^{-6} cm^2 s^{-1}). Past the critical 1:1 stoichiometry the observed diffusion behavior rises more rapidly because now the only effect is to dilute the complex with the faster diffusing host. In this region the observed D is the mole fraction weighted average between pure host and complex. At the highest $[H]_0/[G]_0$ ratios we observe diffusion behavior resembling that of the pure host molecule.

The case for weaker binding ($K_a = 5 \times 10^2$ M^{-1}) is shown in Figure 1b. This chart has some features that may not have been intuitively obvious before a full analysis. If we look first at the behavior of the guest molecule, the curve starts ($[H]_0 = 0$) at the value expected for free guest and the observed diffusion coefficient drops as increasing amounts of host are added. This line resembles a conventional binding curve. The curvature is determined by the magnitude of the association constant K_a, and the limiting diffusion coefficient of the complex (analogous to $\Delta\delta_{max}$ in a chemical shift titration) is never reached. This behavior is seen because with a small K_a there is exchange between uncomplexed and complexed guest in all of the solutions. Only in the presence of a large excess of host will the observed diffusion coefficient approach that of the complex.

The curve traced by the host molecule is an almost featureless, almost flat line. The host species is always present in significant quantities in the uncomplexed form at all $[H]_0/[G]_0$ ratios, and therefore the experiment detects a value between 2 and 3×10^{-6} cm^2 s^{-1} all across the curve. The observed diffusion coefficient of the host is not particularly dependent on solution make up. At lower K_as (not shown) it becomes even more difficult to detect a solution dependence in D_H. This is probably the reason most previous studies of host−guest complexation by diffusion NMR have reported that the diffusion coefficient of the host is unaffected by binding of guest.

Cyclohexylacetic Acid/*β*-Cyclodextrin System. A preliminary study of this system by conventional ^{1}H NMR revealed why the diffusion method is becoming more popular. ^{1}H NMR titrations showed that the cyclohexylacetic acid signals were relatively insensitive to the presence of *β*-cyclodextrin, and the shifts were too small to be useful for measuring K_a. The *β*-cyclodextrin protons H-3 and H-5 moved upfield as cyclohexylacetic acid was added. The other *β*-cyclodextrin protons were almost completely unaffected by the presence of cyclohexylacetic acid. Protons H-3 and H-5 are located inside the cyclodextrin cavity, and so the shifts observed for these protons indicate that the cyclohexylacetic acid is bound as an inclusion complex. The signal from H-5 of cyclodextrin overlapped with that of H-6, so it could not be used for a quantitative analysis. From the behavior of H-3 we determined $K_a = 2190$ M^{-1}. This value is in good agreement with the previously reported value of 1840 M^{-1} in water at 25 °C measured by potentiometry for the conjugate base of cyclohexylacetic acid.[14] Two of the usual problems of the chemical shift titration method are highlighted with this example. The first is that the chemical shift change in the guest molecule is so small that it could not be used to measure the formation constant of the complex. The second problem is that inadequate resolution prevents a complete analysis of the data.

We now refer to the PFG experiments. The results of the diffusion measurements are shown in Table 1. First it can be seen that cyclohexylacetic acid (D_G) diffuses approximately twice as fast as the larger *β*-cyclodextrin molecule (D_H). This is a satisfactory large difference, and so the concept of using D as a probe of K_a is on a sound footing.

Second, the diffusion constant of cyclohexylacetic acid (D_G) is dependent on the composition of the solution. Values near to the figure for pure **2** are seen at high **2**:**1** ratios, and the observed diffusion coefficient decreases

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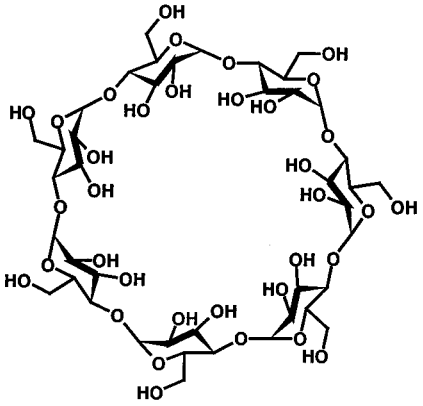
Table 1. Observed Translational Diffusion Coefficients in Mixtures of Cyclohexylacetic Acid/β-Cyclodextrin and Cholic Acid/β-Cyclodextrin

		D_{obs} ($\times 10^6$ cm^2 s^{-1})			
		1 + 2 ⇌ 1:2		**1 + 3 ⇌ 1:3**	
$[H]_0$ mM	$[G]_0$ mM	D_H[a]	D_G[b]	D_H[a]	D_G[c]
0.50	0.22	3.29 ± 0.12	5.39 ± 0.09	3.17 ± 0.09	3.38 ± 0.08
0.50	0.31	3.28 ± 0.08	5.49 ± 0.13	3.13 ± 0.04	3.54 ± 0.03
0.50	0.47	3.28 ± 0.10	5.75 ± 0.07	3.06 ± 0.01	3.60 ± 0.04
0.50	0.63	3.28 ± 0.05	5.80 ± 0.07	3.03 ± 0.01	3.68 ± 0.07
0.50	0.94	3.26 ± 0.07	6.00 ± 0.07	3.01 ± 0.03	3.77 ± 0.05
0.50	1.25	3.24 ± 0.04	6.09 ± 0.06	2.99 ± 0.02	3.96 ± 0.07
0.50	1.88	3.25 ± 0.03	6.31 ± 0.06	2.96 ± 0.07	4.09 ± 0.04
0.50	2.50	3.24 ± 0.07	6.43 ± 0.05	2.94 ± 0.07	4.20 ± 0.03
0	0.50		6.85 ± 0.11		4.51 ± 0.08

[a] Based on observations of the H-2/H-4 protons at 3.4 to 3.6 ppm and the H-5 signal at 3.8 ppm. [b] Based on observations of the 1.99 ppm doublet and the 1.58 ppm mutiplet signal. [c] Based on observations of the 18- and 19-methyl singlets at 0.65 and 0.85 ppm.

with lower mole ratios of **2**. This is as expected (eq 3) for a system in fast exchange. The cyclohexylacetic acid appears to be moving slower in solutions with high host: guest ratio because most of **2** is bound by β-cyclodextrin.



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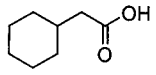

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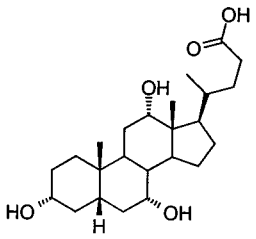

3

Third, the apparent diffusion coefficient of β-cyclodextrin is not constant. Indeed there is a slight trend that D_H is dependent on the concentration of **1**. This result is inconsistent with the assumption that $D_{HG} = D_H$, and clearly for this system the single point approximation is inappropriate for the evaluation of K_a. Attempts to apply the single point method gave values of K_a ranging from 1900 to 800 M^{-1} and which changed systematically according to the solution composition.

A more satisfactory approach is to recognize and take account of the noted solution composition dependence of D_H and use a curve-fitting procedure to find D_{HG} and K_a. In other words the observed diffusion coefficient of the host molecule is treated simply as another exchange

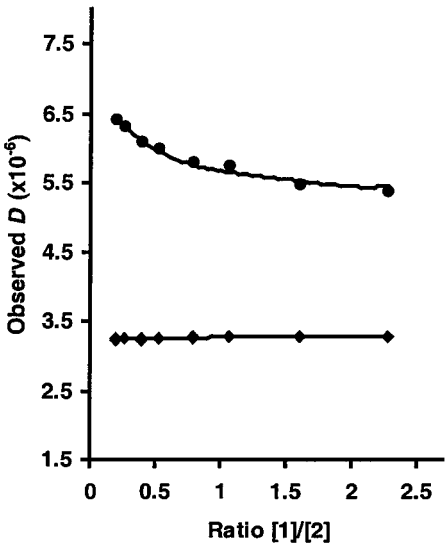

Figure 2. The apparent diffusion coefficients (D_{obs}) of cyclohexylacetic acid (●) and β-cyclodextrin (◆) as a function of solution composition expressed as the concentration ratio [**1**]/[**2**]. The concentration of **1** is held constant at 0.5 mM. The solid lines are the calculated curves for $K_a = 1780$ M^{-1}, $D_G = 7.05 \times 10^{-6}$ cm^2 s^{-1}, $D_H = 3.29 \times 10^{-6}$ cm^2 s^{-1}, and $D_{HG} = 3.23 \times 10^{-6}$ cm^2 s^{-1}. The symbols are drawn to the same scale as the error bars on the D measurements ($\pm 0.07 \times 10^{-6}$).

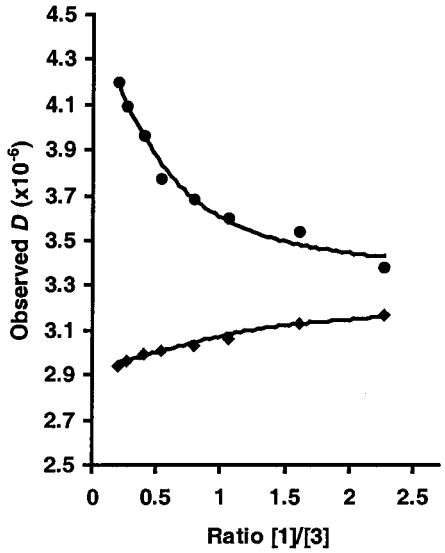

Figure 3. The apparent diffusion coefficients (D_{obs}) of cholic acid (●) and β-cyclodextrin (◆) as a function of solution composition expressed as the concentration ratio [**1**]/[**3**]. The concentration of **1** is held constant at 0.5 mM. The solid lines are the calculated curves for $K_a = 5900$ M^{-1}, $D_G = 4.47 \times 10^{-6}$ cm^2 s^{-1}, $D_H = 3.26 \times 10^{-6}$ cm^2 s^{-1}, and $D_{HG} = 2.93 \times 10^{-6}$ cm^2 s^{-1}. The symbols are drawn to the scale of the error bars on the D measurements ($\pm 0.07 \times 10^{-6}$).

averaged NMR observable. This procedure is exactly analogous to the principle of fitting multiple observed shifts in conventional NMR chemical shift titrations.[15,16]

The result for the cyclohexylacetic acid/β-cyclodextrin system is shown in Figure 2. A satisfactory fit to the experimental data is obtained with $K_a = 1800 \pm 100$ M^{-1}

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and which is in very good agreement with the values determined by ^{1}H chemical shift analysis and by potentiometry. Note that both of the curves shown in Figure 2 are simulated with the same value of K_a and with the same value of D_{HG}. The diffusion coefficient of the complex ($D_{HG} = 3.23 \pm 0.07 \times 10^{-6}$ cm^2 s^{-1}) is slightly slower than that of β-cyclodextrin ($D_H = 3.29 \pm 0.07 \times 10^{-6}$ cm^2 s^{-1}), but the errors are as large as the effect. A noteworthy feature of Figure 2 is the relatively fast diffusion coefficients observed from the guest at all [G]/[H] ratios. This is a result of the modest K_a for this system, so that at all times D_G dominates D_{obs}.

The Cholic Acid/*β*-Cyclodextrin System. We repeated the experiment with a larger guest molecule, expecting that the observed difference between D_H and D_{HG} would be more convincing. Cholic acid was chosen because the complexation of bile acids with cyclodextrins is well studied (ref 17, and references therein). Results for this system are shown in Table 1 and in Figure 3, and indeed a significant solution composition dependence is seen in the diffusion behavior of both species. Figure 3 is presented as an experimental verification of the model system shown in Figure 1b. The steroid/β-cyclodextrin complex **3**:**1** is measurably larger than the simple β-cyclodextrin and this is clearly reflected in the different values $D_H = 3.26 \pm 0.07 \times 10^{-6}$ cm^2 s^{-1} and $D_{HG} = 2.93 \pm 0.07 \times 10^{-6}$ cm^2 s^{-1}. The K_a derived from these data, 5900 ± 800 M^{-1} is the best fit to *both* diffusion curves and this is in good agreement with other studies (e.g., 4100 M^{-1} by fluorescence).[17]

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Conclusion

We have shown that the ^{1}H NMR chemical shift titration method and the diffusion coefficient methods give the same results for K_a. The diffusion coefficient based experiments are time-consuming, but are not dependent on detecting a significant chemical shift change upon complexation.

The results of 'single point' binding experiments need to be viewed with some caution as they are critically dependent on the values used for the pure complexed and noncomplexed species (in this case D_H and D_{HG}). Even if these can be directly measured, they may still be the source of large errors in K_a.

The concept of observing changes in the diffusion behavior of the host as well as the guest has not previously been reported in NMR studies of host−guest complex formation.

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