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NMR diffusion as a novel tool for measuring the association constant between cyclodextrin and guest molecules

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Abstract

In this paper we introduce the use of diffusion measurements by nuclear magnetic resonance (NMR) spectroscopy for determining association constants of weak and very weak interactions between cyclodextrin and guest molecules, as long as both the free and complexed guest molecules are soluble to an extent that allows good sensitivity in the NMR experiment. The experimental setup and data analysis is discussed for three different guest molecules: L-phenylalanine, L-leucine and L-valine, representing different strengths of interaction. The underlying assumptions are discussed and the scope of the method (range of K_a values, requirements to the guest molecule) are discussed. The method's main advantage is its general applicability independent of chromogenic or electrochemical properties of the guest molecule. Whereas calorimetric methods that exhibit a similar generality, are applicable mainly to strong interactions, NMR diffusion measurements are applicable to weaker interactions down to the theoretical limit of 1 M^{-1}, the upper limit for K_a values to be determined by it is approximately 200. A further advantage of the method is the low amount of sample needed. The method is in principle applicable to any case of molecular recognition between a host and guest molecule leading to weak interactions.

Keywords: Translational diffusion; Pulsed-field gradient NMR; Stability constant; Association constant; Equilibrium constant; Cyclodextrin; Guest–host chemistry; Inclusion complex; Supramolecular chemistry; Molecular recognition

1. Introduction

Cyclodextrins are cyclic oligosaccharides composed of α-(1→4)-linked α-D-glycosyl residues, of which α, β- and γ-cyclodextrin, consisting of 6, 7 and 8 glycosyl-units, respectively, are the most studied.[1–4] These cyclodextrins can be described as toroidal, hollow, truncated cones with a hydrophilic exterior and a hydrophobic interior. This unique structure allows molecules (guests) with hydrophobic groups to at least partly enter the cavity and be bound by the cyclodextrin (host) by non-covalent forces only. The resulting entity is known as a guest–host complex or inclusion complex. The inclusion of a guest molecule in a cyclodextrin often alters its chromogenic (e.g., absorbance and fluorescence behaviour) and electrochemical properties. Furthermore, these complexes display very different properties compared to the free guest and host, such as altered solubility, reduced volatility, reduced or enhanced stability, modified chemical reactivity and altered bioavailability. These properties are utilised in many applications in the pharmaceutical, agro-chemical, food and chemical industries, e.g., as a vehicle for drug-delivery of poorly water-soluble drugs, for increasing stability of labile molecules, masking of unpleasant taste or odour and stabilisation of protein solutions against aggregation.[1–3] Moreover, cyclodextrins have gained a position as the most widely used eluent modifier for the separation of structural similar molecules (e.g., enantiomeres) by chromatography and electrophoresis. Additionally, cyclodextrins have gained considerable attention as enzyme models based on their ability to accelerate chemical reactions, e.g., hydrolysis of certain molecules. For the study of non-covalent molecular interactions between molecules, cyclodextrins

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have been and still are a popular choice. Within the field of supramolecular macrocyclic chemistry, the cyclodextrins have gained a unique position as the most studied and commercially successful class of compounds.[4]

Of central importance for the understanding and evaluation of the phenomena of molecular interaction (host–guest complex formation) is the knowledge of the association constants (K_a) and even more important are the thermodynamic parameters, enthalpy, entropy and Gibbs free energy in combination with structural data on the supramolecule formed. The association constant (K_a) between a cyclodextrin and a guest molecule is expressed by:

$$n\mathrm{CD} + m\mathrm{G} \rightleftarrows \mathrm{G}_m{:}\mathrm{CD}_n$$

$$K_a = \frac{[\mathrm{G}_m{:}\mathrm{CD}_n]}{[\mathrm{CD}]^n \cdot [\mathrm{G}]^m} \quad (1)$$

where [CD] is the concentration of cyclodextrin, [G] is the concentration of the guest molecule and $[\mathrm{G}_m{:}\mathrm{CD}_n]$ is the concentration of the inclusion complex. In this paper, however, we will only discuss 1:1 complex stoichiometries.

Thermodynamic parameters can be obtained directly by use of calorimetric techniques (e.g., isothermal titration calorimetry). However, these techniques are limited by the enthalpy of complexation and very weak interactions most often yield unreliable thermodynamic parameters. Alternatively, thermodynamic parameters can be obtained by careful measurement of the association constant at various experimental temperatures. The thermodynamic parameters can be derived from such a dataset by use of the van't Hoff equation in the most simple cases (assuming constant heat capacity, c_p). Thus, new methods for reliable measurement of especially weak association constants are of high importance.

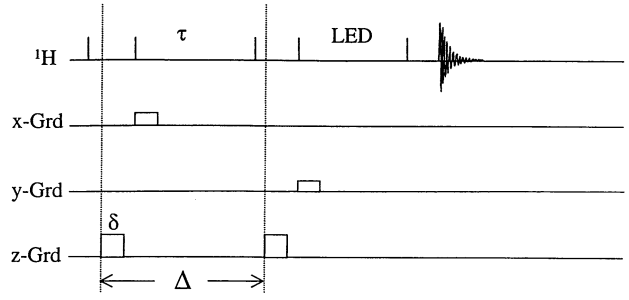

Fig. 1. NMR pulse sequence for diffusion measurements (stimulated echo pulsed gradient spin echo (SE-PGSE) pulse sequence). The first 90° pulse flips the macroscopic magnetisation vector into the xy-plane. A pulsed-field gradient in the z-axis dephases the magnetisation (position encoding). Thereafter magnetisation in stored along the z-axis. A weak pulsed field gradient along the x-axis destroys residual xy-magnetisation resulting from pulse imperfections. After the diffusion time, the magnetisation is flipped back into the xy-plane. A pulsed field gradient along the z-axis rephases the fraction of magnetisation given by Eq. (2) (position decoding). The magnetisation is again stored along the z-axis, while a delay allows for eddy-currents resulting from the gradient pulse to ring down. During this time, a weak pulsed field gradient along the y-axis is used to destroy residual xy-magnetisation. The last 90° pulse flips the magnetisation into the xy-plane and the FID is recorded. δ denotes the gradient duration and Δ denotes the time between the start of the two gradient pulses.

Numerous methods have been used for the determination of association constants in host–guest chemistry including: NMR, calorimetry, spectroscopy, chromatography, capillary electrophoresis, solubility isotherms, potentiometry[5–8] and diffusion measurements by Gouey interferometry.[9] All methods used for the quantification of binding affinity are in one way or the other limited in their use and are varying in accuracy. Several parameters have to be considered, when choosing the most appropriate method for a given set of guest and host molecules. Often, measurement of association constants requires a change of an intrinsic property of the guest molecule or in special cases the host. This includes changes in the chromogenic- and electrochemical properties, retention behaviour, changes in chemical shifts or nuclear magnetic relaxation times, solubility and enthalpy of complexation and is thus highly dependent on the molecules in question. This limits the various techniques to a limited class of molecules with the appropriate physico-chemical properties. Additionally, the choice of solvent and amount and purity requirements for the materials can pose limitations to the methods.[8]

In this paper, we present a method for the determination of association constants between guest and host species based on NMR diffusion measurements.

Theory.—Nuclear magnetic resonance (NMR) has been used widely to determine association constants from chemical shift changes and relaxation time measurements.[10] These methods are, however, not universally applicable since the chemical shift changes are not often significant, especially with aliphatic guests or when association constants are low. Here we investigate the use of nuclear magnetic resonance to quantify the ratio of bound to unbound ligand directly by NMR diffusion measurements. The use of NMR diffusion measurements to determine association constants has been reported for micelle–peptide association and other associations of small molecule with micelles, where there is a large difference in diffusion constant of the binding partners.[11]

In the case of cyclodextrin–guest interaction, this difference is not necessarily that big, but the method can nevertheless be applied.

These measurements, which are described in the next section and shown in Fig. 1, consist of a diffusion delay, flanked by two pulsed-field gradients, where the

Table 1
Association constants (in M^{-1}, unless indicated otherwise) for the interaction of phenylalanine and α-cyclodextrin obtained by different methods

Buffer (pH)	K_a	Method	Reference
Water	15 ± 1	calorimetry	14
Phosphate (13.6)	8 ± 3	calorimetry	14
Phosphate (11.3)	25 ± 1	calorimetry	14
Phosphate (11.0)	15.9 ± 1.1	spectral inhibition titration	13
	15.5 ± 0.2	calorimetry	13
Phosphate (7.2)	42.3	calorimetry	15
Acetate (5.0)	8 ± 5	calorimetry	16
Water	13.6 (kg mol^{-1})	calorimetry	9
	10	diffusion followed by Gouey-interferometry	9
Phosphate (4.0)	14.3 ± 0.4	diffusion followed by NMR	this study

fraction of magnetisation that the second pulse rephases, is described by:

$$A(G) = A(0)\cdot e^{-R(t) - \gamma^2 G^2 D \delta^2 (\Delta - \delta/3)} \quad (2)$$

where $A(G)$ is the signal strength at gradient strength G, $R(t)$ the relaxation attenuation, γ the gyromagnetic ratio (rad T^{-1} s^{-1}), G the gradient strength (T m^{-1}), D the diffusion coefficient (m^2 s^{-1}), δ the gradient duration (s) and Δ the time between the start of the two gradient pulses (s).

The diffusion constant depends on the size of the molecule:

$$D = \frac{kT}{6\pi\eta r} \quad (3)$$

where k is the Boltzmann constant (1.380662×10^{-23} J K^{-1}), T the absolute temperature (K), η the dynamic viscosity (Pa s) and r the radius of the molecule (m). In the case of non-spherical molecules, r is replaced by R_h, the hydrodynamic radius.[12]

If the exchange between free and bound guest is fast on the diffusion time scale (~ 100 ms), the diffusion constant observed in the NMR experiment is a weighted average of the diffusion constant of bound and unbound guest:

$$D_{obs} = \rho \times D_{complex} + (1 - \rho) D_{free} \quad (4)$$

In the case of fast exchange, the fraction of bound guest can be determined by:

$$\rho = \frac{D_{obs} - D_{guest}}{D_{complex} - D_{guest}} \quad (5)$$

where D_{obs} is the apparent (weighted average) diffusion constant of the guest, $D_{complex}$ is the diffusion constant of cyclodextrin and D_{guest} is the diffusion constant of the guest molecule. In the practical application, $D_{complex}$ is not known and cannot be determined. Since our guest molecules are small compared to cyclodextrin, we assume that $D_{complex} \approx D_{CD}$, which will lead to a slight overestimation of the determined value for ρ. The method can be applied to guest molecules of any size—in the case that the size of the guest molecule is of the same order or even bigger than that of cyclodextrin, suitable approximations have to be made.

Furthermore, we assume that the diffusion of one component of the mixture is independent from the others. According to Fick's first law, the flux of solute molecules through a unit area in a system consisting of dissolved guest molecules, dissolved cyclodextrin and dissolved inclusion complex is given by:

$$-\begin{pmatrix} J_{CD} \\ J_G \\ J_{G:CD} \end{pmatrix} = \begin{pmatrix} D_{CD,CD} & D_{CD,G} & D_{CD,G:CD} \\ D_{G,CD} & D_{G,G} & D_{G,G:CD} \\ D_{G:CD,CD} & D_{G:CD,G} & D_{G:CD,G:CD} \end{pmatrix} \cdot \begin{pmatrix} \Delta C_{CD} \\ \Delta C_G \\ \Delta C_{G:CD} \end{pmatrix} \quad (6)$$

where J (mol m^{-2} s^{-1}) denotes the flux of solute molecules through a unit area per unit time, ΔC (mol m^{-4}) denotes the concentration gradient and D denotes the diffusion constants: $D_{CD,CD}$, $D_{g,g}$ and $D_{G:CD,G:CD}$ denote the self-diffusion constants of cyclodextrin, guest molecules and complex, respectively, while the off-diagonal cross-diffusion coefficients denote the diffusion of one component under the concentration gradient of the other.[9] In the absence of any interaction between the molecules and in dilute solutions, the cross-diffusion constants can be assumed to be zero. Both requirements are not fulfilled in our case. Based on the results of Paduano,[9] we nevertheless assume that the cross-diffusion constants are negligible, which is justified by the good agreement between our results and previously published results (see Table 1).

The fraction of guest molecules (ρ) bound to cyclodextrin is defined as:

$$\rho = \frac{[\mathrm{G{:}CD}]}{[\mathrm{G}] + [\mathrm{G{:}CD}]} \quad (7)$$

Combining Eqs. (1) and (7) gives:

$$\rho = \frac{K_a[\mathrm{G}][\mathrm{CD}]}{[\mathrm{G}] + [\mathrm{G}]K_a[\mathrm{CD}]}$$

$$\rho = \frac{K_a[\mathrm{CD}]}{1 + K_a[\mathrm{CD}]} \quad (8)$$

By measuring the apparent diffusion constant of guest molecules and calculating the fraction of bound guest molecules at different concentrations of cyclodextrin and plotting ρ versus [CD], K_a can be determined from fitting the data to Eq. (8).

2. Results

We have applied the method to different guest molecules: the interaction of L-phenylalanine (Phe) and α-cyclodextrin, which is well-studied[9,13–16] to prove the validity of the method. Furthermore we wished to demonstrate the ability to detect even weaker interactions and therefore chose L-leucine (Leu) and L-valine (Val) and α-cyclodextrin. The complex formation between Phe, Leu and Val with α-cyclodextrin was studied with a 2D-ROESY experiment. In contrast to Phe and Leu, no ROESY cross peaks could be observed between Val and α-cyclodextrin (Fig. 2).

The diffusion constants of Phe and α-cyclodextrin in mixtures of Phe and α-cyclodextrin at different ratios were determined as described above. From the apparent diffusion constants of Phe, the fraction bound to α-cyclodextrin was determined. The same was done with Leu and Val. The obtained raw data for Phe are given in Table 2. As can be seen from Eq. (3), changes in sample viscosity will also influence the diffusion constants. Since the viscosity of cyclodextrin solutions is a non-linear function of the cyclodextrin concentration,[17] this has to be taken into account. The viscosity

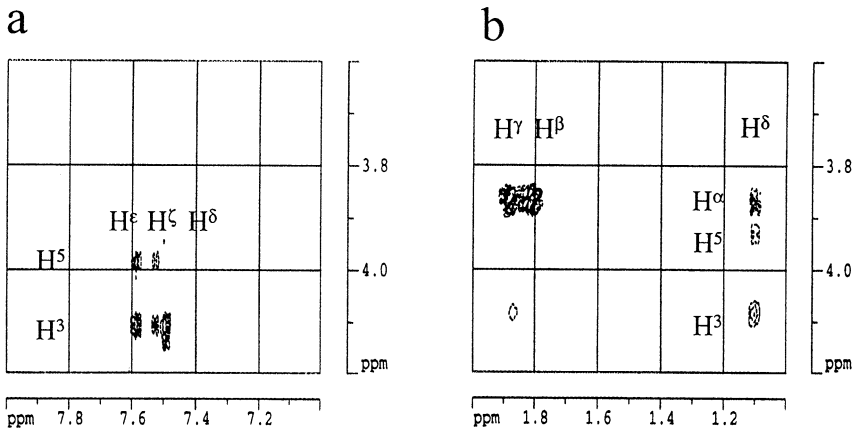

Fig. 2. Regions of 2D-ROESY spectra of α-cyclodextrin and phenylalanine (a) and leucine (b). The dashed lines indicate, that the signal is negative compared to the diagonal signal. The cross peaks arising from through-bond magnetisation transfer between the H^{α} and the side chain protons of leucine can be seen as well.

Table 2
Raw and viscosity corrected apparent diffusion constants (10^{10} D m^2 s^{-1}) of Phe and α-cyclodextrin at different concentrations of α-CD, $[\mathrm{Phe}]_0 = 5$ mM, $T = 290$ K, p$D = 4.0$

$[\mathrm{CD}]_0$ (mM)	$D_{\mathrm{app,Phe}}$	$D_{\mathrm{app,\alpha\text{-}CD}}$	$\eta([\mathrm{CD}])/\eta(0)$	$D_{\mathrm{app,Phe}}$	$D_{\mathrm{app,\alpha\text{-}CD}}$
	Raw data	Raw data		Viscosity corrected	Viscosity corrected
0	1.81		1	1.81	
7.1	1.72	0.95	1.02	1.76	0.97
17.5	1.58	0.90	1.06	1.67	0.96
28.3	1.44	0.84	1.10	1.58	0.92
43.3	1.30	0.82	1.16	1.50	0.95
60.4	1.16	0.77	1.23	1.42	0.95
72.7	1.06	0.75	1.29	1.36	0.96
83.8	1.03	0.72	1.34	1.37	0.96
90.9	0.97	0.73	1.38	1.33	1.00

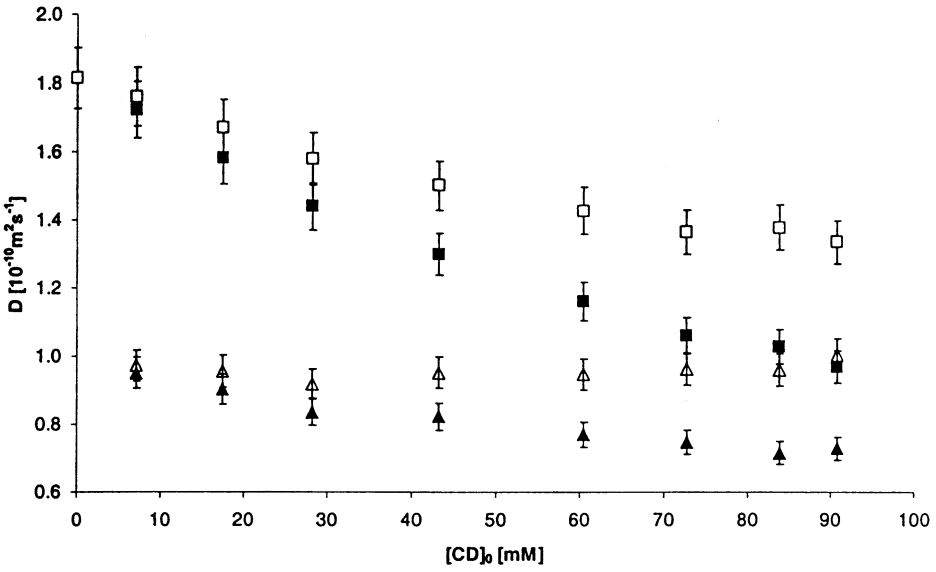

Fig. 3. Observed (full symbols) and viscosity corrected (open symbols) diffusion constants for Phe (■, □), and α-cyclodextrin (△, ▲) plotted vs. the formal concentration of cyclodextrin. The data are given in Table 2. The error level was estimated from repeating a single measurement six times. The standard deviation was found to be 2.4% for the raw diffusion constants. The error bars are drawn at two standard deviations.

of solutions of α-cyclodextrin at different concentrations in the range from 0 to 100 (mM) was measured and fitted to the following empirical function:

$$\eta([\mathrm{CD}]) = \eta(0) + 3.1[\mathrm{CD}] + 11.43[\mathrm{CD}]^2 \quad (9)$$

where η is the dynamic viscosity (Pa s) and [CD] is the concentration of cyclodextrin (mol l^{-1}). All diffusion data were normalised according to Eq. (9). The diffusion constant of Phe at a cyclodextrin concentration [CD] is thus given by:

$$D_{\mathrm{Phe}}([\mathrm{CD}]) = D_{\mathrm{Phe,app}}([\mathrm{CD}]) \times \frac{\eta(0)}{\eta([\mathrm{CD}])} \quad (10)$$

where $D_{\mathrm{phe,app}}([\mathrm{CD}])$ stands for the measured diffusion constant of Phe at cyclodextrin concentration [CD] and $D_{\mathrm{phe}}([\mathrm{CD}])$ stands for the viscosity corrected diffusion constant, respectively. Raw and viscosity corrected diffusion data are given in Table 2 and Fig. 3.

From these data the fraction of bound Phe ρ was calculated according to Eq. (5). Since the diffusion constant of the complex is not known, it was estimated to be the normalised diffusion constant of α-cyclodextrin. Finally, ρ was plotted versus [CD], the concentration of free cyclodextrin, obtained by

$$[\mathrm{CD}] = [\mathrm{CD}]_0 - \rho[\mathrm{G}]_0 \quad (11)$$

The data (see Table 3 and Fig. 4) thus obtained was fitted to Eq. (8) and K_a was obtained from nonlinear regression to be $14.3 \pm 0.4\ \mathrm{M}^{-1}$ for the inclusion complex formation between α-cyclodextrin and Phe at 290 K. This is in good agreement with values given in the literature found by calorimetric[13–16] and diffusion[9] studies (see Table 1).

The same experimental procedure was applied to Leu and Val. An association constant of $6.3 \pm 0.3\ \mathrm{M}^{-1}$ was found for the inclusion complex formation between α-cyclodextrin and Leu at 290 K. To our knowledge, no K_a value for Leu and α-cyclodextrin has been reported

Table 3
Fraction of bound Phe, Leu and Val determined at different concentrations of α-cyclodextrin

[α-CD] [mM]	ρ_{Phe}	ρ_{Leu}	ρ_{Val}
0.0	0.00	0.00	0.00
2.3		0.01	
4.1		0.00	
5.9			0.02
6.8	0.07		
11.1		0.08	
14.4			0.01
16.6	0.17		
20.9			0.01
26.9	0.28		
31.9		0.16	
41.5	0.37		
44.2		0.24	
50.0			−0.04
57.6			−0.04
58.2	0.46		
63.3			−0.04
65.0		0.28	
70.0	0.53		
81.2	0.51		
88.0	0.57		

The estimated error of the data is ±0.035.

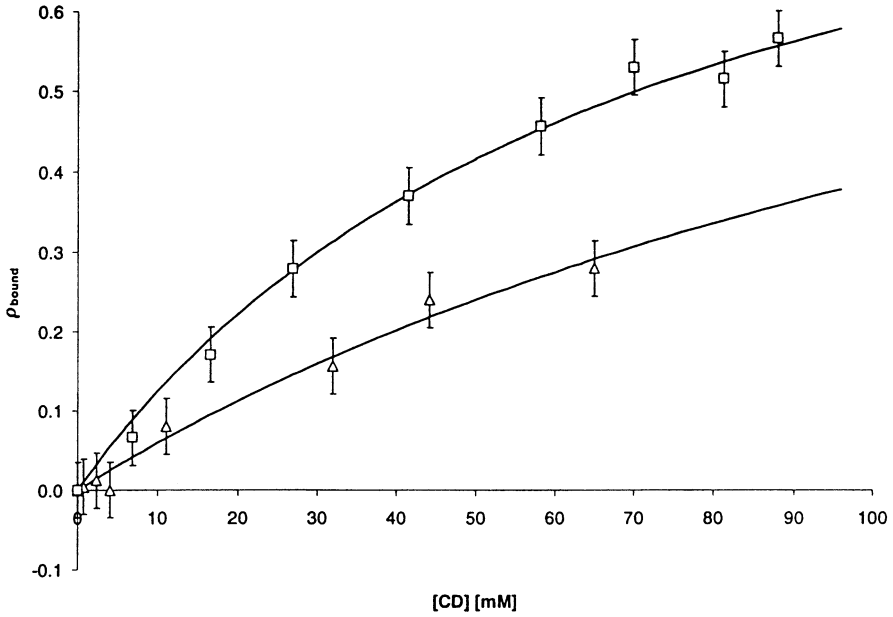

Fig. 4. Plot of ρ_{Phe} (□), ρ_{Leu} (△) and versus the contraction of free α-cyclodextrin. The fitted lines are shown in the graph, the fitted K_a values are: for Phe $K_a = 14.3 \pm 0.4\ M^{-1}$ and for Leu $K_a = 6.3 \pm 0.3\ M^{-1}$.

previously. The experimental data for Leu can be found in Table 3 and Fig. 4.

The diffusion constant of Val was measured in the presence of varying concentrations of α-cyclodextrin (Table 3). The observed differences in diffusion constants of Val at different concentrations of α-cyclodextrin were minute and the estimated ρ values did not yield any significant K_a value. This corroborates the findings from 2D-ROESY NMR spectra where no interaction could be detected.

It should be mentioned again, that the data were obtained applying several simplifications. We assumed that the diffusion constant of the complex is equal to that of cyclodextrin. Furthermore, we assumed that cross-diffusion terms in Eq. (6) can be neglected. These assumptions are discussed below. The third underlying assumption, that the exchange between free and bound guest molecules is fast on the diffusion time scale, can be justified by the fact, that it is already fast on the chemical shift time scale, since we observe only one set of signals.

The fourth underlying assumption is that cyclodextrin is present in its "free", i.e., hydrated form. Previously, aggregates of cyclodextrin in solution were reported.[18,19] These aggregates are very large, up to diameters of 300 nm. Binding of guest molecules to such large aggregates could have an influence on the obtained results. However, judging from the linewidth of the NMR signals of cyclodextrin, these aggregates can only be present in traces in our samples. We assume that the binding of guest molecules does not interfere with the formation of aggregates (if present) and, therefore we neglect the influence of aggregated cyclodextrin on our results.

3. Discussion

We have shown that NMR diffusion measurements are a valuable tool for determining—especially weak—association constants for cyclodextrin–guest complexes. Association constants down to the theoretical limit can be measured. Thus NMR diffusion measurements complement the existing methods which are mainly applicable for strong association constants.

NMR diffusion measurements also complement the "classical" NMR method for stability constant determination, which is based on chemical shift changes. Chemical shift based methods suffer from two weaknesses: first, there has to be a chemical shift change upon complexation. This is not always the case, especially with aliphatic guest molecules, and second, the observed chemical shift change will (in the case of fast exchange between bound and free form) be a weighted average between the chemical shift in the bound and in the free form. In the case of small chemical shift changes and low association constants, chemical shift changes might therefore not be an accurate tool. Compared to NMR-relaxation based techniques diffusion measurements offer the advantage of conceptual and experimental simplicity. For relaxation measurements, each sample needs to be degassed and vacuum-sealed. Moreover, the interpretation of nuclear magnetic relaxation data from ^1H is not straightforward, since many

intra- and intermolecular effects influence the relaxation times and different relaxation pathways have to be taken into account. The analysis of ^{13}C relaxation data is somewhat simpler, since the influence of dipole–dipole relaxation can be separated from other relaxation mechanisms. Nevertheless, an exact analysis of molecular mobility based on ^{13}C relaxation data requires the knowledge of the structure of the complex and its inertial coordinate system and internal motions (e.g., the rotation of cyclodextrin around its guest) have to be taken into account.[20–23] Moreover, obtaining reliable relaxation data from ^{13}C requires either labelled samples or long measurement times.

The method proposed here is generally applicable to measurements of weak guest–host interactions of relatively soluble compounds, since it relies on the change in molecular size upon complexation. Thus, it directly quantifies the amount of free and complexed molecules in contrast to many other methods that are limited to a specific change in the properties of the guest or host molecule, e.g., a change in the chromogenic or electrochemical properties of the guest. Only calorimetry has a similar general applicability towards relatively soluble molecules, however as discussed above, strong interactions are needed to obtain reliable results. The only requirement is that there has to be an NMR signal from the guest molecule (preferably ^{1}H, but the experiment can in principle be applied to other nuclei) that can be detected separately from that of the cyclodextrin–host molecule.

Another advantage of the method is that it requires only a low amount of guest (<10 μmol) and cyclodextrin–host (<100 μmol), which is little compared to many other methods.[8]

Scope and validity.—The method proposed here becomes increasingly inaccurate with higher values of K_a, because the sensitivity of the curve ρ versus [CD] on changes in K_a decreases (see Fig. 5). To assess the upper limit of association constants that can be determined by NMR diffusion measurements, we simulated sets of data for different values of K_a. An experimental error of ± 0.1 for the determination of ρ (compared to ± 0.035 we found in our experimental data) was introduced and the data were subjected to the same treatment as the experimental data. The results are summarised in Fig. 6. They show that above a K_a value of $\sim 200\ M^{-1}$, the method does not yield reliable results any more. This limit can be extended somewhat by reducing the experimental error (increasing sensitivity by recording more transients or increasing the concentration of the guest molecule, recording more datapoints, careful choice of cyclodextrin–guest ratios). The method yields, however, reliable results in the case of very low values of K_a, down to the theoretical limit, which was suggested to be $1\ M^{-1}$,[24] based on the random association constants.

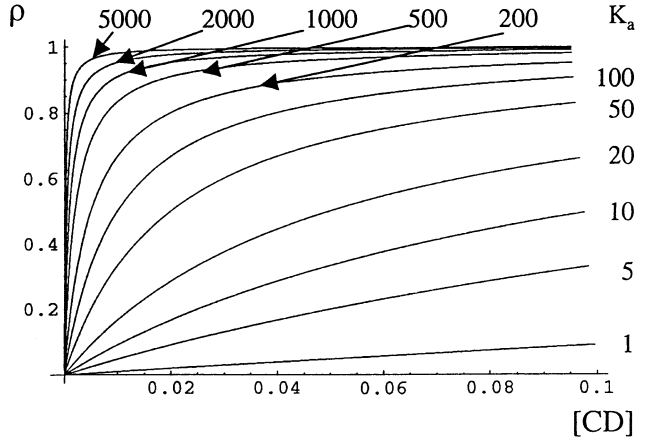

Fig. 5. Plots of ρ vs. the concentration of free cyclodextrin (M) for different values of K_a. It can be seen that with increasing K_a the curve becomes insensitive towards changes in K_a. The curve is calculated based on a concentration of guest molecules of 5 mM.

For very weak association constants the precision of the method compares favourably to other methods judged from the extensive data on cyclodextrin complexes collected by Connors.[6,7]

The method is most sensitive when dealing with guest molecules that are small in size compared to the host molecule. However, it will also work with larger guest molecules, but the precision will decrease with increasing size of the guest molecule. The precision of the determination of ρ depends on the difference between the diffusion constant of the complex and the diffusion constant of the free guest molecule. Depending on the exact shape of the guest molecule and the structural details of the inclusion complex, one can estimate that this difference in translational diffusion constants between a free and a bound guest molecule is $\sim 30\%$ for a guest molecule that is equal in size to the host molecule.[25,12] At such a difference level the method is still applicable. With standard NMR equipment, i.e., available gradient strengths of approximately 0.6 T m^{-1}, longer diffusion times ($> \sim 100$ ms) are needed to obtain a reliable determination of the diffusion constant. If guest molecules exhibit significantly shorter longitudinal relaxation times, dedicated diffusion equipment with stronger magnetic field gradients will have to be used.

With the increasing availability of NMR spectrometers, the proposed method should prove a valuable addition to the existing methodology of determining association constants, especially those of weak interactions.

4. Experimental

99% pure L-phenylalanine, L-valine and L-leucine were purchased from Sigma–Aldrich. Pharmaceutical

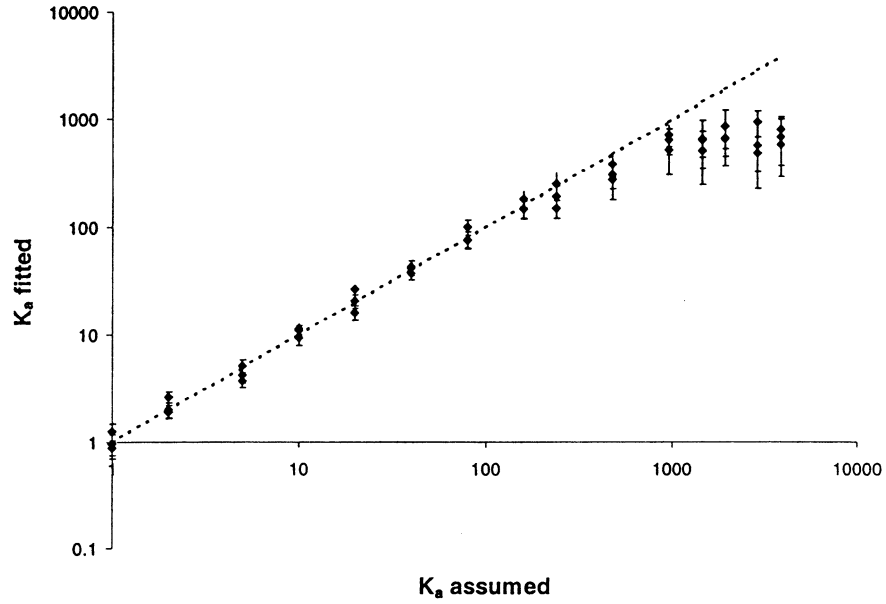

Fig. 6. The K_a value determined from analysing artificial datasets are plotted vs. the K_a values used for generating these datasets. The datasets were produced assuming that ρ can be determined with a precision of ± 0.1, which is approximately three times worse than the precision obtained from our experimental data. Three sets of data were produced for each assumed value of K_a.

grade α-cyclodextrin was purchased from Wacker Chemie Burghausen, Germany. 99% D_2O was purchased from Larodan Fine Chemicals AB, Malmö, Sweden.

Prior to its use in NMR experiments, the cyclodextrins were completely dissolved in D_2O and the solvent was evaporated at 95 °C.

The viscosity of solutions of 0, 25, 50, 75 and 100 mM α-cyclodextrin in 100 mM phosphate buffer at pH 7.28 were measured at rt with a Brookfield digital viscometer model DV-II.

NMR.—All experiments were carried out on a Bruker DRX600 spectrometer equipped with a 5 mm *xyz*-grad TXI probe capable of generating a field gradient of 0.6 T m^{-1}. The sample temperature was kept at 17 °C—below rt—to prevent the occurrence of convection effects. The pulse sequence for diffusion measurements by NMR is shown in Fig. 1.[26,27]

It consists of a 90° pulse followed by a dephasing gradient, thereafter the magnetisation is stored as *z*-magnetisation during the diffusion delay τ. The magnetisation is brought back to the transverse plane and another gradient refocuses the magnetisation of spins that have remained in the same position in the sample during τ. Magnetisation is stored once again in the *z*-direction while an eddy-current delay (LED) allows for ringing down of electronic disturbances due to the gradient pulses.

Weak *x*- and *y*-gradients of 0.06 T m^{-1} are employed during the magnetisation storage times to destroy any transverse magnetisation brought about by pulse imperfection.

Gradient pulses of 1.5 ms duration and five different strengths varying from 0.024 to 0.54 T m^{-1} were employed for dephasing and refocusing. The diffusion delay was set to 500 ms.

2D-ROESY spectra were recorded with a 250 ms continuous wave spin-lock ($\gamma B_1/2\pi = 6$ kHz).

5. Note added in proof

After acceptance of this article, we became aware of a previous publication utilizing pulsed field gradient NMR to demonstrate the binding of alcohols to cyclodextrins.[28] However, "the purpose of this investigation was to explore the applicability of this technique [...] and not to make precision measurements of binding constants". In the present paper, precision measurements of binding constants with elimination of possible sources of error is emphasized and a thorough treatment of scope and validity of the method is given.

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