Rapid Screening of Binding Constants by Calibrated Competitive ^{1}H NMR

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This paper is dedicated to the memory of Dr. J. A. (Tony) Semlyen.

Abstract: A calibrated competitive NMR method has been developed that is appropriate for the rapid screening of binding constants. This method involves the initial characterisation of a receptor–substrate binding event for which the ^{1}H NMR spectrum of a given receptor (calibrant) is modified by the substrate of interest at a range of concentrations. For all subsequent "unknown" receptors, K_a values are then determined by using a competition assay (in the presence of the calibrant receptor) by measuring a single standard ^{1}H NMR spectrum. This enables a rapid assessment of the recognition properties of a library of potential receptors. Only the calibrant receptor needs to be NMR active, while the library of putative receptors, as well as the substrate, can be NMR silent. This method assumes the formation of complexes of 1:1 stoichiometry. To demonstrate this methodology, the binding of a number of crown ether type compounds with K^+ ions has been studied. Comparison of the binding strengths obtained by using this approach with those in the literature shows excellent agreement. A range of new compounds that have recently been synthesised within our group has also been screened in order to illustrate how this approach can rapidly assess binding ability. This method has significance for chemists working in the fields of combinatorial receptor/substrate design and supramolecular chemistry as a means of rapid optimisation of binding strength.

Keywords: crown compounds · high-throughput screening · host–guest systems · NMR spectroscopy · thermodynamics

Introduction

There is currently intense interest in the development of combinatorial libraries of molecules capable of exhibiting host–guest interactions. This is particularly true in the field of medicinal chemistry,[1] but such a development is also of considerable and increasing importance in supramolecular chemistry—for example sensor design.[2] Perhaps the most significant piece of information about any given receptor, whether or not it is part of a combinatorially generated library, is how strongly it binds specific substrates. For this reason, many different techniques for determining receptor–substrate binding affinities have been developed. Increasingly, NMR techniques are being utilised to screen libraries of compounds for drug discovery.[3] Binding can be assayed by using a range of different NMR methods: nuclear Overhauser effect (NOE),[4] chemical shift perturbation,[5] diffusion,[6] relaxation[7] and saturation transfer.[8] These techniques can all provide useful information, although many require quite complex experimental setup.

In supramolecular chemistry, NMR is typically used to monitor receptor–substrate interactions in two different ways—titrations and competition experiments.[9] NMR titrations offer an effective way of using simple ^{1}H NMR spectra to study host–guest systems, and provide information about both complex stoichiometry and binding strength. However, titrations are time consuming to perform, and this makes them difficult to adapt to high-throughput-screening methods. Titration experiments are also generally unsuitable for the study of binding constants $> 10^5\,\text{M}^{-1}$. Competition experiments have therefore been used to overcome some of the problems of NMR titrations. For example, strong complexes ($> 10^5\,\text{M}^{-1}$) can be characterised with reference to weaker ones. Competitive methods have also been used to speed up the measurement of K_a values. Reinhoudt and co-workers were the first to propose methods that studied a change in NMR shift of the substrate in the presence of two receptors.[10] In a key paper, Whitlock and Whitlock used competition experiments for rapid binding constant evaluation.[11] They determined the NMR shifts of the free reference receptor, the fully complexed reference receptor, the free "unknown" receptor and the fully complexed "unknown" receptor. A mixture of reference and unknown receptor was then used to compete for the substrate, and the observed NMR shifts of both receptors allowed the calculation of a binding constant for the "unknown" receptor–substrate complex.[12] Similar competi-

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tion experiments have also been used in cases in which complexation is slow on the NMR timescale.[13] Recently, competition experiments in combination with relaxation parameters have been used to develop a high-throughput approach to NMR-based screening.[14]

In this paper, we report a calibrated competition experiment for the determination of K_a values that offers several advantages over those previously published: a) only the reference receptor needs to be NMR active; this enables NMR-silent receptors (or those which do not respond to the presence of substrate through shifts in their NMR spectra) to be investigated; b) only a single standard ^{1}H NMR spectrum needs to be recorded to determine an approximate binding constant (once the calibration curve has been determined); and c) accuracy is increased by using a calibration curve, rather than simply measuring the shifts of unbound and fully bound reference host. The basis of the method reported here has previously been used by Kempen and Brodbelt for the determination of binding constants by electrospray mass spectrometry,[15] a topic in which there is much interest. NMR, however, offers several advantages over MS for this type of binding study: a) neutral complexes can more easily be studied; b) the temperature can be kept at a constant value (unlike MS, in which heating occurs when the solvent is removed); c) NMR directly reflects the species present in solution; and d) contamination of the instrument is not a problem in NMR methods, whereas it can be in mass spectrometric determinations. We illustrate the application of this new NMR approach for the rapid screening of binding constants for a series of crown ether type receptors, and describe the way in which the method can readily be applied for the characterisation of libraries of potential receptors.

Results and Discussion

Generation of a calibration curve theory: It is first necessary to generate a calibration curve for a reference receptor (calibrant) that binds the substrate of interest. This calibration curve will later enable the easy derivation of binding constants for the library of "unknown" receptors.

$$R_{ref} + S \rightleftharpoons R_{ref}.S \quad (1)$$

$$K_{ref} = [R_{ref}.S]/[R_{ref}]_{free}[S]_{free} \quad (2)$$

$$[R_{ref}]_{free} = [R_{ref}]_{total} - [R_{ref}.S] \quad (3)$$

$$[S]_{free} = [S]_{total} - [R_{ref}.S] \quad (4)$$

$$a[R_{ref}.S]^2 + b[R_{ref}.S] + c = 0 \quad (5)$$

$$a = K_{ref} \quad (6)$$

$$b = -(K_{ref}[S]_{total} + K_{ref}[R_{ref}]_{total} + 1) \quad (7)$$

$$c = K_{ref}[R_{ref}]_{total}[S]_{total} \quad (8)$$

The equilibrium between the reference receptor and substrate is given by Equation (1), and the binding constant K_{ref} is described by Equation (2), here $[R_{ref}]_{free}$ is the concentration of unbound reference receptor, $[S]_{free}$ is the concentration of unbound substrate, and $[R_{ref}.S]$ is the concentration of the complex formed between them. The binding constant K_{ref} should be known from the literature, or measured by using traditional methods. The concentration of the reference complex can then be calculated for samples made with different concentrations of guest as follows. The concentration of unbound reference receptor $[R_{ref}]_{free}$ is related to the total concentration of reference receptor $[R_{ref}]_{total}$ by Equation (3), and in a similar way, $[S]_{free}$ can be related to the total concentration of substrate $[S]_{total}$ by Equation (4). Combining Equations (2), (3) and (4) generates a quadratic Equation (5), in which constants a, b and c are defined by Equations (6)–(8). Solving this quadratic in the normal way enables $[R_{ref}.S]$ to be calculated for different concentrations of receptor and substrate in solution.

These solutions are then made up, and a distinctive NMR shift of the receptor is monitored. A linear relationship should exist between the observed NMR shift of the receptor and the concentration of $[R_{ref}.S]$ as calculated above as long as the stoichiometry of the complex is 1:1. This allows a linear calibration curve to be plotted.

Generation of a calibration curve—Experiment: To illustrate this principle, we chose dibenzo[18]crown-6 as the reference receptor. It is essential that the reference receptor exhibits a significant NMR shift when binding the substrate of interest—in this case K^+. The complex between dibenzo[18]crown-6 and K^+ in methanolic solution is well characterised in the literature, with $\log K$ values in the range 4.80–5.10, and an average value of 4.99.[16] The accurate knowledge of this value is critical, as subsequent K values are determined relative to it. In the case of determining the K values for a library of potential receptors, this method would therefore require that one reference receptor should be first characterised in detail.

A series of samples containing 1 mM dibenzo[18]crown-6 and potassium chloride with a concentration between 0 and 1 mM (in 0.1 mM intervals) were prepared in deuterated methanol. The ^{1}H NMR spectrum of each sample was recorded. As the binding process is fast on the NMR timescale, a peak signal corresponding to the average of complexed and free receptor was observed. These shifts are illustrated in Figure 1. The shifts of the four peaks marked with an asterisk were monitored and plotted against $[R_{ref}.S]$ as calculated above by using a $\log K_{ref}$ value of 4.99.[17] This generated four straight-line calibration curves (one of these is illustrated in Figure 2 for one of the aromatic protons of the receptor) that could then be used to evaluate the binding constants of "unknown" receptors, as described below.

It should be noted that significantly fewer NMR measurements could probably be used to obtain an accurate calibration curve for a given receptor–substrate combination, however, we wished demonstrate the robustness of the procedure, and therefore used 22 points for the calibration.

Calculation of unknown binding constants—Theory: The binding constant of the "unknown" receptor can be described by Equation (9), in which $[R_{unk}]_{free}$ is the concentration of unbound receptor of interest, $[S]_{free}$ is the concentration of

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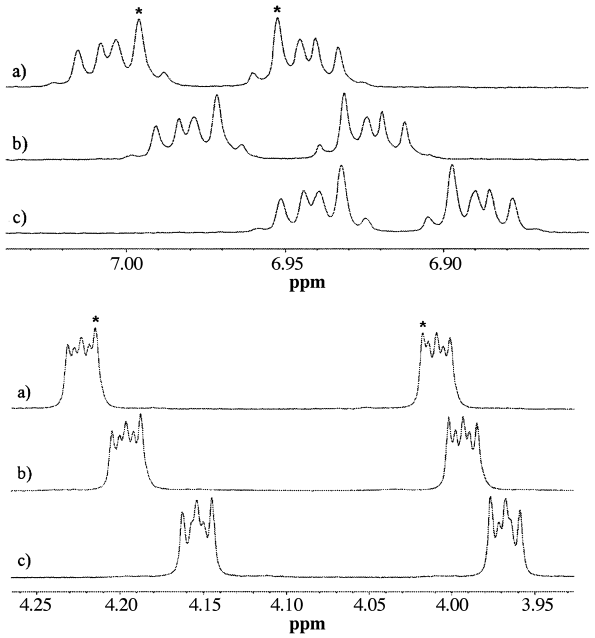

Figure 1. a) ^{1}H NMR spectrum of dibenzo[18]crown-6 (1.0 mM) and KCl (1.0 mM) in MeOD. b) ^{1}H NMR spectrum of dibenzo[18]crown-6 (1.0 mM) and KCl (0.5 mM) in MeOD. c) ^{1}H NMR spectrum of dibenzo[18]crown-6 (1.0 mM) in MeOD.

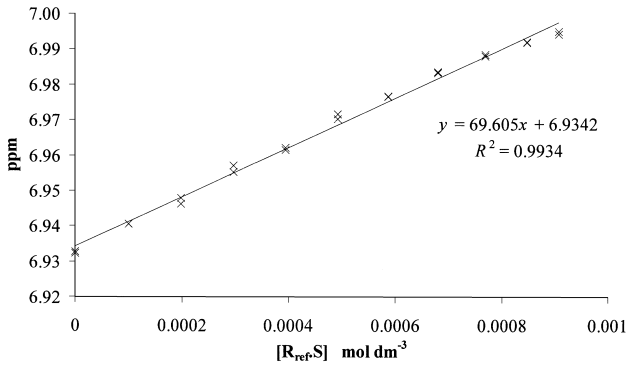

Figure 2. Calibration curve correlating the observed chemical shift with the concentration of complex present in solution.

unbound substrate, and $[R_{unk}.S]$ is the concentration of the complex formed between them. A competition experiment for the substrate is set up between the two receptors R_{ref} and R_{unk}, and therefore, Equation (2) holds for this equilibrium. This allows us to express $[S]_{free}$ in terms of known values by using Equation (10). The other terms in Equation (9) can also be expressed as known quantities as shown in Equations (11) and (12). This enables a relationship between $[R_{ref}.S]$ (as determined from the change in NMR shift by using the calibration curve) and $\log K_{unk}$ to be determined, and hence unknown binding constants can be simply calculated.

$$K_{unk} = [R_{unk}.S]/[R_{unk}]_{free}[S]_{free} \quad (9)$$

$$[S]_{free} = [R_{ref}.S]/K_{ref}([R_{ref}]_{total} - [R_{ref}.S]) \quad (10)$$

$$[R_{unk}.S] = [S]_{total} - [S]_{free} - [R_{ref}.S] \quad (11)$$

$$[R_{unk}]_{free} = [R_{unk}]_{total} - [R_{unk}.S] \quad (12)$$

By using the example of dibenzo[18]crown-6 as a reference receptor ($\log K_{ref} = 4.99$), the relationship between $[R_{ref}.S]$ and $\log K_{unk}$ can be displayed in graphical form (Figure 3). It can be seen that this method will provide accurate binding

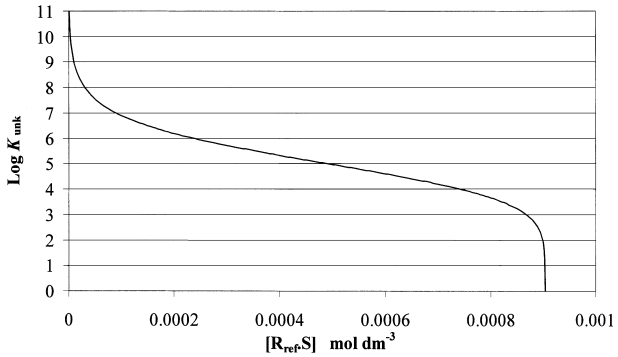

Figure 3. Relationship between $\log K_{unk}$ and $[R_{ref}.S]$ displayed in graphical form.

constants in the range $3 < \log K_{unk} < 7$—in other words, two log units either side of dibenzo[18]crown-6 itself. Outside this range, the method will simply determine binding constants as either large ($\log K_{unk} > 7$) or small ($\log K_{unk} < 3$); however, even this information can be useful for high-throughput screening. For actual determination of binding constants outside the range, a new reference compound should be chosen, which itself has a higher (or lower) binding constant.

Calculation of unknown binding constants—Experiment: In order to validate this method, binding constants were determined for several receptors that had previously been studied. These receptors previously had potassium binding constants determined in methanol at 298 K. A solution was made up in deuterated methanol containing 1 mM concentrations of potassium chloride, dibenzo[18]crown-6 and the receptor of interest. The ^{1}H NMR spectrum of this competition mixture was measured, and the shift of the peaks of the dibenzo[18]crown-6 reference were recorded. The NMR shift of these protons was then used to calculate $\log K_{unk}$ by using the calibration curve (Figure 2, to generate $[R_{ref}.S]$), and then Equations (9) – (12) (to generate $\log K_{unk}$).

It should be noted that this method therefore requires just a single standard ^{1}H NMR measurement and is also not dependent on the receptor of interest being NMR active, or having peaks that shift in a useful way. In addition, the method does not require any computer-based mathematical manipulations of data such as nonlinear regression.

The first crown studied was [18]crown-6 (Scheme 1), which itself shows no useful ^{1}H NMR response to K^+ ions. This crown is the most widely studied of the crown ethers, and numerous binding constants had been previously determined for K^+ binding, from a variety of techniques. The average of these values gave a $\log K$ of 6.17, with all ten values lying between 6.02 and 6.32.[18] We performed our single-point competitive-binding-constant determination and obtained $\log K$ values of 6.18 and 6.21 (we repeated the measurement

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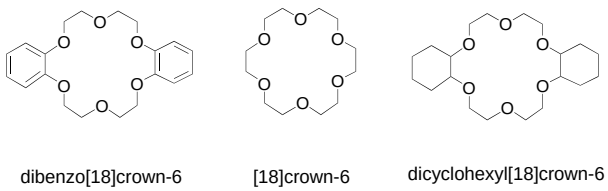


Scheme 1. Crown ethers.

to assess reproducibility), in excellent agreement with the literature values (Table 1).

The second crown ether studied was dicyclohexano[18]-crown-6 (as a mixture of *syn-cis* and *anti-cis* isomers). Only

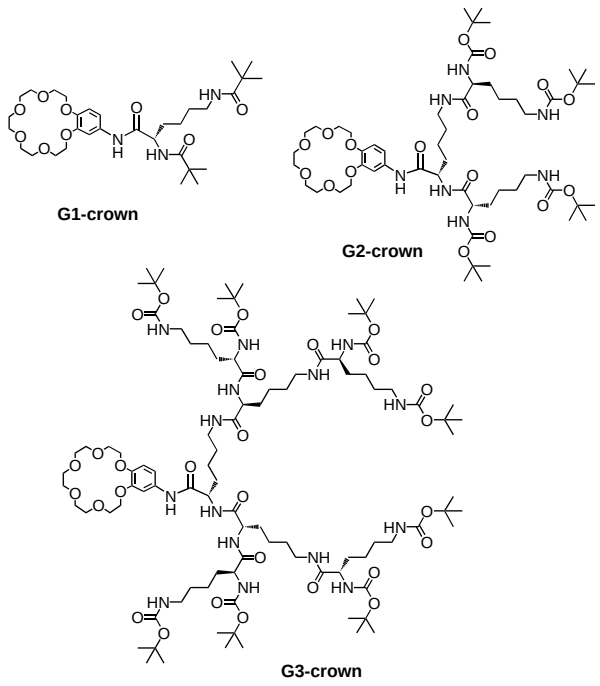
Table 1. Log K values for the receptor–substrate combinations investigated as a validation of this method. Literature values are included for comparison purposes for known systems.

Receptor	Guest	log K (lit.)	log K (exp.)
[18]crown-6	K^+	6.17	6.18/6.21
dicyclohexyl[18]crown-6	K^+	5.64	5.67/5.59
G1-crown	K^+	–	5.01
G2-crown	K^+	–	4.86
G3-crown	K^+	–	4.40
cyclic monomer	K^+	–	3.13/3.15
cyclic dimer	K^+	–	3.27/3.28
cyclic trimer	K^+	–	3.25/3.28

two binding constants are available in the literature for this host, with log K values of 5.63 and 5.65 (calculated by different methods), giving an average of 5.64.[18a] Using the method reported here in two repeat experiments gave log K values of 5.67 and 5.59. The excellent agreement between our method and the literature values indicates that this method, whilst much more rapid than traditional NMR methods, still provides an accurate estimate of binding strength, certainly with sufficient accuracy for rapid screening applications.

In order to illustrate the way in which this method can be applied to a small "library" of compounds, we applied this approach to a series of compounds available in our laboratories. The types of compound studied were novel dendritically modified crown ethers,[19] and novel ether–ester cyclics (Scheme 2). The full synthesis, characterisation and properties of these novel compounds will be reported in due course.

Dendritic crown ethers based on aminobenzo[18]crown-6 functionalised with L-lysine derived dendritic branching,[20] were investigated for their ability to bind K^+ ions. The binding constants were evaluated, as shown in Table 1. It was notable that as the extent of dendritic branching increased, the crown ether bound K^+ less strongly. This could be a consequence of the steric hindrance of the more highly branched molecules—a feature that has been previously observed for host–guest chemistry inside dendrimers.[21] NMR titration experiments were used in an attempt to reproduce these values. However, whilst the general trend G1-crown > G2-crown > G3-crown could be reproduced, it was difficult to obtain accurate log K values for these systems by using a titration technique, because the binding constants were at or above the accurate limit that can be determined by using titration methods. This

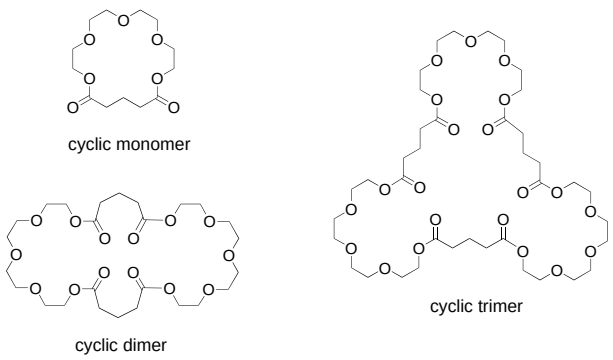


Scheme 2. Dendritic crown ethers.

illustrates the well-known advantage of using competition methods to determine large log K values.

Ether–ester cyclics have been known since the work of Bradshaw and co-workers.[22] Recent synthetic developments (to be reported) have allowed us to synthesise and isolate reasonable quantities of small and medium ring size cyclics, and we wanted to rapidly assay the binding of these different macrocycles with K^+ cations. It might be expected that such structures would act as good analogues of [18]crown-6, or larger cyclic structures such as valinomycin or nonactin.[23]

In this study, the binding constants of three cyclic ether–esters were determined: cyclic-monomer, cyclic-dimer and cyclic-trimer (Scheme 3). Surprisingly, all of these cyclic oligomers were found to be poor potassium binders (Table 1). In fact, the values obtained lie at the lower end of the range that this method can determine, and as such, log K should be considered as <3.5 for all of these complexes. The cyclic dimer and trimer appear to be slightly better at binding K^+, perhaps due to the rigidity of the monomer. Accurate binding constants for these cyclic ether–esters could also have been



Scheme 3. Ether–ester cyclics.

determined by using competitive binding with a reference receptor that has a lower K^+ binding constant; however, that was not done here, as the main aim was to illustrate the ability of this method to rapidly screen the binding affinity of a range of compounds (and hence discard those which were unsatisfactory)—a goal that was met by the experimental procedure reported in this paper.

Conclusion

It is clear that this new *calibrated competitive method* for determining binding constants offers a range of advantages over some of the NMR methods currently used. The number of NMR measurements required is dramatically fewer—of great importance if high-throughput analysis of binding constants is required. In fact, after a calibration curve has been determined, only a single standard ^{1}H NMR measurement is required for each binding constant (although this should be repeated to avoid errors in making solutions). Due to the calibration process, the receptor of interest can be NMR silent or unresponsive, unlike in previous competitive binding assays. In addition, the method depends on simple ^{1}H NMR spectra, rather than more complex acquisitions.

Although dibenzo[18]crown-6 was used as the reference host here, any receptor that exhibits a change in NMR shift between the free and bound states with any guest of interest would be suitable as a reference host. Neither is the method limited to strongly bound complexes. As long as the reference receptor is within two orders of magnitude of the receptor of interest then accurate constants can be obtained. In addition to using two receptors to compete for a given substrate, it is also possible to apply this method to two different substrates binding to a given receptor, as long as one of the substrates has a significant shift in its NMR spectrum.

The method does rest on a number of assumptions, and it is important to bear these in mind when applying it. The reference receptor–substrate complex must be fully and accurately characterised, and furthermore both receptors must bind the guest with 1:1 stoichiometry. This is the most important assumption because the single NMR measurement will not provide any stoichiometric information. While $[R_{ref}.S]$ can always be accurately determined, this can only be transcribed into a K value for the unknown receptor if the stoichiometry of binding is 1:1. Therefore, while useful for rapid screening, it is still recommended that for publication of accurate thermodynamic data, the approach described in this paper should be further backed up by other information, such as Job plot analysis. This method also assumes that there are no interactions between reference receptor and unknown receptor that might alter the NMR spectrum of the reference. As for all NMR methods, this approach is also limited by the necessity to work at NMR concentrations.

Overall, however, this method offers a powerful way of monitoring the recognition capabilities of libraries of receptors or substrates by using simple, single ^{1}H NMR measurements and, as a consequence, could be of broad importance in high-throughput applications and supramolecular chemistry.

Experimental Section

Proton NMR spectra were recorded on a Bruker AMX-500 (^{1}H 500 MHz) at 298 K. Chemical shifts (δ) are quoted in parts per million referenced to tetramethylsilane, which exhibited a singlet at 0.0 ppm. For the purposes of all calculations, chemical shifts were used in Hz units, which are provided with greater accuracy from the NMR spectrometer.

Potassium chloride (99 + %), dibenzo[18]crown-6 (98%), [18]crown-6 (99%) and *cis*-dicyclohexano[18]crown-6 (mixture of *syn-cis* and *anti-cis*) (98%) were purchased from Aldrich and used without any further purification. Deuterated methanol (CD_3OD, 99.8% D) was purchased from Cambridge Isotope Laboratories and was used as received.

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