Influence of Response Factors on Determining Equilibrium Association Constants of Noncovalent Complexes by Electrospray Ionization Mass Spectrometry

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Abstract

A method for determining the equilibrium association constant of a complexation reaction A + B ⇔ AB by electrospray ionization mass spectrometry is described. The method consists in measuring the relative intensities of the peaks corresponding to A and to AB in equimolar A:B solutions at different concentrations C_0. The results are fitted by a non-linear least squares procedure, with the two variable parameters being the equilibrium association constant K_a, and a factor R, defined by: I(AB)/I(A) = R × [AB]/[A]. The factor R is the ratio between the response factors of AB and A, and corrects for the relative electrospray responses of the complex and the free substrate A, mass discrimination of instrumental origin, and/or moderate in-source dissociation. The method is illustrated with the following two systems: complexes between a double-stranded 12-base pair oligonucleotide and minor groove binders, and cyclodextrin complexes with α,ω-dicarboxylic acids. For the oligonucleotide complexes, it is found that the response of the complex is not dramatically different than the response of the free oligonucleotide duplex, as the double helix conformation is disturbed by the drug only to a minor extent. In the case of cyclodextrin complexes, these complexes were found to have a much higher response than free cyclodextrin. This can be due to the fact that cyclodextrin is neutral in solution, while the complex is charged, but it can also stem from the fact that a significant proportion of the complex is in a non-inclusion geometry. The present method requires the exact determination of the concentrations of the reactants and is applicable to 1:1 complexes.

Keywords

Electrospray mass spectrometry, Non-covalent complexes, Binding constants, Oligonucleotides, Cyclodextrins

Introduction

The study of noncovalent complexes by electrospray mass spectrometry (ES-MS) is a constantly growing area of research.[1-3] Characterization of the species that are present in the injected solution can be done by recording ES-MS spectra in soft ionization conditions. One of the major issues in this kind of study is to seek correlation between mass spectrometric results and solution phase behavior. Stoichiometries of the observed complexes can be assessed directly by the measurement of the molecular mass when the individual constituents are known. Moreover, the relative intensities in the mass spectra can be related to the relative abundance of the different species in solution.

For two analytes, A and B, the ratio between the intensities is given by equation (1), where R_A and R_B are the response factors of the corresponding analytes.

$$\frac{I_{(A^+)}}{I_{(B^+)}} = \frac{R_A[A^+]}{R_B[B^+]} \qquad (1)$$

Discrimination can arise from the electrospray process, from the mass analyzer and from the detector efficiency. In the case of noncovalent complexes, the collision-induced dissociation of the complex can also alter the relative intensities.

The influence of the electrospray mechanism on the relative response of analytes was first discussed in the framework of the ion evaporation model by Tang and Kebarle.[4-6] The response

factors are supposed to be proportional to the evaporation rates of the analytes, which depend on the activation free energy for an ion-solvent cluster that leaves a small charged droplet. In 1997, Enke[7,8] proposed the equilibrium partitioning model to account for the concentration dependence of analyte response. The equilibrium partitioning model states that, whatever the exact ion production mechanism is, the molecules that are detected as ions are those that are present at the surface of the droplet. At low concentration, all ions can freely access the surface, which is not saturated. At high concentration, the surface is saturated and the different analytes are in competition for accessing the surface. The response factors will therefore highly depend on the surface activities of the ions. In the current state of the art, it is difficult to quantitatively predict response factors. Qualitatively, a nonpolar analyte is more easily expelled from the solvent droplet than a polar one, and the former has a higher response than the latter.[9] For biomolecules, the electrospray response is very likely to be influenced by the charge state (due to the solvation) and the conformation (due to a different accessibility of polar residues).[6]

For the complexation reaction (2) between substrate A and ligand B, different methods have been applied to determine the equilibrium association constant K_a (3) or the dissociation constant K_d by electrospray mass spectrometry. These methods have been reviewed recently.[10]

$$A + B \Leftrightarrow AB \qquad (2)$$

$$K_a = \frac{1}{K_d} = [AB]/[A][B] \qquad (3)$$

The association constant may be obtained indirectly by determining the concentration of unbound ligand B during a titration experiment.[11,12] The method requires a calibration of $I(\mathrm{B}) = f([\mathrm{B}])$ and the data are treated by the Scatchard method.[13] No assumption is made on the response factors.

Another method for determining K_a or K_d is to measure the relative intensities of the peaks corresponding to the complex AB and to the free substrate A, and to calculate the ratio of the concentrations using equation (4), assuming that the response factors of A and AB are identical.

$$\frac{I(AB)}{I(A)} = \frac{[AB]}{[A]} \qquad (4)$$

The equilibrium constants can be determined with a single mass spectrum[14] or by fitting data obtained by a titration experiment.[15-18] This methodology may be applied to the case of complexes with multiple stoichiometries[18] or when different ligands are in competition for binding to a given target.[14,15] The assumption that the response factors of the complex and the free substrate are the same has been validated by comparison with independent solution-phase data in the case of vancomycin-peptide complexes. This has been attributed to the fact that the peptide is imbedded in the complex and that vancomycin does not change conformation.[14] It has also been shown that source-CID (collision-induced dissociation) of the complex induces an underestimation of the association constant.[14]

A method for determining the ratio between the response factors of the complex and the free substrate is to perform two independent measurements in which the equilibrium is shifted

completely to the left, then to the right by using an appropriate medium. In one study of the dimer $\Leftrightarrow$ hexamer equilibrium of citrate synthase,[19] a correction factor of 0.77 was determined, and the equilibrium association constant in the solution of interest could be subsequently determined. Such a procedure to determine the ratio of the response factors is rigorous, but is not of general applicability.

The determination of binding selectivities by measuring the ratio of two complexes when two ligands are in competition (equimolar mixture) for a given substrate[15,20-31] is also based on a similar approximation: the response factors of the two complexes are supposed to be the same. In the case of a competition experiment between ligands B and C for the substrate A, it is therefore assumed that:

$$\frac{I(AB)}{I(AC)} = \frac{[AB]}{[AC]} \qquad (5)$$

The method has been found to work well for some biological systems (e.g., carbonic anhydrase-inhibitor complexes[20]), but not for others (e.g., protein-CoA ligand binding[21]). The method has also been extensively applied to the study of cation-crown ether complexes.[22-30] Approximation (5) has been tested by several groups by comparison of the relative intensities observed in the mass spectra with theoretical intensities (either determined experimentally by shifting the equilibrium to the right by adding an excess of reactant,[19,22] or calculated from known association constants[23-26]). Approximation (5) was proven to be valid when comparing complexes of the same host with different guest cations, but not in the opposite situation.[23-26]

Another method avoiding any approximation on the response factors has been recently proposed by Kempen and Brodbelt.[32] It consists of monitoring the intensity of a reference complex before and after the addition of a competing host or guest. The calibration curve of the intensity of the reference complex requires prior knowledge of the K_a association constant of the complex. The calibration curve, which is made in a solution containing the reference complex alone, is assumed to be valid in the mixture solution of the competition experiment as well.

The present paper describes a method where the ratio between the response factors of the complex and of the substrate, and the equilibrium association constants with a correction for response effects are determined. The method is solely based on mass spectrometry. It involves no comparison with data obtained by independent methods. We will illustrate the method with two examples: drug-oligodeoxynucleotide complexes and cyclodextrin complexes with α,ω-alkanedicarboxylic acids. Drug-DNA and drug-RNA complexes have been studied intensively by our group[33-35] and others.[15,18,36-39] All reports on estimation of binding constants assume that the response of the complex is equal to the response of the free DNA or RNA oligonucleotide. As the agreement with independent solution-phase methods is good,[34] this approximation seems to be valid, and the results described herein confirm this view. The case of cyclodextrin complexes with α,ω-alkanedicarboxylic acids is more complicated. We reported recently that the signal of the 1:1 complex was due partially to non-specific electrostatic adducts,[40] and that the relative intensities did not reflect the solution-phase behavior. As described below, the response factors will provide information on whether the alkyl chain is included or not in the cyclodextrin cavity.

Experimental section

Materials

Single stranded oligodeoxyribonucleotides d(GGGGAATTGGGG) (M = 3863.53 Da), d(CCCCAATTCCCC) (M = 3486.34 Da), d(GGGGAAAAGGGG) (M = 3824.56 Da), d(CCCCTTTTCCCC) (M = 3468.31 Da) and d(CGCGAATTCGCG) (M = 3646.44 Da) were purchased from Eurogentec (Angleur, Belgium). Complementary (or self-complementary) strands are heated to 85 °C in 100 mM NH_4OAc (pH = 7.2), and then cooled slowly (overnight) to form the duplexes. The extinction coefficient of each duplex was determined by monitoring its denaturation curve by absorption spectroscopy at 260 nm. The extinction coefficients of the single strands are given by Eurogentec (calculated from tables), and the total concentration of strands is calculated from the absorbance at the high temperature limit by Beer's law. The extinction coefficient of the duplex is determined from the absorbance at the low temperature limit. Duplex concentrations were measured immediately before use. The drugs Hoechst 33342 (Fluka, Bornem, Belgium) and berenil (kindly donated by C. Bailly, INSERM, Lille, France) were dissolved in water, then diluted in 100 mM NH_4OAc, and their concentrations were determined just before use by absorption spectroscopy using the following extinction coefficients: $\varepsilon_{\lambda = 348\ nm}$(Hoechst 33342) = 42,000 $M^{-1}cm^{-1}$ and $\varepsilon_{\lambda = 370\ nm}$(berenil) = 34,400 $M^{-1}cm^{-1}$.

The stock solution of α-cyclodextrin (α-CD) was prepared in doubly distilled water at a concentration of 5.0×10^{-3} M. All stock solutions of α,ω-alkanedicarboxylic acids $^{-}OOC\text{-}(CH_2)_n\text{-}COO^{-}$ (5.0×10^{-3} M) were prepared in NH_4OH (pH = 9). All chemicals were purchased from

Aldrich and used without purification. Concentrations were determined by weighing. The equimolar stock solutions (2.0×10^{-3} M) of complexes were prepared by mixing equimolar amounts of α-cyclodextrin and dicarboxylic acid. This stock solution was diluted to different concentrations C_0 with NH_4OH (pH = 9).

Mass Spectrometry

The ES-MS spectra of oligonucleotide complexes were recorded with an LCQ Electrospray-quadrupole ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) operated in the negative ion mode (needle voltage = -3.9 kV). The capillary was heated to 180 °C and the applied potential was –10 V. The skimmer was at ground potential. The tube lens offset was maintained at 40 V. The 80/20 (100 mM aqueous NH_4Ac/methanol) solution was infused at 5 μL/min. Full scan mass spectra were recorded in the range *m/z* [1000-2000].

The ES-MS spectra of α-cyclodextin complexes were recorded with a Q-TOF2 mass spectrometer (Micromass, Manchester, UK) equipped with a Z-spray source. The samples (aqueous solutions at pH = 9) were infused at a flow rate of 5 μL/min. Electrospray ionization was achieved in the negative ion mode by application of -2.5 kV on the needle. The source block temperature was maintained at 80 °C and the desolvation gas was heated to 100 °C. Three different cone voltage values (10, 20 and 30 V) were used. The MS profile determining the transmission of the first quadrupole was defined to achieve maximum transmission at 250 *m/z*, and kept constant for all measurements.

Fluorescence Spectroscopy

Fluorescence titration of the oligonucleotide duplex by the Hoechst 33342 was performed on an SLM-AMINCO 8100 spectrofluorometer (Spectronic Unicam, Cambridge, UK) at 20 °C in an aqueous solution of 100 mM NH_4OAc (pH = 7.0). The concentration of duplex oligonucleotide was 8.1×10^{-9} M. Since melting temperature measurements in 100 mM NH_4OAc extrapolated to a concentration of 8.1 nM give a melting temperature of 35 °C, the duplex can reasonably be assumed to be quantitatively formed at room temperature in the solution used for fluorescence titration experiments. The ligand concentration was varied from 0 to 1.1×10^{-6} M. The solution was excited at 354 nm, and fluorescence emission was measured at 485 nm after mixing for 4 minutes to allow for equilibration between each titrant addition. Each point is the mean value of four data collections. Background fluorescence intensity from the oligonucleotide solution before the addition of drug was subtracted from each point. Fluorescence titration data were fitted by a single site model[41].

Results and Discussion

On the basic assumption

We define a factor R (equation 6) as the ratio between the response factors of the complex AB and of the free substrate A.

$$\frac{I(AB)}{I(A)} = \frac{R_{AB}}{R_A} \cdot \frac{[AB]}{[A]} = R \cdot \frac{[AB]}{[A]} \qquad (6)$$

R = 1 represents the ideal case where the response factors are the same for A and AB. In that case, equation (4) is obtained, and the equilibrium binding constant may be immediately calculated from the relative intensities of A and AB in the mass spectrum. However, if R is larger than unity (i.e., the response of the complex AB is higher than the response of the substrate A), K_a is overestimated if approximation (4) is used. Conversely, if the actual R is lower than unity, this leads to an underestimation of K_a.

Equation (6) implies that the ratio between the response factors is constant over the whole concentration range. This is also an assumption. The electrospray response can indeed deviate from linearity when working with concentrations varying over a very wide range.[7] Nevertheless, this approximation is better than simply considering that R = 1.

Fitting Procedure

Introducing equation (6) into the expression of K_a (3) gives:

$$K_a = \frac{1}{R} \cdot \frac{I(AB)}{I(A)} \cdot \frac{1}{[B]} \qquad (7)$$

If the solution is equimolar in A and B ($[A]_0 = [B]_0 = C_0$), and if we define α as the fraction of bound substrate (equation 8)

$$\alpha = \frac{[AB]}{[AB]+[A]} = \frac{1}{1+\frac{[A]}{[AB]}} \qquad (8)$$

and $C_0 = [A] + [AB] = [B] + [AB]$, we have

$$[A] = [B] = C_0(1-\alpha) \qquad (9)$$

and $[AB] = C_0\alpha$. Successive insertion of equations (9), (8) and (6) in equation (7) gives:

$$K_a = \frac{1}{R} \cdot \frac{I(AB)}{I(A)} \cdot \frac{1}{C_0 \cdot (1-\alpha)} \qquad (10a)$$

$$K_a = \frac{1}{R} \cdot \frac{I(AB)}{I(A)} \cdot \frac{1}{C_0 \cdot \left(1 - \frac{1}{1 + \frac{[A]}{[AB]}}\right)} \qquad (10b)$$

$$K_a = \frac{1}{R} \cdot \frac{I(AB)}{I(A)} \cdot \frac{1}{C_0 \cdot \left(1 - \frac{1}{1 + R \cdot \frac{I(A)}{I(AB)}}\right)} \qquad (10c)$$

This expression can be rearranged to give equation (10):

$$K_a = \frac{1 + R \cdot \frac{I(A)}{I(AB)}}{\left(R \frac{I(A)}{I(AB)}\right)^2 . C_0} \qquad (10)$$

The ratio of the intensities I(A)/I(AB) is measured as a function of C_0 (the concentration of the equimolar solution). Equation (11) relating these two quantities may be found by taking the positive root of equation (10). The experimental results are fitted by a non-linear least squares procedure, introducing equation (11) into SigmaPlot 4.0. The two variable parameters are K_a and R.

$$\frac{I(A)}{I(AB)} = \frac{1 + \left(1 + 4K_a C_0\right)^{1/2}}{2RK_a C_0} \qquad (11)$$

Robustness of the method

Simulations with theoretical points were made to test the robustness of the equation and the influence of experimental errors on the determination of the constant by the method described above. Figure 1 shows plots of equation (11) obtained with three different pairs of parameters (K_a,R). The curvature shows little sensitivity to the balance of K_a and R, and does not change dramatically, although K_a can change by over nearly 2 orders of magnitude. This indicates that large errors in I(A)/I(AB) will result in large errors in the determination of K_a and R by fitting.

The major source of error is however not the experimental error on the determination of C_0 and I(A)/I(AB) themselves, but rather stems from the fact that the solution may not be strictly equimolar. This is illustrated in Figure 2. Fictive experimental points were generated by calculating I(A)/I(AB) for 12 values of C_0 in the range $2.0 \times 10^{-6} - 6.0 \times 10^{-5}$ M, with fixed values of K_a and R ($K_a = 10^6$ M^{-1}, $R = 0.2$). The following datasets were generated by calculating the relative intensities as a function of the concentration in three cases: (i) truly equimolar solution, (ii) solution with an excess of A and (iii) solution with a deficit of A. The datasets were generated manually (except for equation 11, as this equation is valid only for equimolar mixtures). For the equimolar solution (Fig. 2, circles), the fitting of the datapoints with equation (11) gives the correct values of K_a and R. However, when A is in excess compared to B (squares), the intensity ratio I(A)/I(AB) does not sufficiently decrease at high concentration; the curve obtained by fitting with equation (11) goes below the datapoints at high concentration and this results in an overestimation of K_a (and an underestimation of R). The opposite happens when A is in deficit compared to B. The whole procedure was repeated for $R = 1$ and $R = 5$ (data not

shown), and we observed that the larger *R*, the smaller the effect of the non-equimolarity of the solution.

The problem of determining the concentration is common to all methods which give access to equilibrium association constants, but it is clear that the present method is very sensitive to errors in the concentrations of both association partners. It has been shown above that the solution has to be strictly equimolar in A and B to avoid systematic errors in K_a. The advantage of the method is that this kind of systematic error can be detected and evaluated by a careful examination of the results of the fitting procedure. If the fitting curve goes systematically below the experimental points at high concentration, this indicates that A is in excess, and that the value of K_a obtained by fitting is an upper bound to the real K_a. If, however, the curve goes systematically above the experimental points at high concentration, A is in deficit, and the constant K_a obtained by fitting is a lower bound to the real K_a. When applying the above-described fitting procedure, it is therefore recommended to work in a concentration range broad enough (at least one order of magnitude) to detect whether equation (11) is applicable or not. Note that a kind of system for which the equimolarity problem is avoided is for dimerization reactions. We present below the results obtained for two ligand-substrate systems for which the concentrations of the partners could be determined accurately.

DNA-minor groove binder complexes

It has often been assumed in ES-MS that complexes with small drugs that do not disturb the conformation of the host will have the same electrospray response as the host itself.[18] Minor groove binders interact with the floor of the minor groove of DNA through Van der Waals, hydrogen bonding and electrostatic interactions at AT-rich sites, with no dramatic change in the conformation of the DNA double helix.[42-44] We therefore tested this kind of complex with 12-base pair oligonucleotide duplexes to verify the hypothesis according to which R should be close to 1. Figure 3 shows the structure of the drugs and of a 1:1 complex with an oligonucleotide duplex.

Complexes with berenil. Figure 4 shows spectra of equimolar mixtures of the duplex d(GGGGAATTGGGG)•d(CCCCAATTCCCC) and berenil. A small amount of 2:1 complex is detected at high concentration. The contribution of other stoichiometries cannot be taken into account with the present equations, so it must be assumed that the contribution of minor species of other stoichiometries is not significant enough to perturb the determination of K_a. Only the 5-charge state was considered for the determination of the intensity ratio. Fitting of the results obtained by varying the concentration C_0 from 2.0×10^{-6} to 2.0×10^{-5} M gives an association constant $K_a = (2.3 \pm 0.7) \times 10^5$ M^{-1} and a factor $R = 0.8 \pm 0.2$. With a similar duplex having a central sequence AAAA•TTTT, the fitting gives $K_a = (9 \pm 8) \times 10^4$ M^{-1} and $R = 1.6 \pm 1.0$.

Complexes with Hoechst 33342 and comparison with fluorescence. In order to make comparisons with constants measured in solution by an independent method, we have chosen the

drug Hoechst 33342 which, unlike berenil, makes a fluorescent complex with DNA. The complex with the dodecamer $d(CGCGAATTCGCG)_2$ has been studied. Full scan ES-MS spectra were recorded at different concentrations for equimolar drug/duplex mixtures in 80/20 (v:v) 100 mM NH_4OAc/methanol. 20% methanol does not dramatically change the dielectric constant of the medium, and it has been checked by circular dichroism (data not shown) that the B-form of the double helical conformation was preserved. The fitting procedure was applied (Figure 5), considering the 5- charge state. The results obtained by fitting are $K_a = (1.9 \pm 0.4) \times 10^8$ M^{-1}, and $R = 0.5 \pm 0.2$. Considering $R = 1$ (equation 4) would lead to an association constant of $K_a = (5.9 \pm 0.6) \times 10^7$ M^{-1}. No data exist in the literature on association constants determined in NH_4OAc electrolyte, either for the present complex or for related drug-DNA complexes. To determine the association constant by an independent method in a solution with a composition close to the one used in ES-MS, we performed fluorescence titration in 100 mM NH_4OAc. The experiment could not be conducted in the presence of methanol due to quenching effects. The fluorescence titration experiment had to be conducted with a starting concentration of duplex of 8.1 nM to ensure that the equations used are applicable[32]. The association constant measured by fluorescence titration experiments is $K_a = (1.4 \pm 0.2) \times 10^8$ M^{-1}. Despite all the approximations made, this value is close to that determined by the fitting method with correction for the response factors. Assuming that $R = 1$ leads however to an underestimation of the constant by a factor of about 2.

***Meaning of* R.** The relative response factors of two species can be altered by four different effects: (i) discrimination in the ion emission efficiency during the electrospray process, (ii) discrimination due to the transmission of the mass analyzer, (iii) discrimination due to the detector efficiency and (iv), in the case of noncovalent complexes, the collisional activation in the

source of the spectrometer, which has to be minimized so that the complex does not dissociate before mass analysis. The effect of the source conditions on the breaking of duplex DNA and complexes has previously been studied[45] on the quadrupole ion trap mass spectrometer. The experimental conditions chosen here are soft enough to avoid breaking of the complex due to source-CID. The mass range used also prevents ion discrimination during trapping: the instrument automatically selects the RF amplitude that is necessary to trap all ions of the chosen mass range. As the $[duplex]^{5-}$ and the $[1:1]^{5-}$ complex have close values of m/z compared to the mass range, both of their trapping efficiencies can be assumed to be optimum. The detector efficiency depends merely on the charge of the incoming ions, and is supposed to be the same for species of the same charge (5-). There is therefore no other discrimination on the intensities of the duplex and the complex than the different electrospray response factors, and R can be simply interpreted as the ratio between the electrospray response of the complex and that of the duplex.

Influence of the structure of the complex on the response factor. The equilibrium partitioning model states that the response of an analyte is proportional to its affinity for the surface of the droplet.[7] The surface affinity can vary with the size of the molecule, its conformation, its charge, and mostly with the distribution of polar/nonpolar groups on the surface. The difference in electrospray response factors for the duplex and the complex may indicate a conformational change in the double helix upon drug binding. Indeed, crystallographic[46] and molecular modelling studies[47] on Hoechst analogs have shown that the structure of the dodecamer is slightly distorted in the complex compared to the free duplex, and the role of DNA plasticity in minor groove binding has been emphasized.[46] We found that the response of the complex and that of the duplex can differ by up to a factor of two in the case of Hoechst 33342. This implies that even a slight deformation of the molecule can change its electrospray response by a factor of two. The

sensitivity of the electrospray response on conformation is of a major interest: besides the determination of the equilibrium association constants, the present method could allow the detection of substrate conformational changes upon ligand binding; but this point needs further investigation.

Implications for the determination of the absolute values of the binding constants. We have seen that for Hoechst 33342 the response of the complex is about half that of the duplex. If it had been assumed that $R = 1$, this would have also led to an error on the constant by a factor of about two. In fact, such a difference between the actual value of the constant and that determined with assumption (4) is not dramatic, compared to differences that can arise from determination of constants in solution by different methods. For example, significant discrepancies (up to a factor of 40, despite using identical salt and buffer conditions) may be found between constants determined by fluorescence titration and titration calorimetry.[48] The origin of these discrepancies is that the equations used for fitting the titration curves are not valid at large substrate concentrations[32,39]. In titration calorimetry, the concentrations of substrates (micromolar range) have to be higher than in fluorescence titration experiments (nanomolar range)[39] due to sensitivity reasons. In our mass spectrometric experiments, we use concentrations in the micromolar range, but the equation used to determine the association constants is completely different from that of the spectroscopic methods, and equation (11) is valid over the whole concentration range. Mass spectrometry can therefore give more reliable results than calorimetric titrations.

Implications for competition experiments. In the light of the present results, we can now comment on previously published competition experiments[33] between different drugs for a given oligonucleotide duplex, which were based on the assumption that all complexes had the same

response factors. We now see that this assumption may not be true, and that complexes with different drugs may have different response factors (e.g., R = 0.5 for Hoechst 33342 and R ≤ 1.6 for berenil). Nevertheless, the differences in *R* are not so large and competition experiments can still be very useful to make a quick estimation of the relative binding affinities of drugs. If the drugs are similar in shape and in binding mode, the complexes can reasonably be expected to have equal responses.

Charge states of free and bound DNA. At first sight it might seem strange that, in the negative ion mode, the complexes with positively charged drugs show a charge state distribution analogous to that of the free duplex. The most intense charge state for the free duplex is 5-, and the same holds for the complex, even though the drug has two permanent positive charges (Figure 3). This means that in the $[1:1]^{5-}$ complex, there are seven negative charges located on the phosphate groups of the DNA and two positive charges on the drug. Even so, the response factors of the duplex^{5-} and the $[1:1]^{5-}$ complex ions are very similar. This means that only the total charge influences the activation energy for taking the ion out of the droplet. Although the maximum charge states are conditioned by the total number of basic or acidic sites in the positive or negative ion mode respectively, the observed charge state distribution merely depends on the size and shape of the molecule. This is in agreement with the model proposed by J.B. Fenn[49]: "…the spacing of the charges on a desorbed ion must relate to the spacing of the charges on the surface of the droplet…".

Cyclodextrin-diacid complex

Cyclodextrins (CDs) are torus-like macro-rings built up from glucopyranose units. α-CD has six glucopyranose units. We chose to investigate the complexes between α-CD and α,ω-alkanedicarboxylic acids $^{-}OOC–(CH_2)_n–COO^{-}$. In solution, the equilibrium binding constant of α-CD with aliphatic molecules increases with the chain length. This is attributed to the hydrophobic effect[50,51]: in solution, the aliphatic chain is buried into the cavity of the cyclodextrin, either by bending of the chain in a U-motif for inclusion in the cavity with the carboxylate groups on the exterior, or by threading of the guest molecule into the α-CD. In a separate paper,[40] we reported that the relative intensity of the complex compared to the free cyclodextrin did not reflect the trend of the solution equilibrium binding constants. This was supposed to be due to a significant contribution of non-specific electrostatic adducts to the total signal intensity. Here we report the determination of the relative response factors for these complexes compared to the free cyclodextrin.

α-cyclodextrin + α,ω-heptanedicarboxylic acid (n = 7). This diacid will be discussed in detail to illustrate the use of the method in a more complicated case. Figure 6 shows a spectrum of an equimolar mixture (C_0 = 4.0 × 10^{-4} M) of α-cyclodextrin and the diacid. The mass spectrum shows the singly and doubly deprotonated cyclodextrin (the substrate A), the singly charged acid (the ligand B), the doubly charged 1:1 complex (AB), and also a less abundant doubly charged A_2B complex. At the pH used, the diacid is doubly deprotonated in solution. Note that all complexes are doubly charged, although only the singly charged diacid is detected in the

spectrum. The doubly charged acid may not be transmitted by the quadrupole with the MS profile used.[40]

A priori, the response factors of the cyclodextrin and the complex cannot be predicted, but are very likely to differ for three reasons. First, cyclodextrin is neutral in solution, and loses one or two protons during the electrospray process to produce the CD^{1-} and CD^{2-} species. The complex, however, is "pre-charged" by the dianion that is complexed, and indeed appears only as a doubly charged species. Besides, little is known about the conformation of the complex (inclusion or not?).[40] Finally, as will be shown below, collision-induced dissociation (CID) can occur in the electrospray source at the cone voltages used. The factor *R* therefore needs to be determined experimentally.

The fitting procedure was applied considering the cyclodextrin as A and the 1:1 complex as AB. As for the drug-oligonucleotide system, the 2:1 complex A_2B was not taken into account. For the systems studied here, these were only minor species, and a good fitting is obtained with this assumption. Figure 7 A-C shows three attempts of fitting considering the different charge states of α-cyclodextrin, at a cone voltage of 10 V. It can be seen from Figures 7A and 7B that considering only one charge state of the cyclodextrin at a time is not a successful approach. The quality of the fit is poor, and the errors on K_a and *R* are larger than the values themselves. However, when considering the sum of the intensities corresponding to the two charge states of cyclodextrin $I(A) = I(CD^{1-}) + I(CD^{2-})$, the fit is of high quality and the errors on K_a and *R* are reasonable. Considering the charge states in an isolated manner causes problems for this system because the charge state distribution changes with the concentration. Figure 7D shows that the

proportion of CD^{1-} compared to CD^{2-} increases when C_0 increases. This is probably due to the increase in the acid concentration in the solution, which is not buffered. In Figure 7A, the experimental values of $I(CD^{2-})/I(\text{complex})$ do not increase steeply enough with the concentration. This results in an overestimation of the association constant. Conversely, considering CD^{1-} alone leads to an underestimation of the association constant.

The association constant obtained by fitting in Figure 7C at a cone voltage of 10 V is $K_a = (2.6 \pm 0.6) \times 10^3\ M^{-1}$, and the factor $R = 6.6 \pm 1.0$. As R is larger than 1, the response of the complex is larger than the response of the cyclodextrin. As the transmission of the quadrupole cannot be responsible for such a discrimination in the considered mass range, this must be due to a difference in electrospray response.

Two reasons can account for the larger response of the complex compared to the free cyclodextrin. First, as mentioned before, the complex has two negative charges in solution at basic pH. Cyclodextrin is neutral and has to exchange protons to get charged, so that negative ions of cyclodextrin can be more difficult to produce than negatively charged complex. Second, previous results[40] indicated that, in addition to the specific inclusion complex, a significant proportion of the signal detected in ESI-MS could be due to nonspecific electrostatic aggregation between the cyclodextrin and the charged diacid. If a significant proportion of the complex has a non-inclusion geometry, the aliphatic chain could provide the complex with a hydrophobic moiety that would enhance its response dramatically.

The effect of the cone voltage on the determination of the constants has also been investigated. The fitting procedure was applied to the same system at a cone voltage of 20 V. The relative intensity of the complex is slightly smaller than at 10 V (data not shown). Fitting of the results at 20 V gives an association constant $K_a = (4.2 \pm 1.4) \times 10^3\ M^{-1}$, consistent with the value obtained at 10 V within experimental error. The factor R has however decreased to a value of $R = 4.0 \pm 0.8$, a significantly lower value than at 10 V. The response of the complex at 20 V is lower than at 10 V due to its partial dissociation. At 30 V, the abundance of the complex is largely reduced, but no good fitting of the results could be obtained. This illustrates that the above-described fitting method is also capable of correcting for moderate in-source CID of the complex.

Influence of the diacid chain length. The procedure that takes into account the sum of the charge states for the free cyclodextrin has been applied to diacids of different chain lengths. Results are summarized in Table 1. K_a and R values were obtained only for diacids with n = 6, 7 and 8. For the other diacids, the solutions were obviously not equimolar, and this resulted in too large errors. For all diacids, the factor R is much larger than 1. The complexes always respond better than the cyclodextrin. As previously mentioned, this can be due to the fact that the complex bears negative charges in the solution, while the cyclodextrin is neutral. The factor R can also give indications on the conformation of the complex when it escaped the electrosprayed droplet. If the diacid was included in the cavity, the hydrophobic chain would be hidden from the solvent and the responses of all the complexes would be similar. The fact that R increases with the diacid chain length therefore suggests that a significant proportion of the complex has a non-inclusion geometry. The MS-determined equilibrium association constants do not agree with those determined from calorimetry. This is due to the electrospray process itself, as described

previously:[40] the major contribution to the signal of the $[1:1]^{2-}$ complex comes from non-specific, non-inclusion complexes. The fitting method could therefore not be expected to improve the determination of the constant in such case. Rather, the advantage of the method lies in the study of the complex conformation through the value of *R*.

Conclusion

In summary, we reported the equations and methodology for simultaneous determination of the equilibrium association constant (K_a) of noncovalent complexes and the ratio between the response factors of the complex and that of the free substrate (R). The method requires neither the prior knowledge of any association constant, nor any calibration, nor the complete displacement of the equilibrium. The practical limitation is that the concentrations of the reactants has to be determined precisely, but a careful examination of the results allows the detection and evaluation of such an error source. The method is also currently limited to 1:1 complexes (otherwise a different R factor would be needed for each complex of the mixture).

The possibility to determine R experimentally by ES-MS (not by comparison of the MS-determined constants with other methods) can be a very useful tool for fundamental studies of noncovalent interactions. The factor R corrects for any discrimination such as differences in spectrometer transmission, in detection efficiency, different electrospray responses and moderate in-source CID of the complex. When the other discrimination factors are minimized by a careful selection of the experimental conditions, the factor R can reflect the different electrospray response factors of the complex and the substrate. As shown for a drug-oligonucleotide complex, the difference in the electrospray response of the free oligonucleotide duplex and the complex might provide some insight into the effect of complexation on the conformation of the substrate. For cyclodextrin complexes, R indicates whether or not the ligand is included in the cyclodextrin cavity.

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Table

Table 1. Binding constants for complexes of diacids $^{-}OOC–(CH_2)_n–COO^{-}$ and α-cyclodextrin determined by calorimetry and mass spectrometry. The mass spectrometric results are given for a cone voltage of 10 V.

n	K_a Calorimetry [a] (M^{-1})	K_a MS (M^{-1}) [b] (assuming R = 1)	MS (fitting method) K_a (M^{-1})	R
4	no complex	36,000 ± 6,000 [c]		
6	93 ± 1	53,000 ± 5,000	3300 ± 1000	5.0 ± 0.9
7	630 ± 20	67,000 ± 9,000	2600 ± 600	6.6 ± 1.0
8	1790 ± 80	81,000 ± 9,000	2000 ± 400	6.7 ± 0.9
10		100,000 ± 40,000 [d]		
12		91,000 ± 45,000 [d]		

(a) Data from reference [51].

(b) Mean value over the concentration range [$4.0 \times 10^{-4} – 2.0 \times 10^{-3}$ M].

(c) Concentration dependence indicates a too low diacid concentration , and hence an underestimation of the constant.

(d) Concentration dependence indicates a too high diacid concentration , and hence an overestimation of the constant.

Figure legends

Figure 1

Plots of equation (11) with three different pairs of (K_a,R) that almost superimpose in the chosen concentration range.

Figure 2

Results of the fitting procedure for an equimolar mixture ($[A]_0 = [B]_0 = C_0$) (circles), and for a mixture containing an excess of A ($[A]_0 = C_0$ and $[B]_0 = 0.9 \times C_0$) (squares) or a deficit of A ($[A]_0 = C_0$ and $[B]_0 = 1.1 \times C_0$) (triangles). Twelve datapoints were generated for each case, considering $K_a = 10^6$ M^{-1}, $R = 0.2$. These datapoints are then fitted with equation (11) and values obtained for K_a and R are reported in the inset together with their standard errors.

Figure 3

Structures and masses of the drugs Hoechst 33258 and berenil (right), and structure of a 1:1 complex between an oligonucleotide 12-base pair duplex and a minor groove binder.

Figure 4

ESI-MS spectra of equimolar mixtures of berenil and duplex d(GGGGAATTGGGG)•d(CCCCAATTCCCC) at (a) $C_0 = 2.0 \times 10^{-6}$ M and (b) $C_0 = 1.0 \times 10^{-5}$ M.

Figure 5

Determination of K_a and R for the complex between Hoechst 33342 and $d(CGCGAATTCGCG)_2$. Fitting of the experimental results with equation (11), considering I(A) = $I(duplex^{5-})$ and I(AB) = $I(1:1^{5-})$.

Figure 6

ESI mass spectrum obtained for an equimolar mixture (4×10^{-4} M) of α-cyclodextrin (CD) and α,ω-heptanedicarboxylic acid. The cone voltage was set to 10 V.

Figure 7

A-C: Determination of K_a and R for the complex between α-cyclodextrin (CD) and α,ω-heptanedicarboxylic acid considering (**A**) I(A) = $I(CD^{1-})$, (**B**) I(A) = $I(CD^{2-})$ and (**C**) I(A) = $I(CD^{1-})+I(CD^{2-})$. Fittings were made with equation (11). **D**: Evolution of the relative intensity of the two charge states of α-cyclodextrin (CD) as a function of the concentration of the equimolar (CD:acid) mixture.

Figure 1.

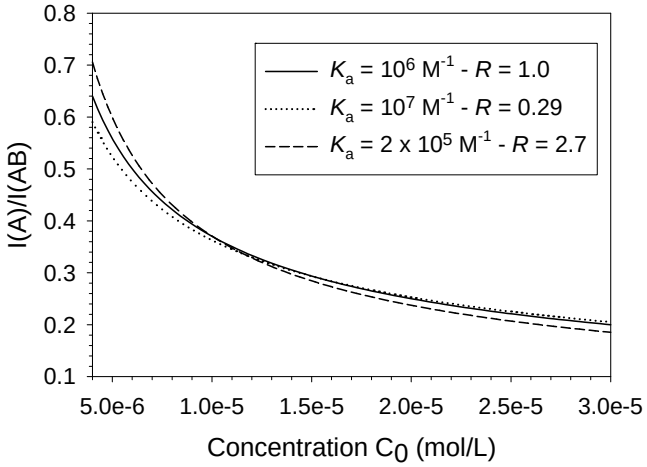

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
I(A)/I(AB)
5.0e-6
1.0e-5
1.5e-5
2.0e-5
2.5e-5
3.0e-5
Concentration C0 (mol/L)
Ka = 10^6 M^-1 - R = 1.0
Ka = 10^7 M^-1 - R = 0.29
Ka = 2 x 10^5 M^-1 - R = 2.7

Figure 2.

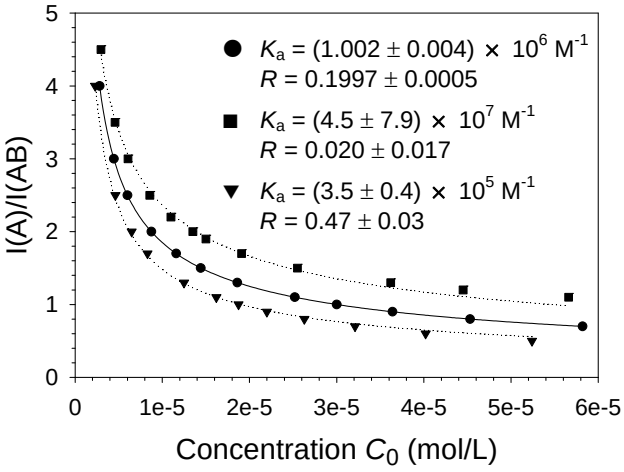

K_a = (1.002 ± 0.004) × 10^6 M^{-1}
R = 0.1997 ± 0.0005
K_a = (4.5 ± 7.9) × 10^7 M^{-1}
R = 0.020 ± 0.017
K_a = (3.5 ± 0.4) × 10^5 M^{-1}
R = 0.47 ± 0.03
I(A)/I(AB)
0
1
2
3
4
5
0
1e-5
2e-5
3e-5
4e-5
5e-5
6e-5
Concentration C_0 (mol/L)

Figure 3

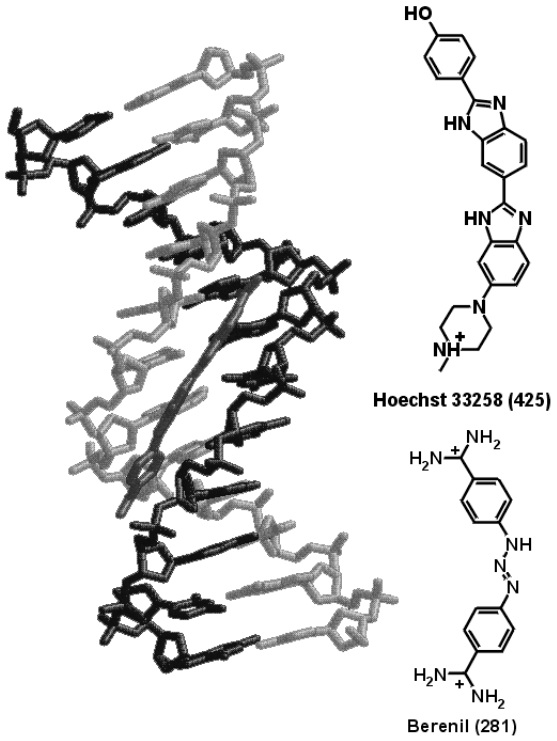

HO
HN
N
HN
N
N
NH
Hoechst 33258 (425)
NH2
H2N
NH
N
N
H2N
NH2
Berenil (281)

Figure 4

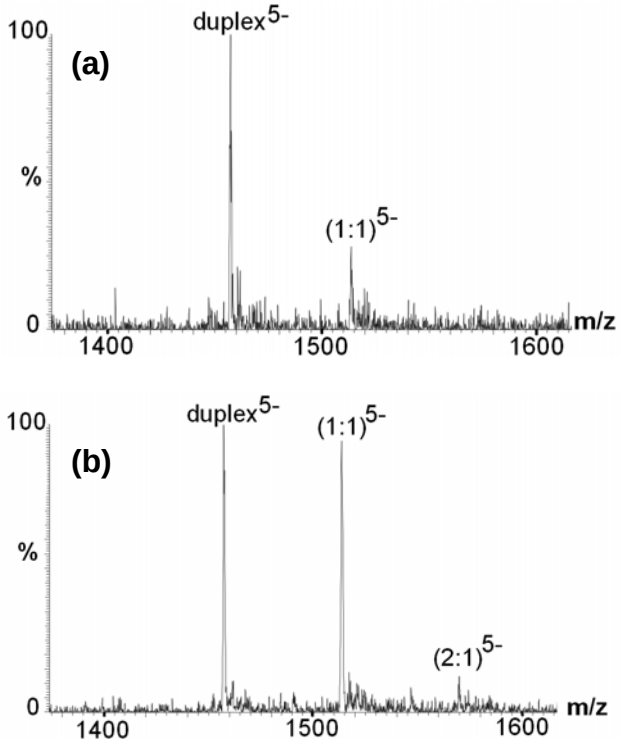

100
(a)
duplex5-
%
(1:1)5-
0
m/z
1400
1500
1600
100
(b)
duplex5-
(1:1)5-
%
(2:1)5-
0
m/z
1400
1500
1600

Figure 5

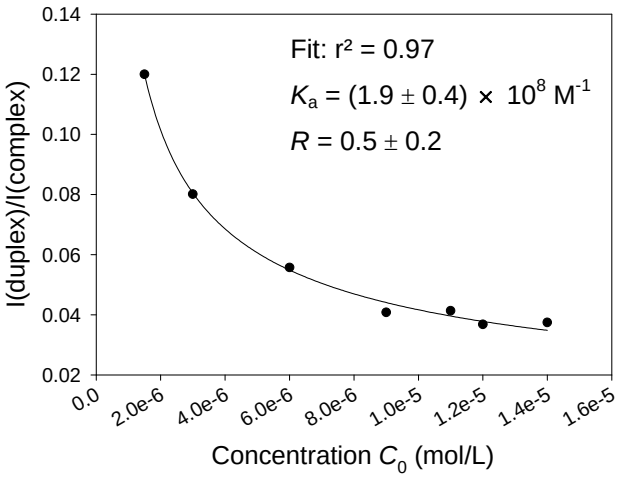

Fit: r² = 0.97
K_a = (1.9 ± 0.4) × 10^8 M^{-1}
R = 0.5 ± 0.2
I(duplex)/I(complex)
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.0
2.0e-6
4.0e-6
6.0e-6
8.0e-6
1.0e-5
1.2e-5
1.4e-5
1.6e-5
Concentration C_0 (mol/L)

Figure 6

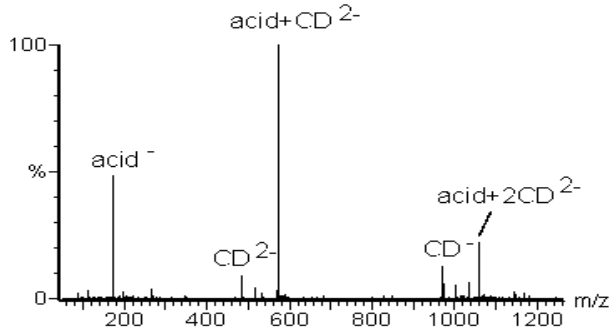
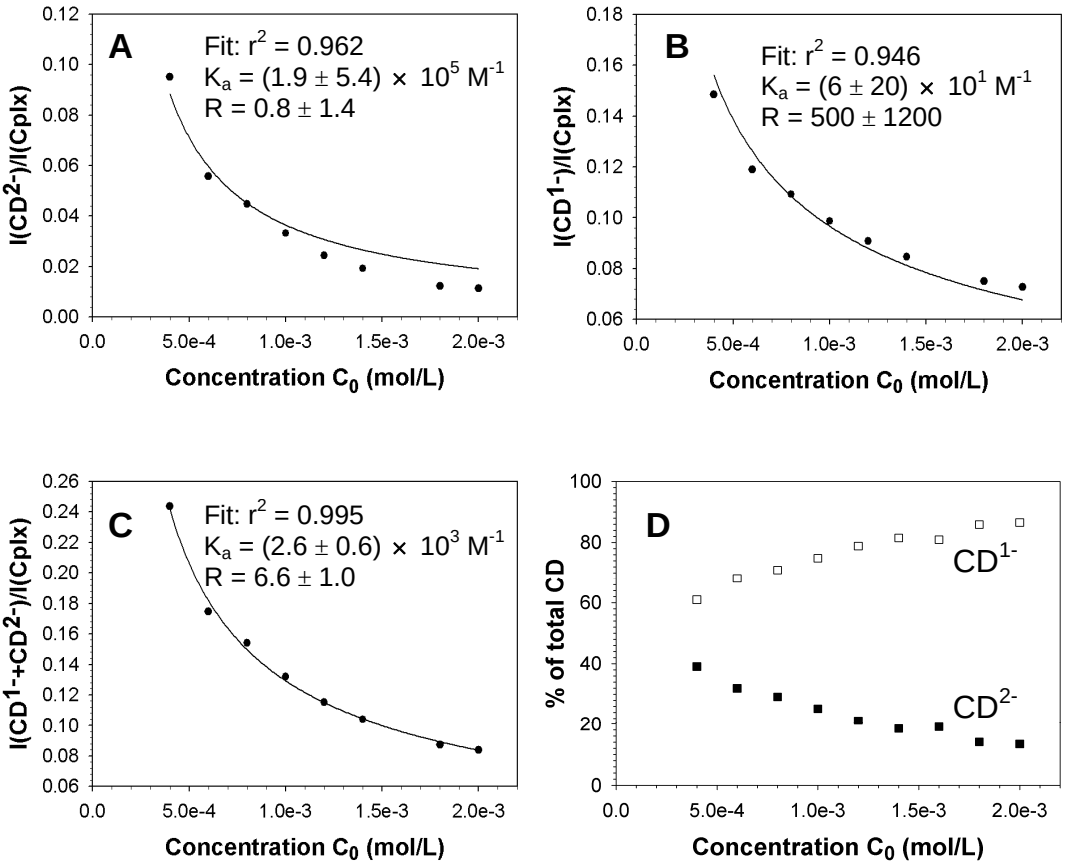

acid+CD 2-
100
%
acid -
acid+2CD 2-
CD 2-
CD -
0
m/z
200
400
600
800
1000
1200

Figure 7


A
Fit: r2 = 0.962
Ka = (1.9 ± 5.4) × 10^5 M^-1
R = 0.8 ± 1.4
I(CD2-)/I(Cplx)
Concentration C0 (mol/L)
B
Fit: r2 = 0.946
Ka = (6 ± 20) × 10^1 M^-1
R = 500 ± 1200
I(CD1-)/I(Cplx)
Concentration C0 (mol/L)
C
Fit: r2 = 0.995
Ka = (2.6 ± 0.6) × 10^3 M^-1
R = 6.6 ± 1.0
I(CD1-+CD2-)/I(Cplx)
Concentration C0 (mol/L)
D
CD1-
CD2-
% of total CD
Concentration C0 (mol/L)