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Determination of Thermodynamic Binding Constants by Affinity Capillary Electrophoresis

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Abstract

A strategy to study thermodynamic binding constants by affinity capillary electrophoresis (ACE) is presented. In order to simplify mathematical treatment, analogy with acid-base dissociation equilibrium is proposed: instead of ligand concentration *[X]*, negative logarithm of ligand concentration (or activity), $pX = -log[X]$, is used. On this base, and taking into account ionic activities, a general procedure for obtaining thermodynamic binding constants is proposed. In addition, the method provides electrophoretic mobilities of the free analyte and analyte-ligand complex, even when binding constants are low and thus, the complexed analyte fraction is also low. This is useful as a base to rationally analyze a diversity of situations, *i.e.*, different mathematical dependencies are obtained when analytes and ligands with different charges are combined. Practical considerations are given for carrying out a full experimental design.

Enantiomeric ACE separation based on the use of chiral selectors is addressed. 2-hydroxypropyl-β-cyclodextrin was chosen as a model ligand, and both enantiomeric forms of four pharmaceutical drugs (propranolol, pindolol, oxprenolol and homatropine methylbromide) were considered as model analytes. Practical aspects are detailed and thermodynamic binding constants as well as free and complexed analytes mobilities are determined.

Keywords

THERMODYNAMIC BINDING CONSTANT; AFFINITY CAPILLARY ELECTROPHORESIS; CHIRAL SEPARATION; CYCLODEXTRIN

1. Introduction

Determination of equilibrium constants (association/dissociation) is a relevant subject in different disciplines. Its knowledge is useful for many application fields: agrochemical, food, pharmaceutical and cosmetic, physiology, medicine, biochemistry, biology and environmental, among others [1,2].

A wide variety of instrumental techniques have been employed for determining equilibrium constants. Basically, all methods consist of setting the initial concentration of some species involved in the equilibrium, then allowing it to proceed to finally, quantitatively determine a given property under the new condition reached using an instrumental technique. A *sine qua non* requirement is that the instrument must be sensitive enough to determine the concentrations generated in the new state. This constitutes the main limitation because of I) the species do not show optical absorption, do not have reduction/oxidation equilibrium response, do not have a net charge to obtain signals by capillary zone electrophoresis, do not present differential retention in chromatography or there is not a selective electrode to potentiometrically characterize them, etc.–, or II) it is not possible to set experimentally a suitable initial condition to generate the required quantity of species within the detection range of the instrument –*i.e.* low solubility of components, insufficient availability of reagents, etc.–[3,4].

Undoubtedly, acid-base dissociation is the most studied equilibrium, thus mathematical handling including exact or simplified calculations, are well known. Hence, it is straightforward to adopt analogous treatment for equilibrium constants of analyte-ligand

systems. Therefore, the equilibrium may be considered in the direction of $AX^{(i+j)}$ complex dissociation, handling $pX = -log\ a_X$ as the variable. The easy understanding promotes the conscious use of these equilibria in experiments, allowing progress toward more complex situations, for example, considering combinations of multiple equilibria or simultaneous equilibria involving mixtures of ligands and *pH*.

In early years, capillary electrophoresis (CE) has been positioned as a useful tool to study chemical equilibria. It was mostly used to determine acidity dissociation constants, in order to characterize molecules, or to optimize separations using *pH* as variable, and based on sound theoretical models [5–7].

Wren and Rowe [8] firstly focused on explaining enantioseparations by CE based in a mathematical model. However, their works were limited to modify chiral selector concentration to achieve successful separations. On the basis of that model other authors proposed a modification to include pH as separation parameter [9]; furthermore they started to employ it aiming to obtain differential association constants. Other papers also discussed the importance of understanding the solution viscosity to obtain valid results [10]. Penn et al. [11] extended previous treatments of enantioselective equilibria in order to develop a systematic and rational approach to optimize CE enantioseparations. Nevertheless, the equations were based in Wren and Rowe's approach, consequently also assumed that free enantiomers and the enantiomer-chiral selector complexes have the same actual mobility which is not necessarily right [12].

Since then, CE and specifically ACE, has become one of the most popular methods to determine constants not only for chiral-analyte systems but for other association equilibria, including 1:1 and 1:2 stoichiometries [13,14]. Those methods are reviewed in several publications [15–19]. Although additional corrections were taken into account (e.g. when the

ligand is an ionic specie), it should be noted that the binding constants measured under the conditions discussed are stoichiometric, not true thermodynamic binding constants, i.e. they are only valid for the particular experimental conditions in which they were determined.

Rigorous works must be based on thermodynamic equilibrium constants, and their determinations require considering the activities of individual forms. In ionic equilibria, the main source of deviation from ideal behavior lies in the interactions induced by charged species; therefore, the activity standard state corresponds to the behavior extrapolated from infinite dilution of the real concentration. The Debye-Hückel theory allows to estimate the activity coefficients of charged species, as a short-cut to calculate activities based on the concentration and ionic strength data.

In this work we propose to use ACE measurements in order to obtain an instrumental response proportional to the ratio associated/dissociated forms of a given compound with reference to a binding equilibrium between analyte and ligands. Data sets of the analytes effective mobilities at carefully selected ligand concentrations provide information of the analyte-ligand system which, in some cases, is not possible to be measured by other techniques.

2. Experimental

2.1. Instrumentation

All experiments were carried out on a Lumex Capel 105M CE system, equipped with UV detector (Lumex Ltd., St. Petersburg, Russia). pH measurements were performed with an Accumet Research AR25 potentiometer (Fischer Scientific, New Hampshire, USA) connected to a Schott Blueline 11-pH glass combination electrode (SI Analytics GmbH, Mainz, Germany).

2.2. Materials

A MilliQ® water purification system (Millipore, Bedford, MA, USA) was used to provide deionized water. Methyl and isopropyl alcohols were HPLC grade (Sintorgan, Buenos Aires, Argentina). Ethyl alcohol 99.5% was obtained from Cicarelli (San Lorenzo, Argentina) and dimethyl sulfoxide p.a. was purchased from Merck (Darmstadt, Germany). Chemical reagents used as BGE components were analytical grade or better. 2-hydroxypropyl-β-cyclodextrin (2-HP-β-CD) -average molecular weight 1460- was purchased from Sigma-Aldrich GmbH (Steinheim, Germany). Racemic propranolol, pindolol and oxprenolol were obtained from Sigma (Steinheim, Germany), and homatropine methylbromide was obtained from USPC Inc. (Maryland, USA). 99.5% benzyl alcohol p.a. was obtained from Biopack (Zárate, Argentina). Fused-silica capillaries (50 μm inner diameter), purchased from Polymicro Technologies (Phoenix, AZ, USA), were cut at a total length of 60 cm (51 cm effective length) for performing ACE analysis, and at a total length of 100 cm (90 cm effective length) for viscosity measurements.

2.3. Procedures

100 mM phosphate buffer was prepared using the needed amount of phosphoric acid and adjusting pH to 2.50 with 1 M sodium hydroxide solution. Background electrolyte (BGE) solutions were prepared by dissolving appropriate amounts of 2-HP-β-CD, transferring into volumetric flasks and diluting with buffer to the final volume to reach the concentrations: 60 , 50 , 40 , 30 , 20 , 10 and 5 mM, corresponding to *pC* 1.22, 1.30, 1.40, 1.52, 1.70, 2.00 and 2.30, respectively. Likewise, 0.5 mg mL^{-1} solutions of each model analyte were prepared by dissolving the solid in the buffer.

All solutions were degassed by immersion in an ultrasonic bath, filtered through a 0.22 μm

membrane and kept at 4°C.

New capillaries were activated for the first use by subsequently flushing at 1000 bar: 1 M NaOH (20 min), water (10 min), 0.1 M HCl (5 min), water (5 min), and BGE (20 min). Between runs, capillaries were preconditioned by flushing 0.1 M NaOH (1 min), water (1 min) and BGE (2 min).

Analytes were hydrodynamically injected by applying 30 mbar during 2 sec. Separations were performed at 25°C, using a potential of 25 kV and UV detection was set at 214 nm. Electropherograms for each racemic analyte were obtained by triplicate in each BGE at all *pC* values.

The electroosmotic flow (EOF) is extremely low at *pH* = 2.50, making extremely slow the mobility of a neutral marker. As an alternative to measure the EOF, advantage can be taken from the linear relationship between the EOF mobility and the current when the capillary dimensions, the composition of the buffer solution and the BGE ionic strength are kept constant [20,21]. Experimentally, a set of analysis injecting benzyl alcohol solution in BGEs with different viscosities were performed by triplicate to calculate the relationship between current and EOF –*i.e.* current and migration time were recorded-. These data were fitted to a linear equation. Thus, analyte migration times in each BGE were measured and simultaneously the currents were recorded. Then, the EOF corresponding to each electrophoretic run can be accurately estimated.

Viscosities of all BGE solutions at 25 °C were obtained by a viscosity *vs* elution time calibration line. These measurements involved the use of the CE instrument with an open fused silica capillary tube operated without applying voltage and consisted of filling the capillary tube completely with a solvent followed by the injection of a small plug of 1000 mg L^{-1} benzyl alcohol (1000 mbar during 1 second) and subsequent application of constant

pressure (99 mbar). During this step, the solvent is continuously flowing through the capillary which makes the plug migrate through the capillary until it is detected as a peak when it reaches the detection window. The procedure is repeated by triplicate, setting temperature at 25°C and registering the benzyl alcohol migration time in each experiment. The theoretical basis of the viscosity determination lies on the Hagen-Poiseuille's law, which relates the dynamic viscosity and the benzyl alcohol migration time when other parameters such as applied pressure, temperature, and capillary tube dimensions (diameter and length), are kept constant. Thus, viscosity *vs* elution time calibration line was constructed by applying the described procedure for several solvents of known viscosities (water, isopropyl alcohol, methanol, ethyl alcohol and dimethyl sulfoxide). After that, the procedure was repeated for each BGE solution, using the benzyl alcohol elution times to easily calculate the unknown viscosity by means of the calibration line without the need of other considerations or approximations. The applied procedure is a modified version to that proposed for Allmendinger et al. [22]. Modifications were introduced because the original method does not consider: I) that CE instrument only controls the temperature of the central zone along the capillary tube, while the extremes are exposed to uncontrolled temperature and II) potential differences in the capillary tube diameter along its length.

The EOF data were subtracted to the analytes apparent mobilities and then, the correction due to viscosities was made to finally obtain datasets of corrected mobilities *vs* ligand concentration for each enantiomer.

3. General Considerations

3.1 Theoretical Background

The binding equilibrium between an analyte, A^{+i}, with ligand, X^{+j}, also known as association, formation or inclusion equilibrium, is usually given in terms of concentration ratio as:

$$A^{+i} + X^{+j} \rightleftharpoons AX^{(i+j)} \qquad k_{f(AX)} = \frac{[AX^{(i+j)}]}{[A^{+i}][X^{+j}]} \tag{1}$$

where $k_{f(AX)}$ is the binding equilibrium quotient, while $+i$, $+j$ and $(i+j)$ are net charges of free analyte, A^{+i}, free ligand, X^{+j}, and complex, $AX^{(i+j)}$, respectively. We propose to handle the mathematical aspects of this equilibrium in analogy to the well-known treatments used for acid-base equilibria, that is, as a dissociation equilibrium, and dealing with thermodynamic constants –*i.e.* in terms of activities:

$$AX^{(i+j)} \rightleftharpoons A^{+i} + X^{+j} \qquad K_{d(AX)} = \frac{a_{A^{+i}} a_{X^{+j}}}{a_{AX^{(i+j)}}} = \frac{[A^{+i}][X^{+j}]}{[AX^{(i+j)}]} \frac{\gamma_{A^{+i}} \gamma_{X^{+j}}}{\gamma_{AX^{(i+j)}}} \tag{2}$$

where, $K_{d(AX)}$ denoted the thermodynamic dissociation constant, also called, instability constant. "*a*" indicates the activity of the specie referred to the subscript, while in the last term "γ" indicates activity coefficient of each specie referred to the subscript. Ion activity coefficients can be estimated by the Debye-Hückel (D-H) theory. Under its extended form the D-H equation can be used for ionic strength up to 0.10 m:

$$-\log \gamma_{\pm z} = p\gamma_{\pm z} = \frac{z^2 A\sqrt{I}}{1 + a_0 B\sqrt{I}} = z^2 p\gamma_{\pm 1} \tag{3}$$

where z is the charge of the considered ion; A, a_o and B are equation parameters; and I is the solution ionic strength calculated as usual..

Applying negative logarithm to Equation 2 and combining it with Equation 3, it is possible to reach a general expression analogous to that used in acid-base equilibria:

$$pK_{d(AX)} = -\log\left(\frac{[A^{+i}]}{[AX^{(i+j)}]}\right) + pX + [i^2 + j^2 - (i+j)^2] p\gamma_{\pm 1} \tag{4}$$

Designating dissociation ratio to the quotient $r = [A^{+i}]/[AX^{(i+j)}]$, then

$$r = \frac{[A^{+i}]}{[AX^{(i+j)}]} = 10^{-(pK_{d(AX)} - [i^2+j^2-(i+j)^2]p\gamma_{\pm 1} - pX)} = 10^{-(pk_{d(AX)} - pX)} \quad (5)$$

where $k_{d(AX)}$ represents the stoichiometric dissociation constant and pk_d was written as:

$$pk_d = pK_{d(AX)} - [i^2 + j^2 - (i+j)^2]p\gamma_{\pm 1} \quad (6)$$

The difference with acid-base equilibria is that pH, regulated by a buffer solution, is measured by means of an electrode giving a value strictly in terms of activities. In this study, X^{+j} represents a ligand, and its concentration is not measured but also calculated from expressions deduced from mass and electroneutrality balances. However, if the analytical concentration of ligand, *C*, is kept two orders/100-folds higher than the analyte concentration, A^{+i}, the known Henderson-Hasselbach approximation is valid, and it can be assumed that ligand concentration, $[X^{+j}]$, is similar to its analytical concentration, *C*, defined in the preparation of solution, *i.e.* $pX \approx pC$. Otherwise, the equations considering mass balance must be used. Therefore, in order to maintain simplicity in this report, ligand concentrations are limited to the validity of $pX = pC$.

When an analyte *A*, is distributed between different forms, properties associated to A^{+i} can be expressed as the linearly weighted sum of the property of each individual species (*e.g.*, solution absorption, solute retention in liquid chromatography or mobility in CE). Thus, the effective mobility in capillary electrophoresis, $\mu_{eff(A)}$, can be expressed as:

$$\mu_{eff(A)} = \alpha_{A^{+i}}\mu_{A^{+i}} + \alpha_{AX^{(i+j)}}\mu_{AX^{(i+j)}} = \frac{\mu_{A^{+i}} + \mu_{AX^{(i+j)}} 10^{-(pk_d - pC)}}{1 + 10^{-(pk_d - pC)}} \quad (7)$$

where α and μ denote respectively, the distribution function and the actual electrophoretic mobility of the species indicated by the subscripts. $\mu_{eff(A)}$ can also be expressed in terms of the dissociation ratio, *r*, as given in Equation 5, or as explicit function of *pC*, as described in the last term of Equation 7.

Different dependencies of the effective mobility as a function of *pC* can be obtained combining analytes and ligands with different charges. In Figure 1 these dependencies are depicted for analytes under cationic, neutral and anionic forms, combined with a neutral (Plot A), cationic (Plot B), and anionic ligand (Plot C). In these representations, mobility values have been taken arbitrarily around typical real values found for a small compound (MW~100-150 Da) with a free mobility of 20×10^{-5} $cm^2V^{-1}s^{-1}$ when it is fully charged, combined with a ligand having the size of a β-cyclodextrin (MW~1150 Da), *i.e.* 4-folds lower mobility for the complex (5×10^{-5} $cm^2V^{-1}s^{-1}$). Finally, a constant of $k_f = 100$ was taken ($pk_d = 2$), by considering that k_f values found in literature [23,24] for these type of compounds range between 60 and 300.

Two main goals can be considered for experiments involving determination of binding constants: modeling the mobility behavior as a function of ligand concentration for optimizing analytical separation, or just the determination of the binding constant required for other purposes. In the development of a separation method, it is possible to choose the more convenient ligand for the experiment. Regarding to this, on the right side of each plot of Figure 1, the maximum possible variation of mobilities (or step-height of the sigmoid curve) is indicated with vertical bars. These maximum variations are given by the mobility differences between free and complexed analyte. If several ligands with different net charge can be chosen, it is recommended to select that which provides larger mobility variation. Figure 2 summarizes the mobility difference magnitude for all possible analyte-ligand charge combinations.

3.2 Practical considerations

The experimental determination of unknown dissociation constants between a ligand, X^{+j}, and

an analyte, A^{+i}, by CE consists in the preparation of a set of BGE solutions with different *pC* values, and the measurement of the migration times for the determination of $\mu_{eff(A)}$ in all of them. Besides, the experimental data set is completed with an additional value which corresponds to the mobility of the fully charged free analyte - *i.e.* in buffer but in absence of ligand-.

By one hand, the mobility for $C = 0\ M$ is the asymptotic value at which the sigmoid curve tends when $pC = \infty$. In practice, the mobility measured in BGE without ligand can be assigned to a very high *pC* value – *e.g.*: $pC = 10$. On the other hand, the mobility value extrapolated to $pC = -\infty$ represents the complex mobility, while the inflection point indicated in all plots of Figure 1 corresponds to $pC = pk_d$ from where thermodynamic pK_d can be obtained based on Equation 6. Thus, the average value of free ion mobility obtained from a high number of replicates in BGE without ligand could be considered as an accurate value of $\mu_{A^{+i}}$, and only two fitting parameters of Equation 7 ($\mu_{AX^{(i+j)}}$ and k_d) have to be assessed.

Ligand concentration range defines the domain in abscissa (x-axis) in which constant determinations will be carried out. This range is, in practice, limited to a certain working window. The lowest possible *pC* value ($pC_{\min}$), is usually limited by solubility of the ligand, X^{+j}. For example, solubility of native β-cyclodextrin in pure water at room temperature is 0.0163 M [25]. This value is indicated as lower limit of the working window in Figure 1 – Plot A. Ligands with higher solubility such as neutral 2-HP-β-CD (~65% w/v at 25ºC, MW:1460 g mol^{-1}) [26] allow to use $pC_{\min} = 0.35$ or even lower. For others, solubility is as high that do not constitute the lower *pC* limit. In the case of charged ligands, pC_{min} is defined by the maximum ionic strength that allows to estimate the required activity coefficients in the framework of a thermodynamic binding constant determination. For instance, in absence of

buffers, the ionic strength generated by a monovalent ligand combined with a single charge counter-ion (e.g. Na^+) should be below 100 mM and, thus, the pC_{min} = -log 0.10 = 1.00. Indeed, this is the lower limit of the working window shown in Figure 1 (Plots B and C). In addition to charged ligands, the use of *pH* buffering compounds will also contribute to the total ionic strength and, consequently, this will shift pC_{min} toward higher values reducing the working window, at least while maintaining the validity of D-H equation. On the other hand, the superior value of the working window (pC_{max}), is limited by the lower ligand concentration fulfilling the condition $[X^{+j}] \approx C$ allowing to affirm that $pX \approx pC$. It can be assumed valid when $C > 100[A^{+i}]$ and, therefore, C_{max} is related to the limit of quantitation of the analyte (C_{LOQ}), which depends on the detection method for the considered analyte. Thus $C_{max} = 100C_{LOQ}$ and $pC_{max} = pC_{LOQ} - 2$.

For CE instrument with UV detection, assuming a C_{LOQ} = 1 mg L^{-1} for a compound of MW=100 Da, $[A^{+i}] = 10^{-5}$ M, $pC_{LOQ} = 5$, consequently pC_{max} = 3. This value is indicated as the superior limit of white zones in Figure 1. The use of improved detectors, offering lower C_{LOQ}, enables lower ligand concentration, leading to wider working *pC* windows toward higher *pC* values. Alternatively, it is also possible to consider more complex expressions that include the mass balance in Equation 7.

Analyzing the sigmoid curves in the framework of the working *pC* window, it can be noted that acceptable non-linear regressions can be obtained only when pk_d is within the working window range, while maximum accuracy can be achieved when experimental mobilities covers the range from above to below the inflection point.

Finally, determinations of binding constants can be performed, either by injecting a compound, A^{+i}, in BGE solutions at different levels of X^{+j}, or by injecting X^{+j} in solutions with different levels of A^{+i}. Mathematics given above do not distinguish the identities of A^{+i}

or X^{+j}, therefore the analysis and treatment are equally valid. The convenience of studying A^{+i} in X^{+j}, or X^{+j} in A^{+i}, must be analyzed based on practical considerations such as solubility, mobility difference between free and associated forms, availability or cost of A^{+i} and X^{+j}, etc.

Results and Discussion

In order to obtain reliable constant values, the only change between experiments must be the ligand concentration while any other property, physical or chemical, must remain constant [15]. Regarding to that, the ionic strength can be set easily constant; nevertheless, the presence of ligands generates changes in separation media viscosity. This effect is more significant for large molecules, such as proteins, or polysaccharides (cellulose or cyclodextrins) particularly at high concentrations hence, a correction must be done. Since electrophoretic mobility has a reciprocal dependence with viscosity, corrected mobilities can be calculated as:

$$\mu^{+} = \mu^{exp}\left(\frac{\eta_{exp}}{\eta_w}\right) \tag{8}$$

where μ^{+} is the corrected mobility, which corresponds to the mobility that would be observed if the separation media had the viscosity of pure water; μ^{exp} is the mobility obtained in real experiments, while η_{exp} and η_w are the viscosities of the separation medium and pure water, respectively. A non-corrected sigmoid curve has smaller mobilities values than the corrected one and calculated pk_d would be higher than real. Some authors have proposed to correct this viscosity effect on the base of the reciprocal relationship of this property with the current [27]. According to this, the viscosity ratio on right term of Equation 8 equals the inverse quotient of currents in CE analysis using these mediums as BGE:

$$\frac{\eta_{exp}}{\eta_w} = \frac{i_w}{i_{exp}} \tag{9}$$

This method, however, has two drawbacks: the current is not only related to viscosity, but also to the EOF which has significant variability due to changes in zeta potential by adsorption of

trace impurities to the capillary wall. Therefore, the use of current ratios makes the viscosity correction to be subjected to the same variability of the EOF. On the other hand, correcting the viscosity to that of pure water would require a current value in pure (deionized) water, which is in practice not possible. In order to overcome those drawbacks, we suggest the use of a correction based on fluid dynamics experiments to obtain precise viscosity correction data independent of the EOF.

Data sets of apparent electrophoretic mobilities at different 2-HP-β-CD concentrations as BGE additive were acquired for propranolol, pindolol, oxprenolol and homatropine methylbromide enantiomers and normalized to the effective mobility in pure water. Different mathematical procedures for fitting these types of data set have been reported and employed for determining k_d [15,28–30]. In this work, however, non-linear regressions between pC and $\mu_{eff(A)}$ according to Equation 7 are preferred due to the simple interpretation of the obtained parameters and their physical meanings.

In Figure 3 experimental results are indicated with symbols while regressions are depicted with continuous lines. For each analyte, plot I refers to the enantiomer with higher mobility and plot II to the slower one. Parameters of the non-linear regressions for all the enantiomers and their standard deviations are gathered in Table 1.

In these cases, pK_d obtained as parameter of the regressions ranged from 1.29 to 1.82, indicating a very weak association between those enantiomeric drugs with 2-HP-β-CD at 25 °C. This range of dissociation values can be traduced in binding constants ranging from 19.5 to 66.1, which are typical values for these complexes [31]. An alternative form to understand these values is considering the CD concentration required to obtain association of 50% of the stronger enantiomer of each pair. According to this, the complex with stronger binding constant is the constituted by propranolol and the chiral ligand, which requires [2-HP-β-CD]

= 15.1 mM to be 50% associated, while at the same concentration the other enantiomeric form is 48.3% associated. The stronger association is reasonable if we take into account that CD cavity is hydrophobic and this is the compound with the larger hydrophobic group (naphthyl). Homatropine methylbromide requires [2-HP-β-CD] = 27.3 mM to achieve 50% association, while at this ligand concentration the other enantiomer is associated in a 42.3%. Pindolol requires a solution with [2-HP-β-CD] = 35.0 mM to be 50% associated, while at this concentration the other enantiomeric form is 49.0% associated. Finally, oxprenolol requires a solution with [2-HP-β-CD] = 50.0 mM to be 50% associated, while at this ligand concentration the other enantiomeric form is 49.4% associated. The analysis of differences in association degrees between enantiomeric forms when one of them is 50% associated with the ligand is an alternative to understand the way in which the values of enantiodiscrimination thermodynamic constants contribute to the separation. It is noticeable that homatropine methylbromide is the compound with larger dissociation difference (7%) when one of the enantiomers is 50% associated. The larger enantiodiscrimination would be because the chiral carbon is adjacent to the hydrophobic group, whereas the chiral carbons are two atoms away from the hydrophobic group entering the cavity for the other analytes.

The *pC* range of the data acquired in this work is the same for all analytes (1.22 to 2.30), since it depends on the solubility and properties of the chosen CD, data set is completed with one more *pC* level in absence of ligand, which was arbitrarily assigned to $pC = 10$. Taking into account that analytes have different dissociation constants, the range of experimental data of mobilities are acquired in different zones of the curves, which affects the precision of the obtained parameters. For example, for propranolol enantiomers whose $pK_d \sim 1.8$, a significant number of mobility points could be acquired in the lower zone of the curve, this is at $pC < pK_d$. This fact leads to a more accurate value of the complex mobility and consequently to a

more precise location of the inflection point. On the other hand it is oxprenolol whose pK_d ~ 1.3 and more mobility values could be measured at pC levels higher than its pK_d. Therefore, certain correlation can be expected between pK_d value and standard deviation of the complex mobility estimation: a larger variability is expected for systems with lower pK_d values.

Conclusions

A method to study association equilibria and determine thermodynamic binding constants by capillary affinity electrophoresis was discussed. The approach was made considering an association with 1:1 stoichiometric relation, and taking into account the fact that an analyte that experiences one or more equilibria, has an effective mobility that is the result of the weighted linear sum of the mobilities of each of its individual species. In addition, the presented model was expressed in analogy to the well known acid-base equilibrium, since it is possible to rationally analyze different situations depending on the properties of analytes and ligands, *i.e.*, a complete and detailed analysis was carried out for all possible analyte-ligand charge combinations. Furthermore, the mobility variation between the free and complexed analyte was summarized for each one; evaluating advantages and limitations.

This study allowed us to establish criteria in order to perform an experimental design, whether the goal is to determine binding constants, or to optimize analytical separations. Additionally, practical aspects to carry out constant determination by the proposed method were described and it was applied to the study of enantiomeric association between 2-hydroxypropyl-β-cyclodextrin and the enantiomers of four pharmaceutical drugs (propranolol, pindolol, oxprenolol and homatropine methylbromide). Thermodynamic dissociation constants for all complexes, as well as the actual mobilities of both free and complexed analytes were obtained. The results and its accuracy were discussed in detail.

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References

[1] R.J. Flaherty, B. Nshime, M. DeLaMarre, S. DeJong, P. Scott, A.W. Lantz, Cyclodextrins as Complexation and Extraction Agents for Pesticides from Contaminated Soil, Chemosphere. 91 (2013) 912–920. doi:10.1016/j.chemosphere.2013.02.005.

[2] M. Kfoury, D. Landy, S. Ruellan, L. Auezova, H. Greige-Gerges, S. Fourmentin, Nootkatone Encapsulation by Cyclodextrins: Effect on Water Solubility and Photostability, Food Chem. 236 (2017) 41–48. doi:10.1016/j.foodchem.2016.12.086.

[3] J. Reijenga, A. van Hoof, A. van Loon, B. Teunissen, Development of Methods for the Determination of pKa Values, Anal. Chem. Insights. 8 (2013) 53–71. doi:10.4137/ACI.S12304.

[4] P. Mura, Analytical Techniques for Characterization of Cyclodextrin Complexes in Aqueous Solution: A Review, J. Pharm. Biomed. Anal. 101 (2014) 238–250. doi:10.1016/j.jpba.2014.02.022.

[5] E. Örnskov, A. Linusson, S. Folestad, Determination of dissociation constants of labile drug compounds by capillary electrophoresis, J. Pharm. Biomed. Anal. 33 (2003) 379–

391. doi:10.1016/S0731-7085(03)00238-3.

[6] J.M. Herrero-Martínez, M. Sanmartin, M. Rosés, E. Bosch, C. Ràfols, Determination of dissociation constants of flavonoids by capillary electrophoresis, Electrophoresis. 26 (2005) 1886–1895. doi:10.1002/elps.200410258.

[7] D. Koval, V. Kašika, J. Jiráek, M. Collinsová, T.A. Garrow, Determination of dissociation constant of phosphinate group in phosphinic pseudopeptides by capillary zone electrophoresis, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 770 (2002) 145–154. doi:10.1016/S1570-0232(01)00595-5.

[8] S.A.C. Wren, R.C. Rowe, Theoretical aspects of chiral separation in capillary electrophoresis: I. Initial evaluation of a model, J. Chromatogr. A. 603 (1992) 235–241. doi:10.1016/0021-9673(92)85366-2.

[9] Y.Y. Rawjee, D.U. Staerk, G. Vigh, Capillary electrophoretic chiral separations with cyclodextrin additives. I. acids: Chiral selectivity as a function of pH and the concentration of β-cyclodextrin for fenoprofen and ibuprofen, J. Chromatogr. A. 635 (1993) 291–306. doi:10.1016/0021-9673(93)80372-F.

[10] S.G. Penn, D.M. Goodall, J.S. Loran, Differential binding of tioconazole enantiomers to hydroxypropyl-β-cyclodextrin studied by capillary electrophoresis, J. Chromatogr. A. 636 (1993) 149–152. doi:10.1016/0021-9673(93)80067-I.

[11] S.G. Penn, E.T. Bergström, D.M. Goodall, J.S. Loran, Capillary electrophoresis with chiral selectors: Optimization of separation and determination of thermodynamic parameters for binding of tioconazole enantiomers to cyclodextrins, Anal. Chem. 66 (1994) 2866–2873. doi:10.1021/ac00090a012.

[12] B. Chankvetadze, W. Linder, G.K.E. Schriba, Enantiomer Separations in Capillary Electrophoresis in the Case of Equal Binding Constants of the Enantiomers with a

Chiral Selector: Commentary on the Feasibility of the Concept, Anal. Chem. 76 (2004) 4256–4260. doi:10.1021/ac0355202.

[13] M.T. Bowser, D.D.Y. Chen, Higher Order Equilibria and Their Effect on Analyte Migration Behavior in Capillary Electrophoresis, Anal. Chem. 70 (1998) 3261–3270. doi:10.1021/ac9713114.

[14] M.T. Bowser, A.R. Kranack, D.D.Y. Chen, Properties of Multivariate Binding Isotherms in Capillary Electrophoresis, Anal. Chem. 70 (1998) 1076–1084. doi:10.1021/ac970854a.

[15] K.L. Rundlett, D.W. Armstrong, Examination of the origin, variation, and proper use of expressions for the estimation of association constants by capillary electrophoresis, J. Chromatogr. A. 721 (1996) 173–186. doi:10.1016/0021-9673(95)00774-1.

[16] Y. Tanaka, S. Terabe, Estimation of binding constants by capillary electrophoresis, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 768 (2002) 81–92. doi:10.1016/S0378-4347(01)00488-1.

[17] K. Ušelová-Včeláková, I. Zusková, B. Gaš, Stability constants of amino acids, peptides, proteins, and other biomolecules determined by CE and related methods: Recapitulation of published data, Electrophoresis. 28 (2007) 2145–2152. doi:10.1002/elps.200600780.

[18] Z. Chen, S.G. Weber, Determination of binding constants by affinity capillary electrophoresis, electrospray ionization mass spectrometry and phase-distribution methods, TrAC - Trends Anal. Chem. 27 (2008) 738–748. doi:10.1016/j.trac.2008.06.008.

[19] C. Jiang, D.W. Armstrong, Use of CE for the determination of binding constants, Electrophoresis. 31 (2010) 17–27. doi:10.1002/elps.200900528.

[20] S. Terabe, H. Utsumi, K. Otsuka, T. Ando, T. Inomata, S. Kuze, Y. Hanaoka, Factors Controlling Electroosmotic Flow in Open-Tubular Capillaries in Electrokinetic Chromatography, J. High Resolut. Chromatogr. Chromatogr. Commun. 9 (1986) 666–670. doi:10.1002/jhrc.1240091115.

[21] S. Terabe, K. Otsuka, T. Ando, Electrokinetic Chromatography with Micellar Solution and Open-Tubular Capillary, Anal. Chem. 57 (1985) 834–841. doi:10.1021/ac00281a014.

[22] A. Allmendinger, L.-H. Dieu, S. Fischer, R. Mueller, H.-C. Mahler, J. Huwyler, High-throughput viscosity measurement using capillary electrophoresis instrumentation and its application to protein formulation, J. Pharm. Biomed. Anal. 99 (2014) 51–58. doi:10.1016/j.jpba.2014.07.005.

[23] S.A.C. Wren, Theory of chiral separation in capillary electrophoresis, J. Chromatogr. 636 (1993) 57–62. doi:10.1016/0021-9673(93)83120-H.

[24] M. V. Rekharsky, Y. Inoue, Complexation Thermodynamics of Cyclodextrins, Chem. Rev. 98 (1998) 1875–1918. doi:10.1021/cr970015o.

[25] M.J. Jozwiakowski, K.A. Connors, Aqueous Solubility Behavior of Three Cyclodextrins, Carbohydr. Res. 143 (1985) 51–59. doi:10.1016/S0008-6215(00)90694-3.

[26] L. Szente, J. Szejtli, Highly soluble cyclodextrin derivatives: chemistry, properties, and trends in development, Adv. Drug Deliv. Rev. 36 (1999) 17–28. doi:10.1016/S0169-409X(98)00092-1.

[27] S.G. Penn, E.T. Bergstrijm, I. Knights, G. Liu, A. Ruddick, D.M. Goodall, Capillary Electrophoresis as a Method for Determining Binding Constants: Application to the Binding of Cyclodextrins and Nitrophenolatest, J. Phys. Chem. 99 (1995) 3875–3880.

doi:10.1021/j100011a065.

[28] M. Fillet, B. Chankvetadze, J. Crommen, G. Blaschke, Designed combination of chiral selectors for adjustment of enantioseparation selectivity in capillary electrophoresis, Electrophoresis. 20 (1999) 2691–2697. doi:10.1002/(SICI)1522-2683(19990901)20:13<2691::AID-ELPS2691>3.0.CO;2-S.

[29] S.N. Krylov, Kinetic CE: Foundation for homogeneous kinetic affinity methods, Electrophoresis. 28 (2007) 69–88. doi:10.1002/elps.200600577.

[30] P. Dubský, L. Müllerová, M. Dvořák, B. Gaš, Generalized model of electromigration with 1:1 (analyte:selector) complexation stoichiometry: Part I. Theory, J. Chromatogr. A. 1384 (2015) 142–146. doi:10.1016/j.chroma.2015.01.055.

[31] S.A.C. Wren, Chiral separation in capillary electrophoresis, Electrophoresis. 16 (1995) 2127–2131. doi:10.1002/elps.11501601343.

Table 1. Parameters resulting from non-linear regressions of effective mobilities ($cm^2V^{-1}s^{-1}$) versus concentration of 2-HP-β-CD expressed as negative logarithm (*pC*), for the two enantiomeric forms of the four analytes.

Analyte	**n**	$\mu_{A^{+i}}$ **x10^5**	$\mu_{AX^{(i+j)}}$ **x10^5**	pK_d	**r^2**
Propranolol 1	31	18.7 (0.4)	1.4 (0.1)	1.79 (0.07)	0.97
Propranolol 2	31		1.6 (0.1)	1.82 (0.07)	0.97
Pindolol 1	30	19.3 (0.3)	0 (2)	1.44 (0.09)	0.98
Pindolol 2	30		0 (2)	1.46 (0.09)	0.98
Oxprenolol 1	22	18.6 (0.2)	0 (2)	1.3 (0.1)	0.98

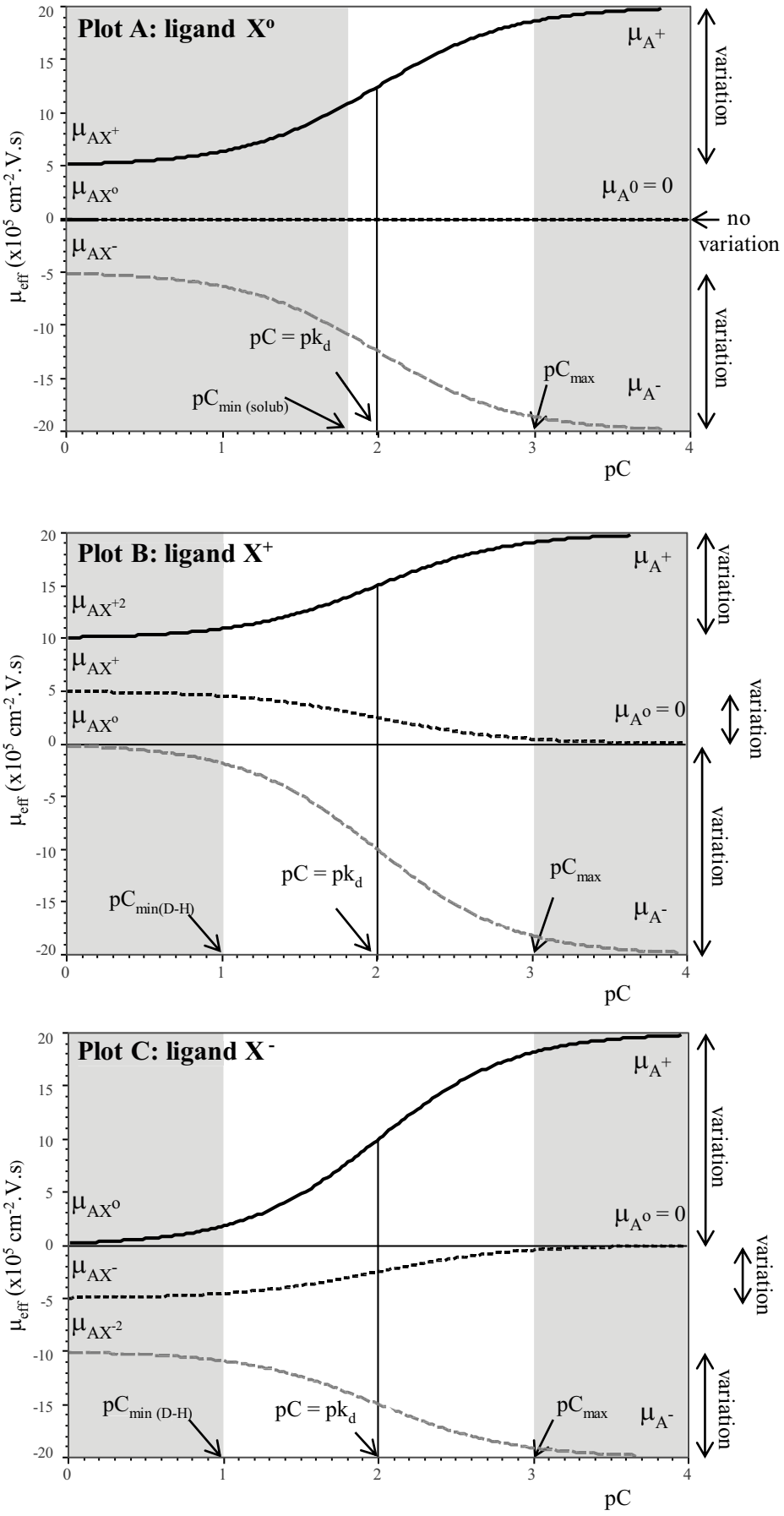
Analyte	n	$\mu_{A^{+i}}$ x10^5	$\mu_{AX^{(i+j)}}$ x10^5	pK_d	r^2
Oxprenolol 2	22		0 (2)	1.3 (0.1)	0.98
Homatropine methylbromide 1	20		2.6 (0.2)	1.44 (0.08)	0.98
		22.1 (0.3)			
Homatropine methylbromide 2	20		3.1 (0.1)	1.56 (0.07)	0.98

Standard deviation values indicated between brackets.

Highlights

- A method to obtain thermodynamic binding constants by ACE is presented.
- The method also provides actual mobilities of free and complexed analyte.
- The study of enantiomeric associations was boarded using 2HPBCD as BGE additive.
- The method was applied to the enantioseparation of pharmaceutical drugs.

Figure 1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μA+
μAX+
μAXº
μA0 = 0
no variation
μAX-
pC = pkd
pCmax
μA-
pCmin (solub)
variation
μeff (x10⁵ cm⁻².V.s)
pC
Plot B: ligand X+
μA+
μAX+2
μAX+
μAXº
μAº = 0
μAX-
pCmin(D-H)
pC = pkd
pCmax
μA-
variation
Plot C: ligand X-
μA+
μAXº
μAº = 0
μAX-
μAX-2
pCmin (D-H)
pC = pkd
pCmax
μA-
variation

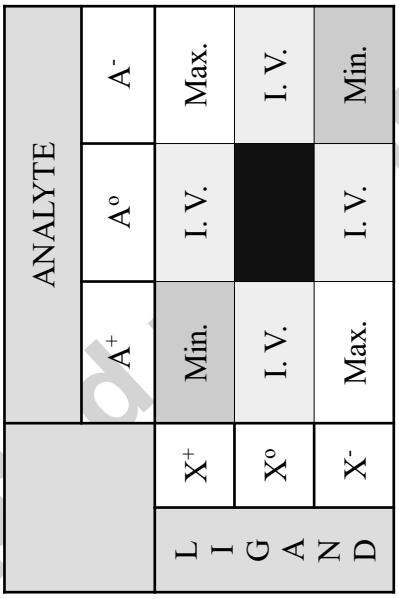
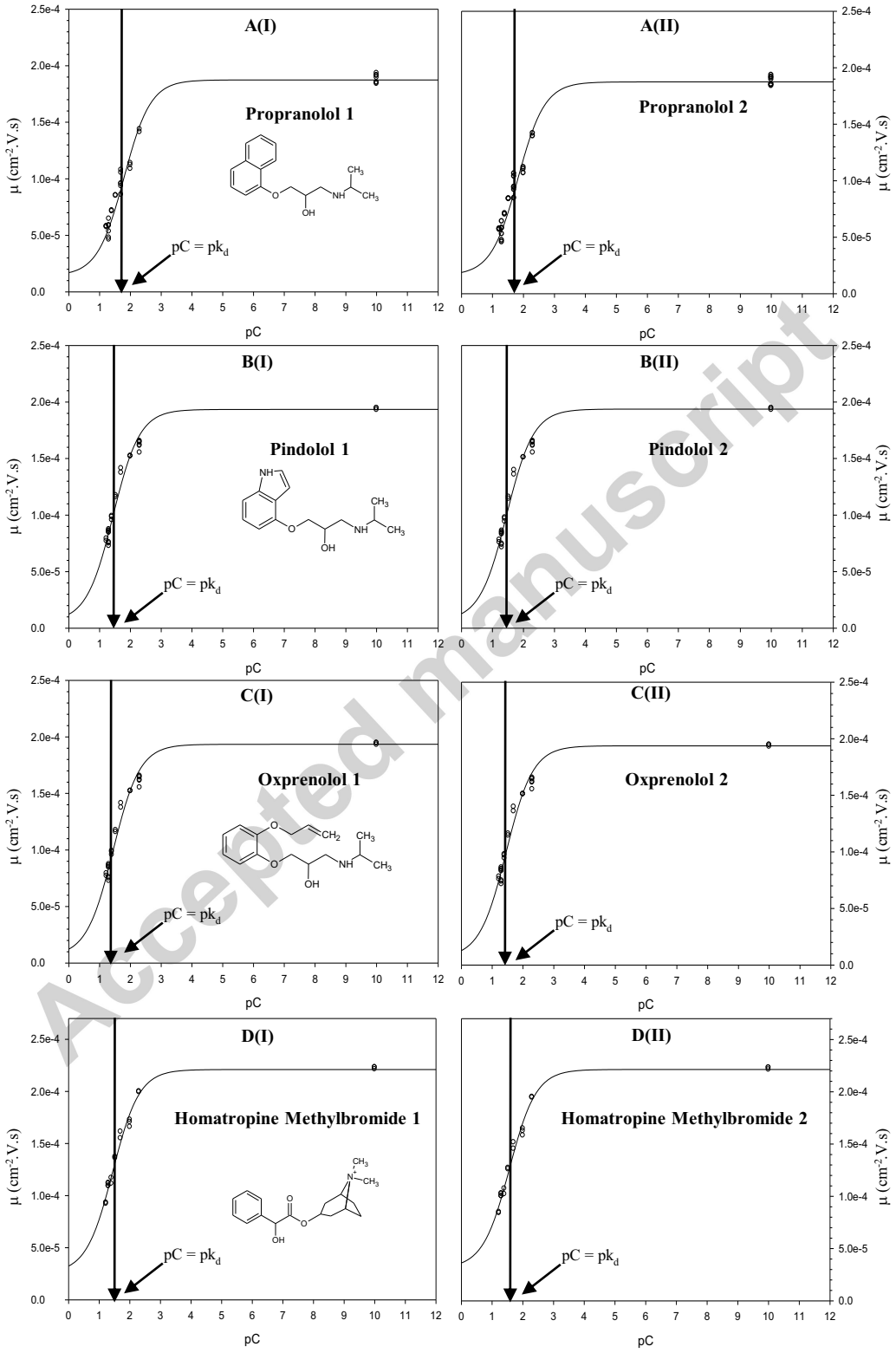
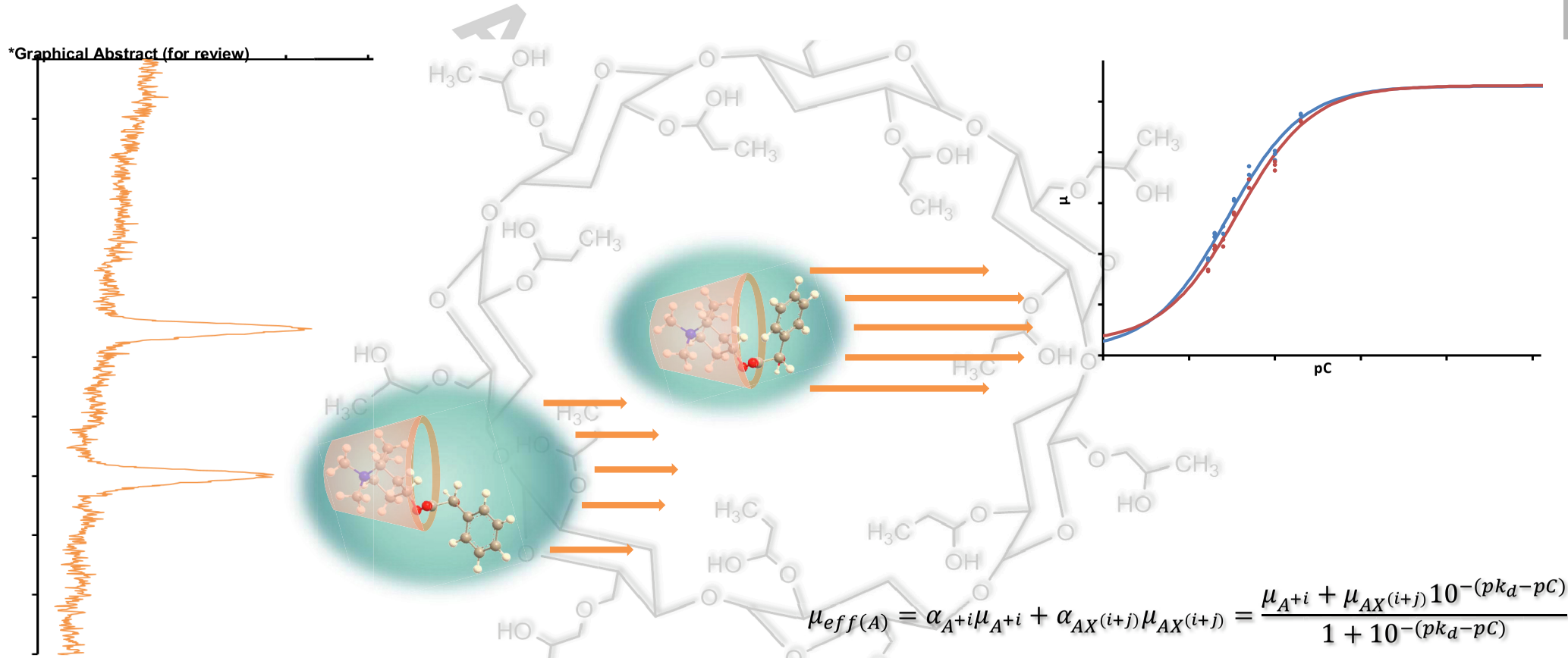


Figure 2

Figure 3



*Graphical Abstract (for review)



$$\mu_{eff(A)} = \alpha_{A^{+i}}\mu_{A^{+i}} + \alpha_{AX^{(i+j)}}\mu_{AX^{(i+j)}} = \frac{\mu_{A^{+i}} + \mu_{AX^{(i+j)}}10^{-(pk_d - pC)}}{1 + 10^{-(pk_d - pC)}}$$