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Enumeration Algorithm for Determination of Binding Constants in Capillary Electrophoresis

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With more accurate simulation models and more efficient algorithms becoming available, the binding constants of an affinity interaction can be obtained from much simpler experiments using capillary electrophoresis. With the enumeration algorithm, all possible combinations of the binding constant and the complex mobility in certain ranges that could result in the experimental migration time of an injected analyte are extracted from a 3-D surface, which depicts the migration times resulting from different values of the binding constant and the mobility of the complex formed between the interacting pair, to form a 2-D curve. When the experimental conditions are changed, the analyte migration time will also change. A new 2-D curve can be constructed from another 3-D surface on the basis of the pairs of binding constants and complex mobility values that could result in the new migration time. Because the true binding constant and complex mobility values have to be the same for both experimental conditions under the same temperature, there has to be a point where both 2-D curves will converge. The coordinates of the converging point give the values for a binding constant and a complex mobility that will fit all 2-D curves generated under certain experimental conditions. *p*-Nitrophenol is used as the analyte, *β*-cyclodextrin is used as the additive, and a one-cell model is used to simulate affinity CE. The experimental conditions that can improve the accuracy of the binding constants are discussed.

The high-throughput evaluation of binding affinities of a large collection of compounds to target biomolecules is one of the bottlenecks in today's drug design, discovery, and evaluation processes. Because capillary electrophoresis (CE) has a well-controlled physical and chemical environment, it can be used to determine binding or dissociation constants of affinity interactions. When CE is used for these studies, it is often referred to as affinity CE or ACE.

Linear and nonlinear regression methods have been used to determine binding constants from ACE experimental data.[1−4] However, because the average mobility of the analyte is used in the regression methods, the errors associated with these methods can be large if the concentration of the additive in the background electrolyte (BGE) is not much higher than the concentration of the injected analyte. In this situation, the mobility of the analyte changes during the course of the electrophoretic migration.[3−5] Moreover, 6 to 10 BGEs with different additive concentrations are normally needed to carry out the experiment for the regression methods, which leads to ∼18 to 30 CE runs if each experiment is done in triplicate. In addition, the correction factor for higher concentration additives and the mobility of free analyte also need to be measured multiple times. Therefore, a faster and more accurate approach to determining binding constants using ACE is essential for high-throughput screening of thousands of possible compounds in a combinatorial library.

Differential equations describing the electrophoretic migration behavior of the analyte in this situation have been derived and the approach to obtain their analytical solutions has been demonstrated.[6] However, finding the analytical solution for such differential equations is often complicated. Computer simulation provides a unique opportunity for us to accurately describe the behavior of analyte in a column by translating the differential equations into finite difference schemes. If the electrophoretic migration behavior can be accurately predicted by computer simulation, the data from an ACE experiment can be used to estimate binding constants.

The accuracy of this approach is dependent on the accuracy of the simulation model. On the other hand, the speed of the simulation is also important due to the fact that thousands of simulation runs are required to determine a single binding constant.

Several computer simulation models of CE (or ACE) are presently available;[7−11] however, none of them is suitable for determining binding constants because of the high demand on computing power and computing time. We have proposed an efficient algorithm to predict the electrophoretic migration process and to determine the migration time on the basis of peak maximums and peak shapes.[12] In this paper, a method for the

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determination of binding constants by computer simulation of ACE is described.

EXPERIMENTAL SECTION

ACE Experiments. The 1:1 interaction between *p*-nitrophenol (analyte, A) and β-cyclodextrin (additive, P) to form a complex (C) is used as a model system. The experiments were carried out on a Beckman Coulter ProteomeLab PA800 (Beckman Coulter Inc., Fullerton, CA) with a built-in PDA detector. A 64.5-cm-long (54.3 cm to detector) × 50-μm-i.d., fused-silica capillary (Polymicro Technologies, Phoenix, AZ) was used. β-Cyclodextrin (Sigma, St. Louis, MO) was dissolved in 160 mM borate buffer (pH 9.1) at various concentrations ranging from 1.0 to 15.0 mM. *p*-Nitrophenol (Fisher, Fair Lawn, NJ) was dissolved in the same borate buffer at a concentration of 2.0 mM.

In each ACE run, a *p*-nitrophenol solution was injected to form a narrow analyte plug in the inlet of the capillary which was filled with the β-cyclodextrin solution at various concentrations. The initial concentrations of the analyte (*p*-nitrophenol) and the additive (β-cyclodextrin) are denoted as $[A]_0$ and $[P]_0$, respectively. A 10 kV potential was applied on the capillary, and a temperature of 20.0 °C was maintained throughout the experiments. Each ACE experiment with a given $[P]_0$ was repeated three times. Methanol was used as the electroosmotic flow (EOF) marker.

Mobility of the Analyte. To measure the electrophoretic mobility of the free analyte ($\mu_{ep,A}$), *p*-nitrophenol was injected into the capillary filled with neat borate buffer ($[P]_0 = 0$), and was driven through by a potential of 10 kV. This measurement was carried out before each ACE run, and a total of 24 measurements were taken.

$\mu_{ep,A}$ is normally calculated using the following equation,

$$\mu_{ep,A} = \frac{L_c L_d}{V}\left(\frac{1}{t_m} - \frac{1}{t_{eo}}\right) \quad (1)$$

where L_c and L_d are the length of the capillary and the length to the detector, respectively, V is the potential, t_{eo} is the migration time of the EOF marker, and t_m is the migration time of the analyte. To accurately determine the mobility, the idle time (t_i, 0.034 min), which is a pause after the instruction is given and before the voltage is applied, and a ramp time (t_r, 0.17 min), which is the time required for the voltage to reach the set value, have to be accounted for. The accuracy of eq 1 can be improved by adding t_i and t_r into the equation, and the free analyte mobility can be obtained from

$$\mu_{ep,A} = \frac{L_c L_d}{V}\left(\frac{1}{t_m - t_i - t_r/2} - \frac{1}{t_{eo} - t_i - t_r/2}\right) \quad (2)$$

Because the physical condition of the BGE changes after β-cyclodextrin is added, $\mu_{ep,A}$ for each ACE run has to be further corrected by either using neutral EOF markers[13,14] or using a correction factor which converts the observed net electrophoretic mobility to an ideal state in which the additive concentration approaches 0.[1,15] In this paper, the $\mu_{ep,A}$ values input into the simulation program is calculated from the $\mu_{ep,A}$ value measured from an additive-free solution multiplied by the ratio of the migration times of the EOF markers, which gives the $\mu_{ep,A}$ value under the specific conditions of the specified additive concentration.

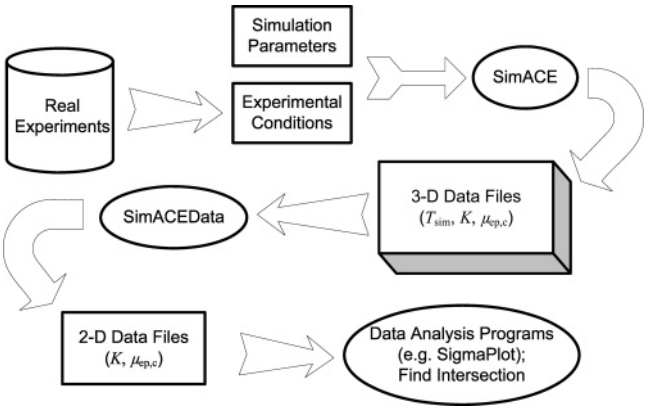

Figure 1. The flowchart of the procedure for determining binding constants.

Simulation Approach. A group of simulation programs, SimACE and SimACEData, written in Microsoft Visual C++ 6.0 are used to determine the binding constants. The complete procedure for determining binding constants is summarized in Figure 1. The first computer program, SimACE, is used to simulate ACE experiments and to generate 3-D surfaces. The second program, SimACEData, is used to extract 2-D curves from data files generated by SimACE.

The ACE simulation program, SimACE, requires all experimental conditions: the length of the capillary ($L_c = 64.5$ cm), the length to the detector ($L_d = 54.3$ cm), the voltage ($V = 10$ kV), the concentration of the injected analyte ($[A]_0 = 2.0$ mM), the concentration of the additive present in BGE ($[P]_0 = 1.0$, 2.0, 3.0, 4.0, 5.0, 10.0, 12.5, 15.0 mM), the migration time of the EOF marker (t_{eo} from each ACE experiment), the corrected electrophoretic mobilities of the free analytes ($\mu_{ep,A}$), and the electrophoretic mobility of the free additives ($\mu_{ep,P} = 0$ for neutral β-cyclodextrin).

RESULTS AND DISCUSSION

The electrophoretic mobility of the analyte in the presence of the additive (μ_{ep}^{A}) can be calculated by the following equation,[15]

$$\mu_{ep}^{A} = f_A \mu_{ep,A} + f_C \mu_{ep,C} \quad (3)$$

where f_A and f_C are the fractions of the free analyte and the complex, and $\mu_{ep,C}$ is the electrophoretic mobility of the complex. If a highly concentrated additive solution is used as BGE, f_C approaches 100%, and $\mu_{ep,C}$ can be assumed equal to μ_{ep}^{A}, which is obtained by measuring the migration time of the analyte peak. However, this is not always achievable due to limits in solubility or the cost of the additives. Therefore, $\mu_{ep,C}$ is often the other unknown in addition to the equilibrium constant (K).[1,2] The existence of the two unknowns and the complicated differential

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equations make the mathematical approach to determine binding constants a challenging task.[6]

The approach described in this work utilizes the tremendous power of modern computers to implement an enumeration algorithm for obtaining the binding constant of affinity interactions from less restrictive experimental conditions. The general idea of the enumeration algorithm is that all possible solutions to a given problem are investigated in the process of finding the real solution(s). In our case, all possible combinations of the complex mobility and the binding constant that could result in an analyte migration time are investigated in the effort to find the values that are common in all conditions.

ACE Simulation. This enumeration approach itself is not related to any particular simulation program, but the accuracy of the determined binding constants relies heavily on the accuracy of the simulation model. The simulation program used in this approach must have the ability to not only simulate the electrophoretic migration, but also consider the binding interaction between the analyte and the additive. The simulation program does not need to generate the shape of the entire analyte peak; instead, only the simulated migration times of the peak maximums are required.

Our previous work has shown that the shape of an analyte peak is directly related to the relative mobilities of the free analyte, the free additive, and the complex.[12] The peak maximums often reside at the rear or front edge of the initial analyte plug according to the relative mobilities. The migrational behavior of peak maximums has been described mathematically, and a computer program was written to predict the migration times of the peak maximums within required accuracy. This simulation program is very fast, because only one cell is monitored rather than the entire capillary. It is suitable to be used in this enumeration approach. This model will be referred to as the one-cell model in the rest of the discussion, and the program is used to simulate the ACE process in this paper.

3-D Surfaces. When the experimental conditions and required values for various constants are sent to the simulation program, migration times are generated and plotted according to the changes of the binding constant and the complex mobility. When the generated migration time (T_{sim}) agrees with the experimental migration time (T_{exp}), the pair of K and $\mu_{ep,C}$ values are collected for further consideration.

The binding constant and the complex mobility of a given binding interaction can be estimated in certain ranges, which may be wide in the beginning but can be narrowed quickly as we get more knowledge about this binding interaction. The simulation program has the ability to scan the K vs $\mu_{ep,C}$ plane at user-defined intervals on both axes within the preestimated ranges. Each pair of K and $\mu_{ep,C}$ given to the simulation program will generate a simulated migration time. After scanning the entire K vs $\mu_{ep,C}$ plane, a 3-D surface can be constructed with K, $\mu_{ep,C}$, and the simulated migration time (T_{sim}) as the x, y, and z axes, respectively. An example is shown in Figure 2, which is plotted using SigmaPlot (SPSS Science).

The computer program, SimACE, is used to generate 3-D surfaces. In addition to all experimental conditions, users have to set the ranges and intervals of K and $\mu_{ep,C}$ (or the mobility difference, $\mu_{ep,A} - \mu_{ep,C}$) to be scanned by SimACE. Because the

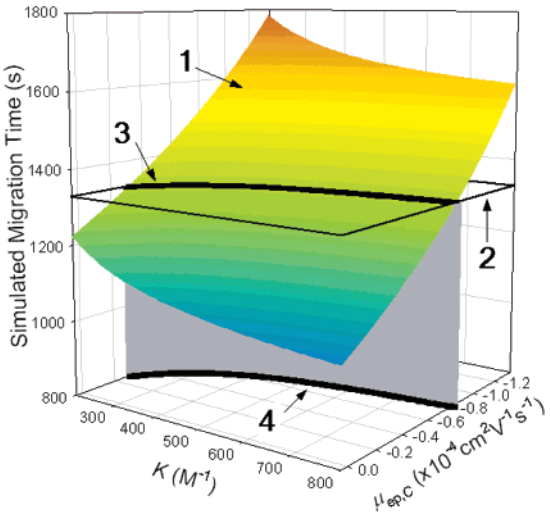

Figure 2. A 3-D surface which is cut through by a plane to produce a 2-D curve, which is then projected onto the bottom plane: (1) The 3D surface, (2) the cutting planes, (3) the intersection between 1 and 2, and (4) the projected intersection curve.

previous studies have shown that the binding constant for the 1:1 interaction between *p*-nitrophenol and *β*-cyclodextrin was between 500 and 600 M^{-1}, the range and interval of K were set at ~200 to 800 and 5 M^{-1}, respectively. The range of $\mu_{ep,A} - \mu_{ep,C}$ was $(-2.4 \text{ to } -1.0) \times 10^{-4}$ cm^2 V^{-1} s^{-1} with an interval of $5 \times 10^{-7}$$cm^2$ V^{-1} s^{-1}.

Because of the highly efficient algorithm, it usually takes only a few minutes on a Pentium III (866 MHz) computer to generate a 3-D surface as shown in Figure 2 that contains over 33 000 simulated ACE runs. Therefore, this method is still feasible even if a very large range needs to be tested when the binding constant is completely unknown.

Multiple ACE runs were carried out for each initial additive concentration. All of these runs have to be simulated separately. We cannot run just one simulation with the average experimental migration time and the average EOF time of the ACE runs of the same concentration settings. Because the migration times for the EOF markers for individual ACE runs are different, the migration time of the analyte plug can change significantly. Thus, taking averages would give rise to a new source of error.

Each simulation run will generate one output data file. The output is customizable, and it must contain at least three columns: the simulated migration time, the binding constant, and the complex mobility from which the simulated migration time is generated.

2-D Curves. The 3-D surface described in Figure 2 can be intercepted with planes of constant migration times. In this work, the planes are selected on the basis of the experimental migration time,

$$z \equiv T_{sim} = T_{exp} \pm \text{error} \qquad (4)$$

where error results from the measurements made in the experiment, as well as the simulation program itself. The intersection between these planes and the 3-D surface contains all pairs of K and $\mu_{ep,C}$ which can generate migration times equal to the experimental migration time with some associated errors. The

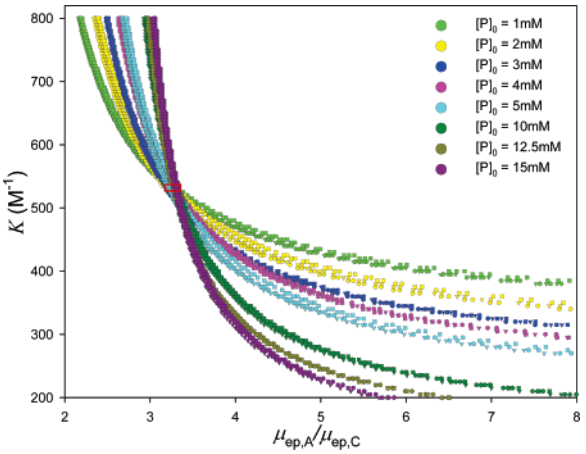

Figure 3. 2-D graph generated from SimACE and SimACEData with $\mu_{ep,A}/\mu_{ep,C}$ as the x axis. [p-Nitrophenol] = 2mM. Each curve is composed of data points. Each color of the curves corresponds to one additive (β-cyclodextrin) concentration. Three shapes (circle, square, and triangle) in one color correspond to three ACE runs under identical experimental conditions. The red rectangle indicates the intersection of eight sets of curves.

projection of all those points within the intersection on the K vs $\mu_{ep,C}$ plane gives a 2-D plot (Figure 2).

The data extraction program, SimACEData, is used to extract 2-D curves from the output data files. By default, the first column of a data file is the simulated migration time. Any row with a first-column value close to the experimental migration time within user-defined errors will be collected into a new output file, which can be imported into any data analysis programs, such as SigmaPlot, to plot the 2-D curves.

The Intersections. If another ACE experiment is performed with identical conditions except for the initial concentrations of the analyte or additive, the migration time resulting from the same K and $\mu_{ep,C}$ values will be different from the previous one, creating a different 3-D surface. A new 2-D curve can be obtained on the basis of the new experimental migration time. Because the two experiments used the same analyte and additive, the binding constant and the complex mobility should have the same values. Therefore, the two 2-D curves have to intersect. The coordinate of this intersection gives the values for the binding constant and the complex mobility. If more experiments are performed, the 2-D curves extracted from all 3-D surfaces should all intersect at one point, because there is only one true binding constant for a given binding interaction under specified conditions.

Because the conditions of the capillary and its environment could change from one run to another, the calculated $\mu_{ep,C}$ as well as $\mu_{ep,A}$ may vary slightly for any two ACE runs. The ratio of $\mu_{ep,A}$ to $\mu_{ep,C}$, instead of $\mu_{ep,C}$, is used as the x axis. The free analyte mobilities obtained from the runs just before an ACE run is used to calibrate the changes caused by slight temperature change, solvent evaporation, or other changes in capillary conditions. Figure 3 shows all 2-D curves generated by the simulation programs for the set of experiments with the initial concentration of p-nitrophenol at 0.2 mM with the x axis of $\mu_{ep,A}/\mu_{ep,C}$.

To obtain the binding constant and complex mobility, only two of these 2-D curves are needed, as demonstrated in Figure 4. Each of the curves can be fitted by the following equation,

$$y = \frac{a + x}{b + cx} \tag{5}$$

where a, b, and c are three constants defining the curve. The solutions of x and y of any two equations give the values for $\mu_{ep,A}/\mu_{ep,C}$ and K.

Depending on the information on the binding isotherm carried by the two experiments, however, errors on the obtained values can be different. When the experimental conditions that produced the two curves are significantly different, as depicted in Figure 4A–C, the intersections are very clear; however, when the

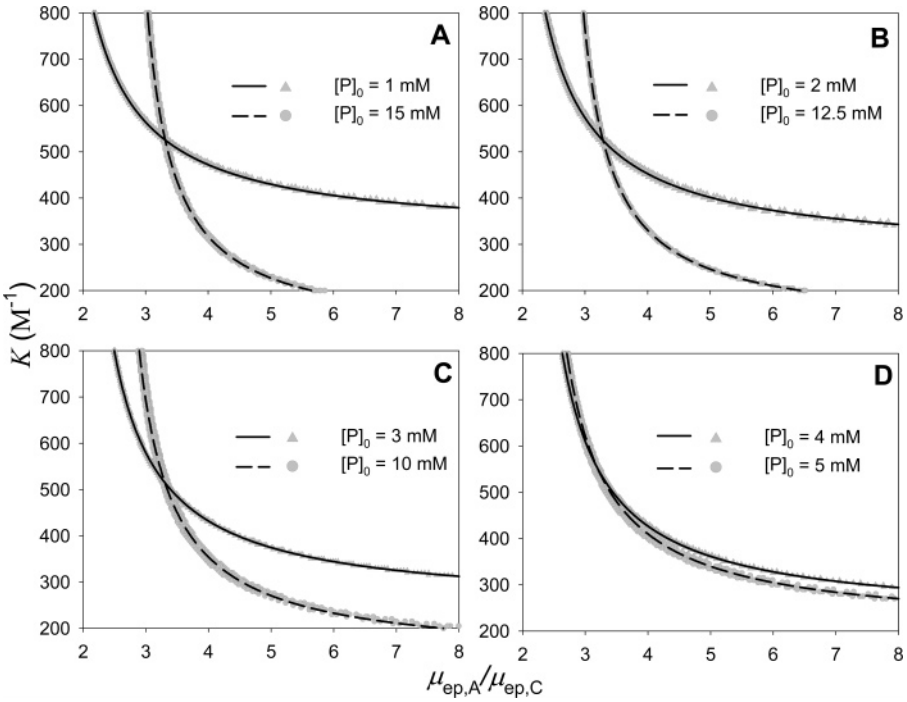

Figure 4. The intersections of two sets of curves: (A) 1 and 15 mM, (B) 2 and 12.5 mM, (C) 3 and 10 mM, and (D) 4 and 5 mM.

Table 1. Solutions, K (M^{-1}) and $\mu_{ep,A}/\mu_{ep,C}$, of Every Two Equations Representing the Curves Shown in Figure 3

Additive Concentrations (mM)	Additive Concentrations (mM)							
	1	2	3	4	5	10	12.5	15
1		513, 3.37	543, 3.15	**526, 3.31**	**529, 3.28**	**530, 3.27**	**529, 3.28**	**525, 3.31**
2			550, 3.12	513, 3.37	**521, 3.31**	**525, 3.28**	**524, 3.29**	**519, 3.33**
3				461, 3.69	497, 3.42	**515, 3.31**	**514, 3.31**	**508, 3.34**
4					550, 3.20	**539, 3.25**	**533, 3.27**	**524, 3.32**
5						**534, 3.26**	**529, 3.28**	**516, 3.33**
10							511, 3.31	482, 3.40
12.5								436, 3.50
15								

experimental conditions are similar, as shown in Figure 4D, the two curves become closer in shape, and the intersection can be less clearly defined. As discussed in two earlier papers,[3,4] the experiments have to cover a significant portion of the binding isotherm for an affinity interaction in order to accurately determine the binding constant and complex mobility. As shown in Figure 5, the concentrations used to generate curves in Figure 4A−C

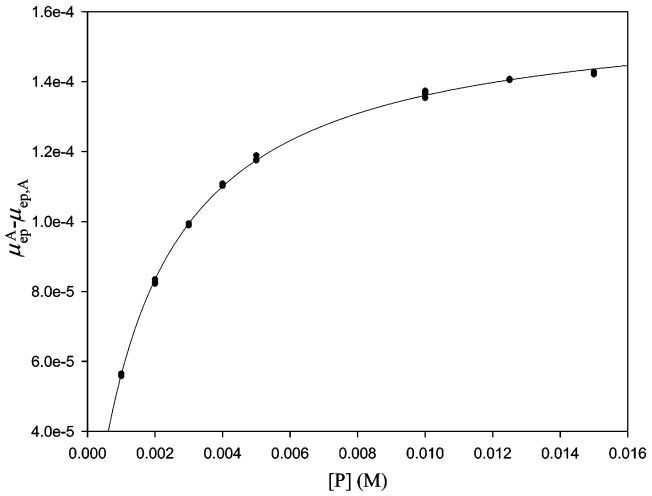

Figure 5. The binding isotherm of *p*-nitrophenol (A) and β-cyclodextrin (P).

are at significantly different portions of the binding isotherm, while the concentrations used in Figure 4D are too close to provide enough information. It should be noted that the constants could still be obtained in this case, albeit with a larger uncertainty, because the simulation program describes the analyte migration at any given moment, from a plug of pure analyte to that of fully equilibrated with the additive molecules in the BGE.

Table 1 lists the solutions of every two equations representing the curves. The solutions shown in the shaded cells should not be used because these pairs of curves are too similar to give clear intersections. The average K and $\mu_{ep,A}/\mu_{ep,C}$ in the remaining cells are 524 ± 8 M^{-1} and 3.30 ± 0.03, respectively.

Although two sets of experiments with two different additive concentrations are often enough to determine the binding constant and complex mobility, more experiments could provide added assurance and help to estimate errors in the obtained constants. Figure 6 shows three curves generated from the experimental

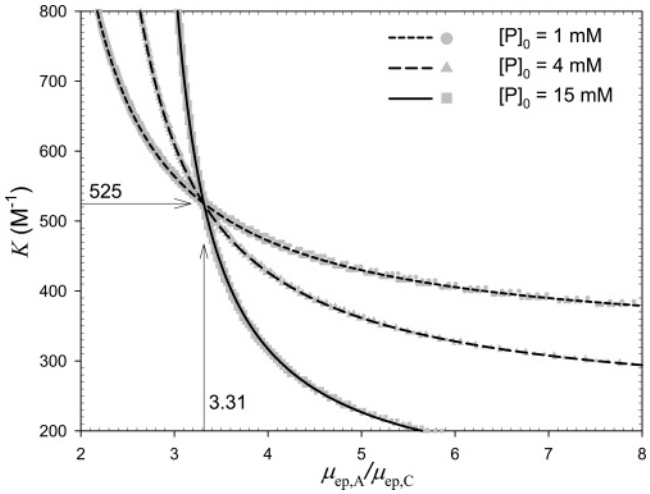

Figure 6. The intersection of three sets of curves: 1, 4, and 15 mM. Three different sets of solutions (K and $\mu_{ep,A}/\mu_{ep,C}$) can be obtained from the three equations representing the curves. The average K and $\mu_{ep,A}/\mu_{ep,C}$ were 525 M^{-1} and 3.31, respectively.

migration times obtained when the additive concentrations are 1, 4, and 15 mM. Three different sets of solutions can be obtained from the three equations representing the curves.

Enumeration Method vs Regression Methods. For the purpose of comparison, the binding constant for the 1:1 interaction between *p*-nitrophenol and β-cyclodextrin was also determined using the regression methods.

The binding constant obtained from nonlinear regression on the same set of data is 533 ± 7 M^{-1}, and from the three linear regression methods, *x*-reciprocal, *y*-reciprocal, and double reciprocal,[1] the values are 526 ± 6, 547 ± 10, and 534 ± 6 M^{-1}, respectively. These values are slightly different due to different variances within these regression methods.[3] All of these values agree with the one generated by the enumeration method.

There are three advantages of the enumeration method over the regression methods.

First, in theory, the enumeration method is based on the implemented simulation model of ACE and takes into account

every moment of the analyte migration. The migration rate could change due to the changes in local conditions, such as the concentrations of the free analyte and free additive, before equilibrium is reached.

On the other hand, all four regression methods are based on the average analyte mobility, assuming the analyte migrates with a constant velocity throughout the capillary. If $[A]_0$ is not much smaller than $[P]_0$ or the length of the injection plug is not infinitely narrow, the migration rate of the analyte plug could differ significantly from the average velocity, especially at the beginning of the ACE process when the equilibrium is not reached. Therefore, the binding constant and the complex mobility obtained from the enumeration method should be more accurate and less dependent on experimental conditions as long as the conditions are given to the simulation program.

Second, using the enumeration method, it is possible to obtain a binding constant with only two or three sets of the concentrations of the analyte and the additive as demonstrated in Figures 4 and 6. While using the regression methods, ~6 to 10 different concentrations of the additive are required. The enumeration method has the potential to be used for high throughput screening of combinatorial libraries.

Finally, the enumeration method is applicable to other types of interactions. The regression methods generally cannot be used for higher order binding interactions except for highly cooperative or noncooperative binding.[16] On the other hand, as long as the accurate simulation programs can be written, which in turn depends on the accuracy of the equations describing the analyte behavior, the enumeration method can be used. For a 1:2 interaction, one could in many cases use low concentrations of a higher order species so that the interaction is limited to a 1:1 ratio to obtain a first-order binding constant, then use higher concentrations of that species for the interaction to obtain the second binding constant.

CONCLUSION

The enumeration algorithm for determining binding constants itself is sound and solid. As long as the computer-simulation model is accurate, the algorithm can generate accurate results. Although the one-cell model for ACE simulation can only provide the peak positions, because of its single-cell-based calculation, the simulation is extremely fast. Thousands of ACE runs can be simulated within a few minutes, making the enumeration algorithm feasible for the determination of binding constants while simplifying the experimental procedures of ACE. Accurate results can be obtained regardless of the ratio of the initial additive and analyte concentrations.

To minimize the adverse effects of changeful capillary conditions, each set of experiments should be done continuously, and the experimental conditions must be controlled carefully. The simulation programs written in-house are combined with commercial data analysis programs to estimate binding constants by plotting 3-D surfaces, extracting 2-D curves, and finding the intersections among the 2-D curves. Using the K vs $\mu_{ep,A}/\mu_{ep,C}$ plane can help to eliminate the effect of the differences in capillary conditions during the ACE experiment.

Computer-aided research has become an increasingly important part of chemistry today. The idea of using the enumeration algorithm in determining physical and chemical properties by computer simulation has the potential to solve other chemistry problems when a direct mathematical approach is too difficult or not accurate enough when certain assumptions have to be used. The enumeration approach introduced in this paper is equally applicable to binding studies using techniques such as NMR, chromatography, and optical methods. It can also be used in all processes that can be described by rectangular hyperbolas, such as Michaelis−Menten kinetics.

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