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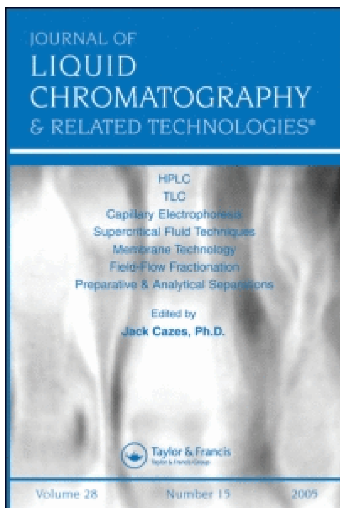

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
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Xiuli Lin[ab]; Christa L. Colyer[a]
[a] Department of Chemistry, Wake Forest University, Winston-Salem, North Carolina, USA [b] School of Pharmaceutical Sciences, Shandong University, Jinan, P. R. China

Online publication date: 22 June 2010

To cite this Article Lin, Xiuli and Colyer, Christa L.(2008) 'Chromatographic and Electrophoretic Methods for the Determination of Binding Constants for Dye-Protein Complexes', Journal of Liquid Chromatography & Related Technologies, 31: 11, 1620 — 1640

To link to this Article: DOI: 10.1080/10826070802126312

URL: http://dx.doi.org/10.1080/10826070802126312

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Journal of Liquid Chromatography & Related Technologies®, 31: 1620–1640, 2008
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ISSN: 1082-6076 print/1520-572X online
DOI: 10.1080/10826070802126312

Chromatographic and Electrophoretic Methods for the Determination of Binding Constants for Dye-Protein Complexes

Xiuli Lin[1,2] and Christa L. Colyer[1]

[1]Department of Chemistry, Wake Forest University, Winston-Salem, North Carolina, USA
[2]School of Pharmaceutical Sciences, Shandong University, Jinan, P. R. China

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Abstract: Basic knowledge of equilibrium conditions and the association behavior of any dynamic chemical system is important if one is to evaluate and understand that system. Binding constants for molecular associations can be determined by a variety of different approaches, each with its own advantages and disadvantages. This review examines various chromatographic and electrophoretic methods that have been developed to study dye-protein interactions. An overview of each technique is presented, along with a discussion of its strengths, weaknesses, and potential applications. Examples are provided that illustrate the use of these methods in determining the overall extent of dye-protein binding.

Keywords: Association constants, Capillary electrophoresis, Chromatography, Dye-protein interactions, Noncovalent binding

INTRODUCTION

Importance of Dye-Protein Interactions

The function of living systems is highly dependent on noncovalent molecular interactions. Characterization of these interactions is fundamental

Correspondence: Christa L. Colyer, Department of Chemistry, Wake Forest University, Winston-Salem, NC 27109, USA. E-mail: colyerCL@wfu.edu

to the understanding of biochemical and physiological processes.[1] Virtually all biomolecular processes involve molecular recognition and ligand binding by proteins. Selective and sensitive probes for such interactions are provided by a large number of dyes that form noncovalent fluorescent complexes with biomolecules. A review by Colyer,[2] in 2000, examined labeling dyes that are suitable for excitation and emission in the UV-visible and NIR regions of the spectrum, and that can be used for a variety of protein analytes. These dyes are believed to bind rapidly to proteins through electrostatic and/or hydrophobic interactions. Most of them require less stringent conditions than covalent derivatization of proteins and so provide the analyst with greater flexibility in sample preparation and handling methods. The association of proteins with dyes can be applied to protein purification and separation, and enzyme immobilization and activity control. For example, NIR dyes give stable and highly fluorescent complexes, which are well-suited for dynamic monitoring of protein changes at the single cell level.[3] Perhaps the best studied dye-protein system to date is the Cibacron Blue F3GA–HSA system, although this dye also interacts with other proteins.[4–8] Cibracron Blue, an anthraquinone dye, is widely used in affinity chromatography for purifying nucleotide-binding enzymes, and a number of kinases and dehydrogenases were purified on adsorbents with the immobilized dye.[4,5]

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Understanding the interactions between dye and protein can help us to recognize the structure, function and activity of the protein. A comprehensive understanding also requires quantitative data in the form of binding or equilibrium constants. Note that the terms equilibrium-, affinity-, complex-, binding- and stability- constants are used synonymously in the literature as they pertain to dye-protein interactions. The association constant K can be mathematically related to other analogous constants that are frequently used in its place. These include the dissociation constant (i.e., the reciprocal of K or $1/K$), the partition coefficient, P (that is, the ratio of the concentration of a solute in two phases or pseudophase, $K = V(P - 1)$ where V is the molar volume of the substrate or pseudophase), the partition ratio, k' (that is, the ratio of the amount of solute in one phase relative to the amount in the other phase: $K = k'\Phi$, where Φ is the phase ratio), and so on.

Numerous approaches have been explored and found useful for binding constant determinations. These include such techniques as spectroscopy, separations, calorimetry, potentiometry, solubility, and reaction kinetics.[1,9,10] Each approach has advantages and disadvantages, although separations-based and spectroscopic methods are perhaps the most widely used. An example of a spectroscopic determination is provided by the study of NN127 and SQ-3, two squarylium dyes, which were shown to have enhanced fluorescence upon complexation with

proteins such as BSA, HSA, b-lactoglobulin A and trypsinogen.[11] In this example, electrostatic interactions were found to be involved, and dye/protein stoichiometries in the noncovalent complexes were found to be 1:1 for SQ-3, although various possible stoichiometries were found for NN127 depending upon pH and protein concentration. Association constants on the order of 10^5 and 10^7 were found for noncovalent complexes of SQ-3 and NN127 respectively, with HSA[11] by spectroscopic methods.

Although chromatography has often been the preferred separations-based method for evaluating molecular associations,[10] the advent of commercial capillary electrophoresis (CE) instruments has meant that many of the separations-based methods used for determining binding constants have been adapted for CE. These methods, specifically as they pertain to dye-protein binding, are reviewed herein.

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General Theoretical Considerations

According to the multiple equilibria theory, the reversible binding of a ligand (such as a drug molecule or in this review, a dye "D") on a macromolecule (for example, protein "P") is governed by the equation:[12,13]

$$r = \frac{[D]_b}{[P]_t} = \sum_{i=1}^{m} n_i \frac{K_i[D]_f}{1 + K_i[D]_f} \tag{1}$$

where r is the ratio of the number of bound dye (dye–protein complex) molecules to total protein molecules; $[D]_f$, $[D]_b$ and $[P]_t$ are the concentrations of free dye, dye-protein complex and total protein, respectively; m is the number of types of independent binding sites; n is the number of sites of class i, and K_i is the corresponding binding constant. In order to obtain valid binding parameters by this formula, the equilibria between the constituents must be maintained without disruption throughout the chromatographic or capillary electrophoresis process used to study the equilibria.

COMMON METHODS FOR CALCULATING BINDING CONSTANTS BY CHROMATOGRAPHIC AND CAPILLARY ELECTROPHORESIS TECHNIQUES

There are two methods for calculating binding constants of dye-protein complexes that can be used both by chromatography and capillary electrophoresis. In the following discussion, the electrophoretic mobility in CE replaces the elution rate in chromatography, where appropriate.

Frontal Analysis (FA)

Frontal analysis (FA) affinity chromatography was first used in 1975 by Kasai and Ishii to study biological interactions.[14] Frontal analysis[15–20] is an accurate method to quantitatively compare different chromatographic adsorption systems. The use of CE-FA was inspired by the use of FA in size exclusion chromatography,[21,22] and was introduced by Kraak et al.[21] The principle of frontal analysis is as follows: A mixture of dye and protein is introduced into a size exclusion column or a fused silica capillary. The free and bound components are separated in the LC column or capillary. Provided the dye-protein complex and free protein have the same mobility and the free dye mobility differs from that of the dye-protein complex, two plateaus (which are attributed to free dye and to the dye-protein complex, which co-elutes with free protein) will result. The free dye concentration can be determined by an external calibration procedure, whereby plateau heights corresponding to a series of injections of fixed, known concentrations of dye alone are used to construct a calibration curve. By comparison of the free dye plateau height (designated as h_s) obtained during separation of a dye-protein sample to that obtained for the dye-only calibration standard (designated as h_c), it is possible to determine the free dye concentration $[D]_f$ as a fraction of the total dye concentration $[D]_t$ (assuming a linear response of the detector to dye concentration):

$$[D]_f = \frac{h_s}{h_c}[D]_t \quad (2)$$

From the free dye concentration thus determined, it is possible to find the concentration of dye bound to protein, as

$$[D]_b = [D]_t - [D]_f \quad (3)$$

and $[D]_b$ can be used to calculate the fraction r of dye molecules bound per protein molecule, as shown in Eq. (1).

One disadvantage of frontal analysis is the relatively large sample volume – typically between 20–200 nL[23,24] depending on the diameter of the capillary – required for each study. Another disadvantage of CE-FA had been its restriction to studying UV-absorbing compounds only, although recently this has been overcome by the introduction of CE-FA methods employing laser-induced fluorescence detection[25] and contactless conductivity detection.[26] However, it also has a distinct advantage in that frontal analysis can simultaneously provide information on both the association constant for a solute and its total number of binding sites in a column or capillary. This feature makes frontal analysis valuable in monitoring the stability of affinity columns during their use in the

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long-term studies.[27] More specifically, an attractive feature of CE-FA is that it is insensitive to changes or fluctuations in migration times, EOF, and applied voltage.[24] Investigations of the relationship between the ligand density of Cibacron Blue-immobilized porous and non-porous sorbents and protein-binding capacities and association constants was carried out by Wirth et al.[28] using a FA chromatographic method. In this case, the magnitude of the association constants for the lysozyme-Cibracron Blue system were found to be dependent on the ligand density of the sorbent. With decreasing ligand density, the protein-ligand interaction became stronger; that is, K_a values became larger.

The Hummel-Dreyer Method (HD)

The original Hummel and Dreyer method was developed on soft gel columns and the separation was based on size exclusion.[29] The adaptation of this new method to HPLC greatly improved the resolution and rapidity while reducing the injection and elution volumes, which is advantageous in the case of expensive or limited products. Like the FA method, the Hummel-Dreyer method assumes that the dye-protein complex and free protein have the same mobility and that the free dye mobility differs from that of the dye-protein complex. The principle of the Hummel and Dreyer method is as follows: the dye is dissolved in the run buffer (or mobile phase) at varying concentrations, creating a high detector background response. A fixed, discrete amount of protein sample is dissolved in the run buffer before being injected into the column or capillary. As the protein migrates or travels through the dye-containing buffer or mobile phase, it will become labeled with dye to an extent governed by the association constant. A positive peak (dye-protein complex plus any co-migrating free protein) and a negative peak (related to the depletion of dye from the mobile phase) will result. The area of the negative peak is directly related to the amount of dye bound to the protein, and hence, bound dye concentrations can be measured by this method. The HD method assumes that the concentration of free dye in the buffer $[D]_f$ is constant and equal to the total concentration of dye added to the running buffer $[D]_t$. An external calibration method involves the injection of buffer alone (not containing dye or protein) into a column or capillary filled with buffer (or mobile phase) and dye. The resulting negative or vacancy peak area A_c of this calibration standard is compared to the negative peak area arising from the injection of a protein sample A_s in order to calculate the bound dye concentration:

$$[D]_b = \frac{A_s - A_c}{A_c}[D]_f = \frac{A_s - A_c}{A_c}[D]_t \quad (4)$$

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Analogous to the FA method described previously, the calculated bound dye concentration in HD can be used in conjunction with Eq. (1) to determine the fraction r of dye molecules bound per protein molecule. A particular advantage of the Hummel and Dreyer method is the control of the free dye concentration as an independent variable, on which depends the bound protein ratio. HD can be used to determine the number of binding sites and their association constants, even in the case of low affinity.

FA and HD both rely on the fact that the dye-protein complex must be resolved from the free dye, so the two methods can be used to validate each other. Both methods have been implemented with CE to determine association constants and stoichiometries of noncovalent complexes of the red luminescent squarylium dye Red-1c with bovine serum albumin (BSA) and β-lactoglobulin A by Yan and Colyer.[25] For example, the CE-HD results shown in Fig. 1 led to the determination of an association constant of $4.7 \times 10^5\ M^{-1}$ for the Red-1c – BSA complex at pH 9.5, and although this confirmed the results of the analogous CE-FA study, the CE-HD results were less precise. Furthermore, the binding of some

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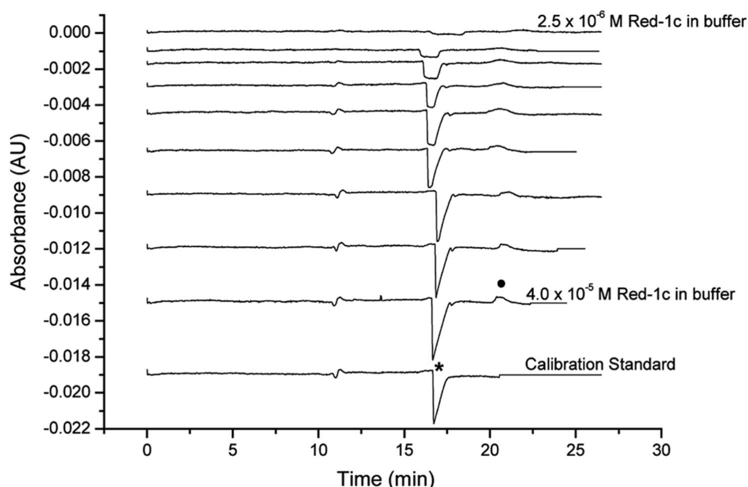

Figure 1. Electropherograms of BSA – Red-1c complexes in 100 mM boric acid buffer (pH 9.5) with 100 mM added KCl, with a negative peak due to vacancy of free dye (*) and a positive peak due to protein—dye complex along with free protein (•), according to the CE-HD method. Experimental conditions: 50 µm I.D. × 50 cm (45.4 cm effective length) uncoated capillary; 0.2 s injection at 5 psi of 4×10^{-6} M BSA sample; running buffer containing Red-1c, ranging in concentration from 2.5×10^{-6} M (top) to 4×10^{-5} M (2nd from bottom) in increments of 0.5×10^{-6} M; 10 kV separation voltage with resulting current 85 µA; absorbance detection at 607 nm. Electropherograms are vertically offset for clarity. *Source*: Reproduced from ref.[25] with permission from Elsevier.

sulfonic textile dyes to trypsin were investigated by Schneedorf et al.[30] using the gel permeation equilibrium technique of Hummel-Dreyer.[29] In acid media, Schneedorf et al. found apparent dissociation constants of 46, 6, and 61 mM for trypsin with flavianate, orange II, and orange G, respectively.

CHROMATOGRAPHIC TECHNIQUES FOR CALCULATING BINDING CONSTANTS

The use of an immobilized ligand in a chromatographic system for the study of biomolecular interactions is a method known as analytical or quantitative affinity chromatography. This may be performed on either a low-performance or high-performance support. When using silica or another HPLC-type matrix, the method is called high-performance affinity chromatography (HPAC). A book edited by Chaiken gives a general overview of the experimental approaches used in analytical affinity chromatography.[31]

Affinity chromatography can be used to measure equilibrium constants and kinetics of biological interactions. In affinity chromatography, immobilized ligands affect the partitioning of the solute (a soluble biomolecular analyte of interest) between the mobile and stationary phases, and hence, affect the analyte's retention time. Additionally, the kinetics of the binding interactions and the rate of diffusion of the biomolecular analyte with respect to the immobilized ligand influence the peak breadth. As such, careful consideration of peak shapes and retention times in affinity chromatography can lead to the determination of binding constants and rate constants for the interaction of the solute with immobilized ligand. The interaction mechanism between dye ligand and protein molecule includes the complex combination of electrostatic, hydrophobic and hydrogen bonding.[32]

A number of ligands can be effective for affinity chromatography, although commercially reactive dyes are ideal due to a variety of factors, such as their low cost, stability, ease of immobilization on the solid support, and high binding capacities.[33] Roschlan and Hess first established the utility of reactive dye affinity chromatography as a protein purification process in 1972,[34] when they immobilized Cibacron Blue by direct covalent binding onto Sephadex G-200 in order to purify yeast pyruvate kinase. Triazinyl-based reactive dyes are probably still the most widely used in protein purification. For example, reactive chlorotriazines such as anthraquinone dyes can serve as immobilized ligands for a broad range of proteins.[35]

Analytical affinity chromatography can be performed by either of two different procedures: zonal elution or frontal analysis. The latter,

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suitable for measuring equilibrium constants over a wide range of concentrations, was discussed under Frontal Analysis. The former, suitable for measuring equilibrium constants at low concentration, is discussed presently.

Zonal Elution

In zonal elution experiments the solute is introduced as a (short) pulse, and the elution volume is determined from the first moment of the appearance of the effluent peak. Current zonal elution theory[36,37] is based entirely on the assumption that one is operating in the linear region of the equilibrium isotherm. Separation techniques must be extremely rapid in the case of loosely bound complexes, which is prejudicial to their efficiency, in particular for zonal chromatography. Thus this method cannot be used with ligand – protein associations with constants inferior to $10^6\ M^{-1}$.[38] Although zonal elution methods are suitable for both electrophoretic and chromatographic assays, direct separation of free- and protein-bound fractions is seldom employed in either case. Furthermore, the more common zonal elution format for electrophoresis – namely, the mobility shift assay – has been used extensively for a number of solute-protein systems,[39] but not for the study of dye-protein interactions. As such, we have chosen to restrict our discussion of zonal elution methods to this section on chromatographic techniques, which specifically involve dye-protein binding.

The adsorption of BSA onto an immobilized blue triazine dye was studied by zonal and frontal elution chromatography to exemplify the use of the preceding theory by Arnold et al.[36,37] From this work, frontal elution was found to be the preferred technique for measuring the kinetics of biological molecules exhibiting strong interactions. Zonal methods have relied on the assumption of a linear equilibrium isotherm, an assumption that is unjustified for most biological systems. For highly nonlinear isotherms, the frontal elution method is preferred. A theory for nonlinear zonal elution has been presented by Liu and Stellwagen,[40] who studied the interactions between Cibacron Blue-modified Sepharose and lactate dehydrogenase using the method of zonal chromatography analysis. In their work, it was found that both the average valence and the association constant decrease with an increase of the phosphate concentration. They attributed this phenomenon to the strong binding of dye–ligand to the Sepharose matrix surface at high salt concentration. The high ionic strength of the dye–ligand and the agarose matrix resulted in the decrease in ligand density accessible to protein adsorption.[40,41]

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The Langmuir and Freundlich Isothermal Models used in Dye-Ligand Chromatography

Reactive dye affinity chromatography is a robust and economical alternative to traditional affinity chromatography.[42] To successfully design an affinity step, a thorough understanding of the fundamental mechanisms underlying such separations is an important requirement.[43] The adsorption isotherms and interaction mechanisms involved in dye-ligand chromatography are of great importance since they may predict the dynamic behavior of scaling-up and controlling the system.[44] The model usually employed for affinity dye-ligand systems is the Langmuir and Freundlich isothermal model. The Langmuir model[45] represents a simplified case of protein adsorption. It is based on the assumption of homogeneity such as equally available adsorption sites, monolayer surface coverage, and no interaction between adsorbed species. The model is described by the following equation:

$$q^* = \frac{q_m c^*}{K_d + c^*} \tag{5}$$

where K_d represents the dissociation constant, c^* is the concentration of the protein in the mobile phase (bulk solution), q^* is the amount of protein adsorbed onto the matrix and q_m is the maximum capacity of the matrix.

The Freundlich isotherm[46] describes reversible adsorption and is not restricted to the formation of the monolayer. This empirical equation takes the form:

$$q^* = k(c^*)^n \tag{6}$$

where q^* and c^* are as defined for Eqn. (5), and where k is the velocity constant of the Freundlich isotherm and n is the Freundlich isotherm exponent (dimensionless), which is correlated with the type of interactions (attractive or repulsive) between the adsorbed chemicals.[45] A Freundlich isotherm implies that no theoretical limit exists for a maximum capacity of the adsorbent.

Katsos et al.[47] studied the binding and elution of l-glutamate oxidase (GOX) by way of affinity adsorbents prepared by the immobilization of a number of commercial textile dyes and biomimetic dyes bearing a ketocarboxylated-structure as their terminal biomimetic moiety on cross-linked agarose gel. Here, the dye-protein affinity followed the Langmuir isotherm model. Various other practical examples of the use of the Langmuir isothermal model[48] can be found in the scientific literature and include the adsorption of lysozyme onto Blue-Sepharose,[49] and the adsorption of BSA, gamma-globulin and alcohol dehydrogenase onto Cibacron Blue immobilized onto Sepharose CL-6B.[50] A single-step dye

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affinity chromatographic separation method was developed by Ouyang et al.[42] utilizing commercially available triazinyl-based reactive dyes, which were screened for their binding affinity to enzymes of interest. These were secreted alkaline phosphatase (SEAP) from a CHO cell culture, and glucoamylase from *Aspergillus niger* fermentation. The Langmuir isotherm was followed in the case of SEAP adsorption on the dye-ligand affinity resin and elution of the adsorbed enzymes from the dye ligands by enzyme substrates or salts was used to obtain relatively pure enzyme products in this work.

CAPILLARY ELECTROPHORESIS TECHNIQUES FOR CALCULATING BINDING CONSTANTS

The use of CE for binding studies of pre-equilibrated samples, which has taken place since the early 1990s both in the frontal analysis and in the zone electrophoresis formats,[21,51–53] builds on theory developed for moving boundary electrophoresis and affinity chromatography.[54–57] Currently, several CE methods are available to measure binding constants, e.g., affinity capillary electrophoresis (ACE), Hummel–Dreyer method (HD), vacancy affinity capillary electrophoresis (VACE), vacancy peak method (VP) and frontal analysis (FA).[21,22,58–60] With the exception of VACE, these methods were primarily developed for high-performance liquid chromatography and have since been transferred to CE.[61,62] A thorough comparison of the various CE binding study methods and their limitations was presented by Busch et al.[22,58] and Tanaka and Terabe,[63] and so only those methods employed for the study of dye-protein interactions in particular will be elaborated upon here.

Nonequilibrium Capillary Electrophoresis of Equilibrium Mixtures Method (NECEEM)

In conventional CE methods that involve injection of a discrete plug of premixed probe-target (dye-protein in this case) sample solution, equilibrium conditions are quickly abandoned once the probe-target complex is resolved from free probe and from free target as they migrate through the capillary in separate bands. In this instance, once equilibrium conditions have been violated, it is more appropriate to utilize the method of nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM), as developed by Krylov and coworkers,[64–66] to study probe-target binding. Additionally, NECEEM can provide kinetic data regarding the probe-target complexation reaction and so is a valuable tool in the study of biomolecular interactions. Although Krylov's original work focused

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on protein-DNA complexes, the utility of NECEEM principles for quantifying a variety of dye-protein interactions have been carried out most recently in our laboratory.

The method is based on the assumption that the noncovalent complex is initially in equilibrium with the free forms of the probe and target molecules (protein and dye in this case) when the mixture is introduced as a discrete sample zone into the capillary. Upon application of an electric field to initiate separation, the dynamic equilibrium process of complex association and dissociation cannot be sustained as the free dye and free protein zones are physically removed from the complex zone in the evolving separation. For example, as some of the complex dissociates, the resulting free dye will migrate away from the zone of the complex and towards the zone of the free dye, while the resulting free protein will migrate away from the zone of the complex and towards the zone of the free protein. The positions of these zones relative to one another depend upon their mobilities under the chosen separation conditions. Dissociated dye and protein molecules 'leaving' the zone of the complex are responsible for the fronting and/or tailing observed around the peak attributed to the complex. These various zones are observed in Fig. 2 for the noncovalent dye Red-1c with β-lactoglobulin B. Areas below the free dye and the dye-protein complex in the electropherogram (abbreviated A_D and $A_{P\text{-}D}$, respectively, in Fig. 2) are proportional to the concentrations of free dye and intact protein—dye complex at the detector. Also evident in Fig. 2 is a third region of interest between the free dye and complex peaks. The area (denoted A_{dissoc}) below the exponential curve

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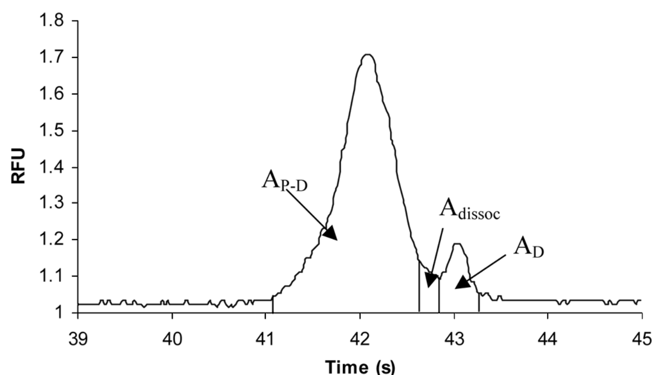

Figure 2. NECEEM electropherogram of pre-column labeled 75 μM β-lactoglobulin B with 5.0 μM Red-1c. Separation conditions employed by microchip CE system: 100 mM boric acid buffer with 100 mM KCl (pH 9.41); 30 s injection at 500 V; 7 cm separation channel length (10 mm from injection point to detector); 2.5 kV separation voltage.

in this region is attributed to dye liberated from the complex as it migrates through the capillary and experiences dissociation to an extent governed by its dissociation constant K_d.

It has been shown[64–70] that the association constant can be determined experimentally by recording a single electropherogram and measuring the areas under the free dye, dye-protein complex, and dissociation regions, according to the expression:

$$K_a = \frac{1 + \left(\frac{A_{P-D} - A_{dissoc}}{A_D}\right)}{[P]_o\left(1 + \frac{A_D}{A_{P-D} + A_{dissoc}}\right) - [D]_o} \qquad (7)$$

where $[P]_o$ is the initial, total protein concentration, $[D]_o$ is the initial, total dye concentration, and all other symbols are as previously defined.

Several assumptions are inherent to Eqn. (7). First, the equation assumes that the fluorescence quantum yield of the noncovalent complex does not differ from that of the free dye. Since this is often untrue for good noncovalent probes when complexed with their protein targets, a suitable modification of Eqn. (7) becomes:

$$K_a = \frac{1 + \left(\frac{A_{P-D} \cdot (\varphi_D/\varphi_{P-D}) + A_{dissoc}}{A_D}\right)}{[P]_o\left(1 + \frac{A_D}{A_{P-D} \cdot (\varphi_D/\varphi_{P-D}) + A_{dissoc}}\right) - [D]_o} \qquad (8)$$

where φ_D/φ_{P-D} stands for the relative fluorescence quantum yield of the dye alone φ_D and the dye-protein complex $\varphi_{P\text{-}D}$. The relative fluorescence quantum yield can be determined experimentally by a number of different methods, such as the comparative method of Williams et al.[71] Second, inherent to Eqn. (7) is the assumption that the protein and dye interact on a one-to-one basis, but simple algebraic manipulation of the equation permits its use with other binding stoichiometries. Unpublished work from our own laboratory has utilized the NECEEM method both with conventional and microchip CE systems to evaluate the binding constants of a squarylium dye Red-1c with BSA and β-lactoglobulin B, which were found to range from $3.02 \times 10^4\ M^{-1}$ to $1.39 \times 10^6\ M^{-1}$.

Mobility Moment Analysis Method

Although NECEEM permits the determination of kinetic constants of interactions along with equilibrium constants, it requires peak identification and quantification. Estimation of contributions to a composite peak from the free form of the analyte and the dissociated complex during separation in not always a trivial matter. To overcome such

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limitations, the method of mobility moment analysis is described by Shimura et al.[72] In this work, ovalbumin and concanavalin A labeled with the succinimidyl ester of Alexa Fluor 488 carboxylic acid dye was chosen as an example of a weakly binding system. By Shimura's method, mixtures of Alexa Fluor-labeled protein and its unlabeled, interacting partner (ovalbumin) at various concentrations were injected into an uncoated capillary. The changes in the mobility moments of the fluorescent-labeled molecules were used to determine the dissociation constants of the complexes. Since peak identification is not necessarily required by this method, it has the advantage of utility for heterogeneous samples, and so its potential for dye-protein studies in the future appears great.

Dye-Displacement Capillary Electrophoresis Based Method

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Since many of the traditional electrophoretic methods for binding constant determinations are time-intensive and require a relatively large amount of material, they are not well-suited for use in high-throughput applications. Alternatively, a NIR dye-displacement capillary electrophoresis based method was evaluated by Sowell et al.[73] for determining binding constants of drugs toward subdomain IIIA of human serum albumin. The dye-displacement technique is based on a competitive interaction between the drug of interest and a dye probe. In this example, a heptamethine cyanine dye was used in competition with a variety of drugs such as ketoprofen, ibuprofen, quinidine, naproxen, imipramine, and clofibrate for binding at subdomain IIIA of serum albumin. Preequilibrated samples containing a drug, the dye, and HSA were introduced into the capillary under zone electrophoresis conditions. Drugs interacting with the subdomain site II were capable of displacing the dye from HSA, as illustrated in Fig. 3. Spontaneous dissociation of the dye from HSA during electrophoresis was assumed to be negligible.[73] Based on a calibration curve prepared using drug compounds with known HSA binding constants, the affinity of new compounds was estimated from a single experimental run.[73] Thus, the authors assert that the method is faster, consumes less reagent, and is more amenable to high-throughput formats than other CE-based affinity methods. It should be noted, however, that the assay is only feasible for probing affinities for one particular HSA binding site.

Partial Filling Multiple Injection Affinity Capillary Electrophoresis (PFMIACE) Method

Recently, partial filling multiple injection affinity capillary electrophoresis (PFMIACE) was introduced by Zavaleta et al.[74] to estimate

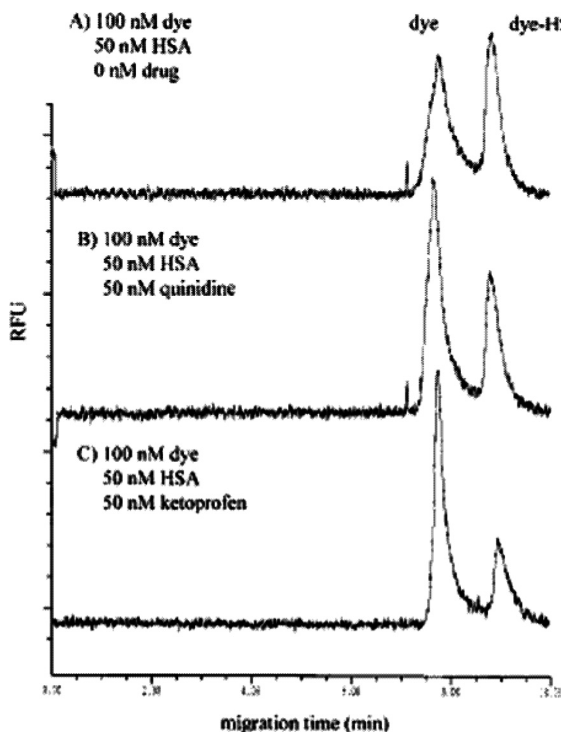

Figure 3. Electropherograms illustrating the competitive binding of ketoprofen and quinidine. (A) 100 nM dye and 50 nM HSA; (B) 100 nM dye, 50 nM HSA, and 50 nM quinidine; (C) 100 nM dye, 50 nM HSA, and 50 nM ketoprofen. *Source*: Reproduced from Ref.[73] with permission from Wiley-VCH Verlag GmbH & Co.

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binding constants of receptors to ligands. This work is an extension of the earlier methods of multiple-injection ACE[75] and of partial-filling ACE.[76] As illustrated in Fig. 4, the PFMIACE method involves the sequential injection of multiple samples of receptor (or ligand) prior to their electrophoresis in a buffer solution with an increasing concentration of ligand (or receptor). The glycopeptide antibiotics vancomycin (Van), tcicoplanin (Tcic) and ristocctin (Rist) were used as model receptors and the enzyme carbonic anhydrase B was used as a model ligand in Zavaleta's work. By either of two variations of the PFMIACE method, changes in the migration time of the receptors were used to estimate binding constants. The purported advantages of this method over more traditional ACE methods include smaller sample requirements and faster analysis times, since a single electrophoretic run per ligand concentration is sufficient to yield multiple binding constants concurrently, and this, in

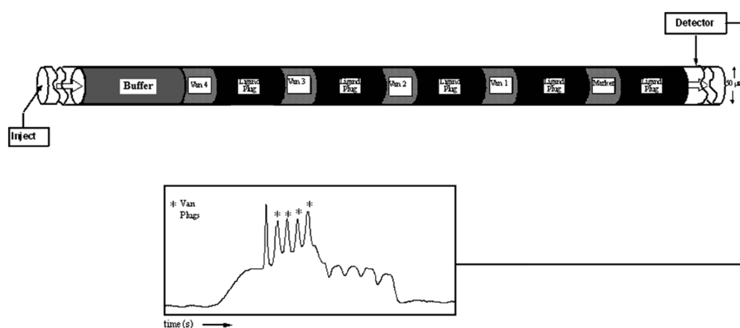

Figure 4. Schematic of partial-filling multiple injection affinity capillary electrophoresis (PFMIACE) experiments. *Source*: Reprinted from ref.[74] with permission from Elsevier.

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turn, leads to less opportunity for instrumental error or variability in migration times. Presumably this method could be extended for use with dye-protein systems in the future.

CONCLUSIONS AND PERSPECTIVES

The many papers devoted to an examination of the development of theoretical frameworks and limitations of affinity CE methods also illuminate both the widespread use of these methods and the requirement for careful data interpretation, especially when deriving quantitative estimates of binding interactions. Between these studies of pure applications and pure theoretical deliberations are needed more systematic studies comparing theory and practice and possibly involving the simulation of peak profiles in order to establish reliable guidelines for CE-based binding studies. The appealing simplicity of the experimental procedures and their broad versatility should ensure an even more widespread use of the methods for affinity studies in other disciplines such as molecular biology, immunology, toxicology, and related sciences.

Chromatography and electrophoresis have been useful not only for preparative purposes but also for the characterization of biomolecules. Binding constant calculations facilitated by chromatography and electrophoresis can provide essential information related to the structure or function of biomolecules, and can allow us to probe the very fundamental nature of biomolecules forming the basis of every biological process. Various factors may affect the accuracy and the reproducibility of binding constant determinations, and these must be carefully controlled. For example, the temperature of the mobile phase in chromatography,[77] or

inside the capillary in electrophoresis[61] must be controlled, since a decrease in temperature can induce an increase in the binding constants. In certain cases, self-association of proteins may have an influence on dye-protein interactions, since this influences the free protein concentration, and a decrease in the binding constants is generally observed when the protein concentration increases.[22,78] Progress in HPLC technology and automation have reduced columns sizes and, hence, the volumes of eluent. This more closely mimics the sample needs in CE, and is advantageous in the case of expensive dyes or hard-to-obtain macromolecules.

Beyond the realm of small molecules and biomolecules, CE analyses of cells and subcellular particles are still in their infancy. A central issue is that the heterogeneity of cells such as bacteria and viruses is detrimental to reproducible analyses, and it is clear that for such systems any attempts at quantitative binding assays using migration shifts or peak area measurements by CE will be challenging. However, proteins on the surface of molecular assemblies and microbes of interest can serve as useful handles by which analysts may manipulate these important analytes. By exploiting noncovalent interactions between protein-based analytes and new fluorescent probes, much is to be gained in terms of analytical sensitivity, selectivity, and separation efficiency.

Understanding the nature of interactions and the composition of the resulting complexes of molecular or cellular analytes with dyes represents an important area of study that can be facilitated by CE and chromatography. The ability to quantify the strength of noncovalent interactions between a specific fluorescent probe molecule and protein analytes of interest, for example, can serve to inform the choice of separation conditions best suited to both separation efficiency and sensitivity. Also, it is anticipated that a quantitative measure of dye—protein association constants will permit the intentional design of probes in order to take advantage of observed selectivities for handling particularly complex forensic, environmental, or clinical samples.

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