

REVIEWS

Recent Advances in Affinity Capillary Electrophoresis (2007)

XIAOJUN LIU, FROSEEN DAHDOUH, MARISOL SALGADO, FRANK A. GOMEZ

Department of Chemistry and Biochemistry, California State University, Los Angeles, 5151 State University Drive, Los Angeles, California 90032-8202

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ABSTRACT: Papers detailing the use of affinity capillary electrophoresis (ACE) in examining binding parameters between biological species are summarized in this review. The works cited in this review were published during 2007 and were drawn from the major chemical and biochemical journals and especially the major analytical chemistry literature. © 2008 Wiley-Liss, Inc. and the American Pharmacists Association *J Pharm Sci* 98:394–410, 2009

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INTRODUCTION

The human body, in its grand complexity, is like no other biological species on earth. With its countless chemical and biological components and number of reactions and events occurring simultaneously, it is impossible to imagine how anything as ingenious could be constructed by man. Given the intricate nature of how these different components are intertwined and timed to switch on and off, react and maneuver within cells, fluids and organs of the body, their influence on a person's health are tremendous.

Molecular recognition is the starting place of essentially all biological processes and, hence, much of medicine. All cellular functions in the human body depend on protein–protein, protein–small molecule, protein–nucleic acid, or other intermolecular interactions. These biological interactions are essential to life and

are involved in all of the enzyme-based reactions involved in cell division, cell death and cell transformation. Hence, biological interactions are important in the initiation, progression, and effects of all human disease including Parkinson's, Alzheimer's, AIDS-HIV, and cancer.

There are a variety of techniques available to measure affinity parameters between biological species including equilibrium dialysis, radioimmunoassay, fluorescence quenching, ultracentrifugation, nuclear magnetic resonance and slab gel electrophoresis. If the amount of bound and free ligand in solution can be distinguished, these techniques can provide reasonable estimates of binding constants (K_b).

One method that has been shown to be a versatile microanalytical technique to estimate this extent of interaction is affinity capillary electrophoresis (ACE). In only 15 years, ACE has emerged as a useful and sensitive technique for studying bimolecular noncovalent interactions and for determining binding and dissociation constants of formed complexes. In 1992, the first reports detailing the use of ACE to measure affinity parameters between

Correspondence to: Frank A. Gomez (Telephone: 323-343-2368; Fax: 323-343-6490; E-mail: fgomez2@calstatela.edu)

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biological species were published.^{1–7} Since then, many interactions including protein–ligand, peptide–metal, peptide–peptide, protein–peptide, protein–antibody, polymer–peptide, antibody–antigen, enzyme–drug, and polymer–cyclodextrin have been examined successfully using ACE.^{8–10}

In general, there are three different types of ACE. In the first type, affinity ligand and receptor are first premixed and an aliquot of the solution analyzed by CE. This form of ACE has been used successfully in the analysis of DNA–protein, DNA–DNA, and DNA–small molecule interactions because many of these interactions have appreciably slow k_{on} (association rate constant) and k_{off} (dissociation rate constant) rates and premixing of ligand and receptor are required for formation of complex. In type two, the mobility of a receptor (or ligand) changes upon binding to a ligand (or receptor) that is present in the electrophoresis buffer. The change in migration time can be correlated to a binding constant via Scatchard analysis or other form of analysis. In type three, the ligand is immobilized to the capillary wall, and beads or other matrix are used as solid support. Receptor present in solution then flows through the capillary and binds to the immobilized ligand. This technique is used primarily to capture and enrich a substance of interest as well as for cleaning, desalting, and removing unwanted impurities. For example, there is considerable work in the use of immunoaffinity CE (IACE) for the capture and enrichment of numerous analytes. The advantages of this technique are small sample volumes, high-throughput, and sample automation. Moreover, the technology has progressed such that limits of detection (LOD) compare favorably to those found in HPLC and even the enzyme linked immunosorbent assay (ELISA).

The physicochemical properties of small molecules with potential biological activity are critical parameters that have a major impact on determining the extent a molecule will be absorbed in a living organism, as well as on formulation and drug delivery. In the pharmaceutical sciences the ability to determine these properties of drug compounds is critical. Due to the high cost of drug discovery and development, techniques that allow for rapid screening of potential products are highly prized. A study in 2003 reported that the cost for discovering, developing and launching (including marketing and other business expenses) a new drug (including other prospective drugs that failed) rose over a 5-year period to

nearly \$1.7 billion.¹¹ ACE has successfully been applied in the pharmaceutical industry for the analysis of basic drugs, pharmacokinetic profiling, bioavailability determinations, plasma protein binding studies and drug activity level determination. This review will focus on the chemical literature in 2007 and will highlight those representative publications that are most relevant to research in the pharmaceutical area.

DISCUSSION

Protein–Drug Interactions

The use of open-tubular capillary columns of lengths greater than 20 cm is a general requirement in ACE when using commercial instruments. Only homebuilt CE instruments have the versatility to use much shorter capillaries or, in the case of microchips, channels of less than 2 cm in length. The use of standard length columns is not amenable in some ACE studies and especially when the binding interaction has fast kinetics. Yang et al.¹² have employed high-speed CE with laser-induced fluorescence (LIF) to examine the binding and inhibition of SH2 domain proteins using fluorescently labeled phosphopeptides. In a typical assay SH2 domain proteins are mixed with fluorescent peptide(s) and are separated by CE. The protein–peptide complex and free peptide are detected and their peak height used to generate a binding isotherm. Multiple SH2 proteins could be detected simultaneously. In addition, one inhibitor could be screened against several SH2 domains in a multiplexed assay. The use of high-speed CE in examining binding interactions has great potential when kinetics dictate a change in standard ACE conditions.

The use of proteins as chiral selectors is well known in enantiomeric separations. Fagerstrom et al.¹³ have used cellulose cellobiohydrolase I (Cel7A) to investigate the effect of pH on affinity and enantioselectivity for a series of propranolol derivatives. The extent of interaction is correlated to structural changes in the ligands and, therefore, to the importance of hydrophobicity and electrostatics. It was found that the affinity increased at pH 7.0 compared to 5.0. Further studies involved inhibition of the enzyme catalysis to prove that the ligand interaction with Cel7A occurred at the active site.

Chloroquine (CQ) is an inexpensive and useful antimalarial drug used in the prophylaxis and treatment of malaria. Huang et al.¹⁴ have developed

a sensitive method for the estimation of CQ and its separation from other pharmaceuticals using CE and end-column electrochemiluminescence (ECL) detection. The technique was subsequently used to investigate the interaction of human serum albumin (HSA) to CQ ($K_b = 7.7 \times 10^3 \text{ M}^{-1}$) and in determining the number of binding sites. The CE-ECL technique may be further applied to other pharmacokinetic studies.

Protein-carbohydrate complexes are critical in growth control, cell adhesion and cell differentiation as well as autoimmune diseases, inflammation and viral infection. Understanding the binding between carbohydrates and proteins is still problematic and especially in determining the structure of the carbohydrate ligand, binding sites on the protein and in protein conformational changes on binding. Fermas et al.¹⁵ have employed frontal analysis CE (FACE) with electrospray ionization mass spectrometry (ESIMS) to characterize the antithrombin-heparin pentasaccharide complex. This work is a follow-up to their earlier work employing ACE to examine the binding of sulfated oligosaccharides to antithrombin and other proteins¹⁶ that did not provide structural data about the bound carbohydrate sequence. It provides information on specific carbohydrate ligands a difficult task since most biologically active carbohydrates (polysaccharides and glycoproteins) are frequently available as heterogeneous mixtures. FACE is a mode of ACE whereby a receptor and ligand are preincubated prior to injection onto the capillary column. Upon electrophoresis the free and bound forms of the protein are separated. The heights of the peak fronts represent the free protein and the free and bound forms of the protein and can be used to estimate a binding constant. Coupling to MS allowed for the determination of the binding stoichiometry (1:1 and in agreement with previous studies) and the characterization of the specific bound carbohydrate in the complex. This work opens the way to identify specific biologically active oligosaccharide sequences in mixtures that bind to proteins.

Drug binding to serum proteins is an important area in drug discovery and development given the numbers of proteins (over 60) in human plasma. Hence, there is a need to develop novel techniques to evaluate the extent of drug-plasma protein interactions. Martinez-Gomez et al.¹⁷ have used CE frontal analysis to evaluate the extent of interaction between 17 antihistamines and HSA and α -acid glycoprotein (AGP). Wide-ranging

behavior with respect to their affinities was observed. The interaction of antihistamines with HSA is predicated by the hydrophobicity and the polar surface area of the compounds. Binding of antihistamines to AGP is unclear and depends on steric parameters and hydrogen bonding character of the compounds. Values for K_d ranged from 7×10^2 to $4 \times 10^4 \text{ M}^{-1}$.

Phage display technology is a technique used to display peptide or protein sequence on the surface of the phage. Yu et al.¹⁸ have developed a new method to screen candidate drug targets in a phage displayed cDNA library. After four rounds of screening, the PCR products of specific phages were sequenced and were found to be identical sequences for albumin and cytochrome *c* oxidase subunit III. Albumin, for example, is synthesized by liver cells and secreted into the plasma which serves as a carrier for drugs and internal biological molecules and should interact with dexamethasone. The authors used frontal analysis CE (FACE) to examine this interaction and determined a binding constant of $1.15 \times 10^3 \text{ M}^{-1}$ which validated the weak interaction between dexamethasone and albumin.

Protein-Ligand and Small Molecule Interactions

Some guanine-rich DNA sequences are known to self-assemble into specific structural motifs called G-quartets (G4). These motifs occur due to the planar arrangement of four guanine bases that can interact with adjacent guanines via Hoogsteen-like hydrogen bonds. Szilagyi et al.¹⁹ have developed a capillary gel electrophoresis (CGE)-based method to verify G-quartet formation using a thrombin-binding aptamer and its scrambled sequence counterpart. Unlike earlier studies, CGE was successful in separating the linear and folded forms of the oligonucleotide which was subsequently used to prove formation of the DNA-protein complex. This observation was contrary to earlier studies that reported some instability of the aptamer-thrombin complex during electrophoresis.

When limited quantities of material are available for use in standard ACE due to expense, synthetic difficulty, and/or time constraints, there is a need for either alternative assay techniques or a variation in the ACE technique. A variation in ACE is to employ partial-filling strategies termed partial-filling ACE (PFACE). Here, zones of ligand (or receptor) are used in place of the column being completely filled with analyte thereby reducing

the amounts of sample required for the binding assay. Zaveleta et al.^{20,21} have developed two variations of PFACE called partial filling multiple injection affinity capillary electrophoresis (PFMIACE) to determine binding constants between vancomycin from *Streptomyces orientalis*, teicoplanin (Teic) from *Actinoplanes teicomyceticus* and ristocetin (Rist) from *Nocardia lurida* to D-Ala D-Ala terminus peptides and carbonic anhydrase B (CAB, E.C.4.2.1.1) to arylsulfonamides. In the first variation, the capillary is partially filled with ligand at increasing concentrations, a standard, three or four separate plugs of receptor each separated by small plugs of buffer, a plug containing a second noninteracting standard, and then electrophoresed in buffer. Upon electrophoresis, equilibrium is established between the ligand and receptors causing a shift in the migration time of the receptors with respect to the noninteracting standards. This change in migration time is utilized to estimate multiple binding constants for the same interaction. In the second technique, separate plugs of sample containing noninteracting standards, peptide one, buffer, and peptide two, were injected into the capillary column. The capillary is partially filled with a series of buffers containing an antibiotic at increasing concentrations and electrophoresed. Peptides migrate through the column at similar electrophoretic mobilities since their charge-to-mass ratios are approximately the same but remain as distinct zones due to the buffer plug between peptides. Upon electrophoresis, the plug of antibiotic flows into the peptide plugs affecting a shift in the migration time of the peptides with respect to the noninteracting standards due to formation of the antibiotic-peptide complex. A similar shift in migration time is used to estimate K_b . In these techniques, smaller amounts of sample, than that employed in standard ACE methods, are used which should make it amenable to studies requiring high-throughput binding assays.

Over the past two decades CE has become a staple in many analytical laboratories where analysis of small quantities of materials must be accurately, efficiently and expeditiously assessed. Unfortunately, CE also suffers from several weaknesses as an analytical technique. Adsorption of charged species to the capillary wall can occur in the absence of efforts to minimize adsorption and can change the magnitude of electroosmotic flow (EOF). The presence of Joule heating and other effects of using high voltage

create variances in EOF sometimes yielding irreproducible migration times for analytes. This disadvantage can be especially troubling in the pharmaceutical industry where quality control is a priority and where method development is critical in product manufacture, analysis and marketing. Fortunately, various chemometric-based techniques including multivariate experimental design and response surface methodology (RSM) have been devised to aid in optimizing the performance of a system. Hanrahan et al.²² used a Box-Behnken design to predict the significance of capillary length, voltage and injection time on the binding of a small ligand to carbonic anhydrase B (CAB, E.C.4.2.1.1) using ACE. In this work, the design was an efficient option in RSM and an alternative to central composite (CC) designs. By combining a fractional factorial with incomplete block designs the approach avoided the extreme vertices yielding a rotatable design with only three levels per factor. In this approach, statistical analysis results were used to create a mathematical model for response surface prediction at the target response ($K_d = 1.19 \times 10^{-6}$ M). Figure 1 shows a representative response surface showing the correlation between injection time, capillary length and K_d . The adequacy of the model was validated by experimental runs with the predicted model solution (Fig. 2). Here, a K_d of 1.29×10^{-6} M was obtained (an 8.4% discrepancy difference from the target response). This use of chemometrics in ACE was a novel approach that can be extended to drug design as it yielded a large amount of information while minimizing the number of experimental runs (Tab. 1).

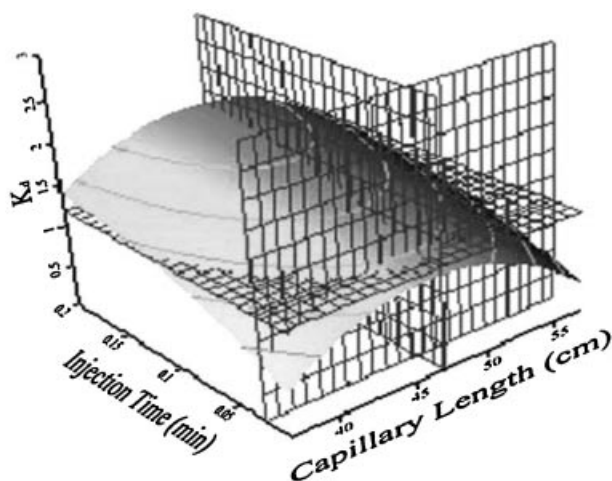


Figure 1. Representative response surface generated plot showing injection time versus capillary length. (Reprinted with permission from Ref. 22.)

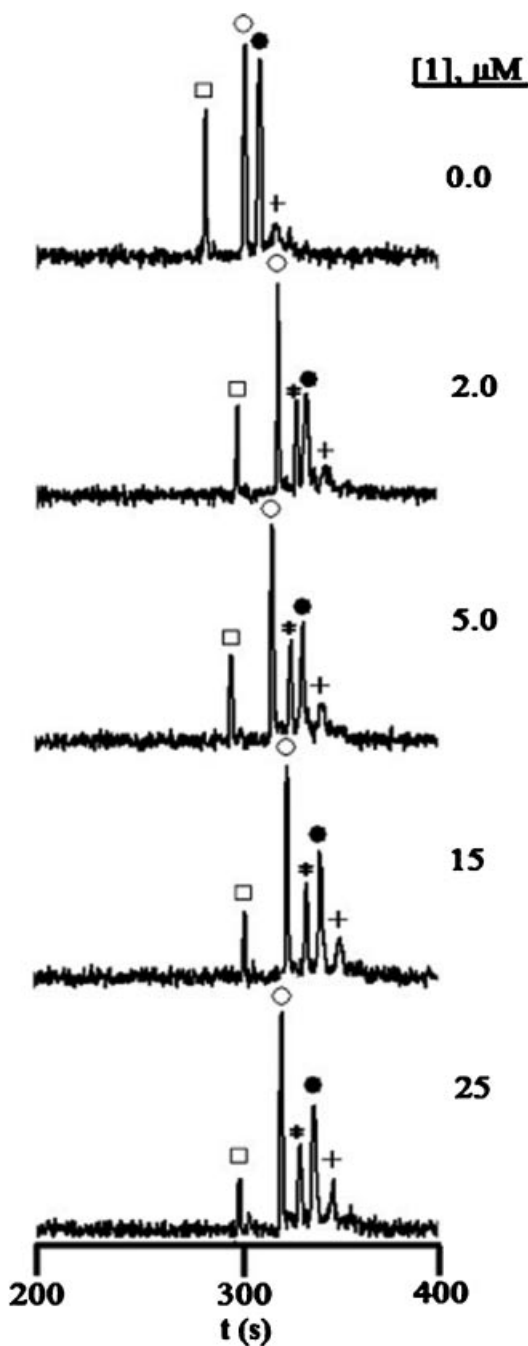


Figure 2. A representative set of electropherograms of CAB (darkened circle) in 192 mM glycine–25 mM Tris buffer (pH 8.3) containing various concentrations of 1 using the FTFACE technique. The total analysis time in each experiment was 7.0 min at 11 kV (current 2.8 μ A) using a 47-cm (inlet to detector), 50- μ m i.d. open, uncoated quartz capillary. MO (open square) and HHM (open circle) were used as internal standards. The asterisk (*) and cross (+) are discussed in the text. (Reprinted with permission from Ref. 22.)

Ramirez and Gomez²³ developed a variation of ACE called voltage gradient partial-filling ACE (VGPFACE) and demonstrated its efficacy in determining binding constants between carbonic anhydrase B (CAB, E.C.4.2.1.1) and arylsulfonamides, and vancomycin from *S. orientalis* and teicoplanin from *A. teicomyceticus* to small peptides. Two variations of VGPFACE were described. In the first technique, the capillary is partially filled with ligand at increasing concentrations followed by a sample containing receptor and two standards and electrophoresed in buffer using a voltage gradient that is systematically increased from 0 to 25 kV over the duration of the experiment. Upon continued electrophoresis, zones of solution overlap, and equilibrium is established between the ligand and receptor, causing a shift in the migration time of the receptor with respect to the standards. This change in migration time is utilized to estimate a binding constant. In the second technique, a multiple-injection sequence is used whereby the capillary is partially filled with ligand at increasing concentrations, a standard, three or four separate plugs of receptor each separated by small plugs of buffer, and a plug containing a second standard; this is then electrophoresed in buffer with a similar voltage gradient. Upon continued electrophoresis, a similar equilibrium is established and a value for K_b is obtained for the interaction. Voltage gradient ACE techniques offer a number of advantages over traditional ACE techniques including lower sample consumption, shorter experimental time, less propensity for instrument error and peak variability.

Besides estimating binding parameters, ACE can also be utilized to examine other physicochemical properties. Seguí-Lines et al.²⁴ have used dynamic ligand exchange (DLE)–ACE to assess conformational stability and activity of holoproteins to different ligands. Off-line sample treatment is not required since ligand exchange and protein folding processes occur during electromigration of proteins through the capillary column. This work has potential in drug screening.

Earlier work from the Krylov labs²⁵ employed a kinetic CE (KCE) technique known as nonequilibrium CE of equilibrium mixtures (NECEEM) to select smart DNA aptamers for the MutS protein. Here, K_d values of aptamers over a range of two orders of magnitude were discovered. Drabovich et al.²⁶ have expanded on this work by using these three smart aptamers in developing a multiprobe affinity technique of proteins with a dynamic

range of more than four orders of magnitude with a detection limit of 0.1 nM MutS. Figure 3 details the NECEEM method and the dynamic range obtained for the interaction between aptamer and MutS.

For decades mercury has been considered the cause of many neurological problems as well as myocardial infarction and in the development of some kinds of autism. One mechanism by which mercury and its derivatives can interact with humans is via HSA. HSA is a transport protein for many endogenous and exogenous compounds and represents a class of blood components to which mercury is bound after exposure to mercury sources. Although there are a number of traditional analytical techniques to measure the amount of bound mercury to proteins including HSA, these techniques suffer from nonspecific adsorption, excessive time and large sample size. Li²⁷ has examined the interaction of mercury, methylmercury, ethylmercury, and phenylmercury to HSA using a new hybrid technique called CE on-line coupled with electrothermal atomic absorption spectrometry (CE-ETAAS). The stoichiometry, binding modality, thermodynamics and kinetics for the interactions between HSA and mercuric species were obtained and two types of binding to HSA were identified. This work is the first study detailing the thermodynamics and kinetics for the interactions between mercurial species with HSA and should be a basis for further understanding of the toxicological effects of mercurial species.

ACE was used to examine the interaction between fluoroquinolones and HSA.²⁸ As noted in the Ostergaard paper,²⁹ the authors incorrectly applied the well-known equation for the retention factor (k) to capillary electrophoretic affinity studies. Fluorescence quenching technique was also used to examine the interaction and was compared to the CE method. The results of these techniques were comparable. Thermodynamic parameters (ΔG , ΔH , and ΔS) of the interaction were determined to all be negative and it was concluded that van der Waals interactions and hydrogen bonds were most important in the binding processes of the ligand to HSA.

The binding of polyamidoamine dendrimers (PAMAM G4,) consisting of an ethylenediamine core and branched units made from methyl acrylate and ethylenediamine, to serum albumins (HSA and bovine serum albumin [BSA]), was studied via ACE, circular dichroism, calorimetry, zeta potential and fluorescence polarization.

Shcharbin et al.³⁰ showed that the albumins and PAMAM G4 formed complexes with stoichiometry of 4–6:1 for G4:HSA and 4–5:1 for G4:BSA.

Protein–Antibody and Protein–Metal Interactions

The use of CE and its application to immunoassays is well documented and was originally demonstrated in the classical papers of Regnier et al.^{31–34} in the early 1990s. Many groups have expanded on this original work and have showed the utility of CE in both enzymatic and nonenzymatic microreactions. Giovannoli et al.³⁵ have developed a new noncompetitive CE immunoassay based on analyte immobilization. In this work HSA was covalently attached to the capillary column and a mixture of preincubated HSA and excess FITC-labeled monoclonal and polyclonal antibodies was electrophoresed through the column. Free labeled antibody binding sites are captured by the HSA on the capillary and the labeled immunocomplex detected. Limits of detection (LOD) (14.0 and 9.0 nM for the FITC-pAb and FITC-mAb, respectively) were impressive. The noncompetitive format was applied to measure the HSA concentration in spiked samples of human urine with acceptable recoveries.

The use of platinum-containing species as anticancer agents is very well documented. Determining which compounds might be most effective is dependent on target functionality, cellular uptake and pharmacokinetic properties. Besides determining which metal to use, the proper choice of ligand is critical to the success of the anticancer agent. Aleksenko et al.³⁶ have examined platinum (II) complexes with amino alcohol ligands and assessed their binding to HSA by ACE. These compounds are different from early platinum species in that equilibrium exists between ring-opened and ring-closed forms in solution. They found a slight variation in binding constants for the ring-opened and closed compounds and attributed this similarity to a fast equilibrium between the two forms thereby making differences in binding to HSA minimal.

Peptide–Peptide and Peptide–Drug Interactions

The completion of the human genome has enabled the discovery of many new proteins providing scientists with the opportunity to parlay these findings into potential effective pharmaceuticals. These proteins are frequently limited in

Table 1. Applications of Affinity Capillary Electrophoresis

Interacting Molecules	Detection Scheme	References
SH2 domain proteins—fluorescently labeled phosphopeptides	CE with laser-induced fluorescence anisotropy detection with excitation from an argon laser ($\lambda_{exc} = 488$ nm)	12
Propranolol derivatives—cellulose cellobiohydrolase I (Cel7A)	CZE with UV detection at 214 or 254 nm	13
Chloroquine (CQ)—human serum albumin	CE coupled to end-column electrochemiluminescence (ECL) detection	14
Antithrombin—heparin pentasaccharide	CZE with UV detection at 214 or 280 nm coupled to mass spectrometry	15
Antihistamines—human serum albumin (HSA) and α -acid glycoprotein (AGP)	CZE with UV detection at 225 nm	17
Albumin—dexamethasone	CZE with UV detection at 214 nm	18
Thrombin-binding aptamer—proteins	CZE with UV detection at 254 nm	19
Carbonic anhydrase B (CAB, E.C.4.2.1.1) to arylsulfonamides	CZE with UV detection at 200 nm	20, 21
Carbonic anhydrase B (CAB, E.C.4.2.1.1)—benzenesulfonamide	CZE with UV detection at 200 nm	22
Carbonic anhydrase B (CAB, E.C.4.2.1.1)—arylsulfonamides	CZE with UV detection at 200 nm	23
Holoprotein—ligand	CZE with UV detection at 200 nm	24
DNA aptamers—MutS protein	Fluorescence but check conditions	26
Human serum albumin—mercury compounds	CE coupled to electrothermal atomic absorption spectrometry (ETAAS) detected at 253.7 nm	27
Human serum albumin—fluoroquinolones	CZE with UV detection at 280 nm	28
Serum albumins—dendrimers	CZE with UV detection at 214 or 280 nm	30
Human serum albumin (HSA)—FITC-labeled monoclonal and polyclonal antibodies	CE with laser-induced fluorescence anisotropy detection with excitation from an argon laser ($\lambda_{exc} = 488$ nm)	35
Pt—human serum albumin	CE with diode-array detection	36
Vancomycin from <i>Streptomyces orientalis</i> and teicoplanin from <i>Actinoplanes teicomyceticus</i> —small peptides	CZE with UV detection at 200 nm	23
Vancomycin-PEG—peptides	CZE with UV detection at 200 nm	38
Vancomycin from <i>Streptomyces orientalis</i> , teicoplanin (Teic) from <i>Actinoplanes teicomyceticus</i> and ristocetin (Rist) from <i>Nocardia lurida</i> -D-Ala D-Ala terminus peptides	CZE with UV detection at 200 nm	20, 21
Vancomycin and ristocetin—peptides	CZE with UV detection at 214 nm	39
Integrin fragments—RGD-based peptides	CZE with UV detection at 200 nm	40
Anti-D-arginine L-RNA aptamer—peptides	CZE with UV detection at 250 nm	45

Triton X-100 (TX) to amphiphilic copolymers of sodium 2-(acrylamide)-2-methylpropanesulfonate and <i>N</i> -dodecylmethacrylamide (C ₁₂) (p(A/C ₁₂ (x)), where x is the mol% content of C ₁₂)	CZE with UV detection at 214 nm	47
Azobenzene-modified polyacrylate (AMP)-β-cyclodextrin (CD) and CD-containing polymers (PolCD)	CZE with UV detection at 365 nm	48
Azobenzene-modified poly(acrylic acid)s (AMPs)-surfactants (tetraethylene glycol monododecyl ether and octadecyl ether)	CZE with UV detection at 365 nm	49
Human rhinovirus (HRV)-receptor V123 decorated liposomes	CE with laser-induced fluorescence anisotropy detection with excitation from an argon laser (λ _{exc} = 488 nm)	50
Probe DNA-poly(dimethylacrylamide) conjugate	CE with laser-induced fluorescence anisotropy detection with excitation from a mercury laser (λ _{exc} = 465–495 nm)	51
Polymer-cations	CE with contactless conductivity detection	52
DNA-polymers	CE with laser-induced fluorescence anisotropy detection with excitation from an argon laser (λ _{exc} = 488 nm)	53–55
Single-stranded DNA (ss-DNA) and several divalent cations (Ca ²⁺ , Mg ²⁺ , Mn ²⁺ , and Ni ²⁺)	CZE with UV detection at 240 or 260 nm	56
DNA-metal cations	CZE with UV detection at 254 nm	57
Diastomeric anti-benzo(<i>a</i>)pyrene diol epoxide (BPDE)-derived deoxyguanosine (dG) adducts-8E11 mAb	CZE with UV detection at 280 nm	58
Alkyl(methyl)-methylimidazolium-based ionic liquid cations-neutral cyclodextrins	CZE with UV detection at 200 or 214 nm	59
Pyridine derivatives-cyclodextrins	CZE with UV detection at 214 nm	60
Bile salts-cyclodextrins	CZE with UV detection at 192, 195, and 200 nm	61
Metal cations-humic acids	CE coupled to ICP-MS	62

their chemical and physical stability hindering their usage. A method to stabilize them is through chemical modification to form a conjugated protein. Polyethylene glycol (PEG), for example, has been effectively utilized in protein conjugation studies to yield potentially stable and robust therapeutic drugs.³⁷ Hernandez et al.³⁸ have derivatized the glycopeptide antibiotic vancomycin from *S. orientalis* with several PEG species (PEG = 550, 750, 1100, 2000, 5000, and 8000 g/mol) at the N-terminus of the glycopeptide backbone and has assessed their binding to several D-Ala-D-Ala terminus peptides using ACE. They found that derivatization of vancomycin with PEG has little effect on the affinity of D-Ala-D-Ala peptide ligands.

A number of analyses have been used to examine ACE data. Besides the classic Scatchard equations, other forms of analysis have provided extent of binding as well as stoichiometry of binding. Zhang et al.³⁹ have used the retention factor (k) to examine the extent of binding between the glycopeptides antibiotics vancomycin and ristocetin and the protein carbonic anhydrase B. Their results were compared to other forms of analysis used in ACE and were found to be reliable.

The use of mass spectrometric (MS) techniques to evaluate binding parameters between two species is a growing area of study. MS offers several advantages over other analytical methods including speed, sensitivity, and exact stoichiometric measurement. Raji et al.⁴⁰ have utilized electrospray MS (ESI-MS) to evaluate binding affinities between integrin fragments and RGD-based peptide ligands. Frontal analysis CE (FACE) was used as a complementary solution phase technique to measure binding constants and was compared to the ESI-MS technique. In fact, in separate experiments, MS/MS results compared more favorably to CE than ESI/MS. Reasons for this discrepancy in data were attributed to three factors: (1) the strengthening of electrostatic forces upon transfer of the complex from solution to gas phase; (2) the inability to accurately determine the response factors of the complexes; and (3) the possible alteration of equilibria due to concentration gradients in shrinking droplets. This work should serve as a precaution to others interested in utilizing ESI-MS for binding constant measurements given the great differences in results obtained when compared to other traditional analytical techniques.

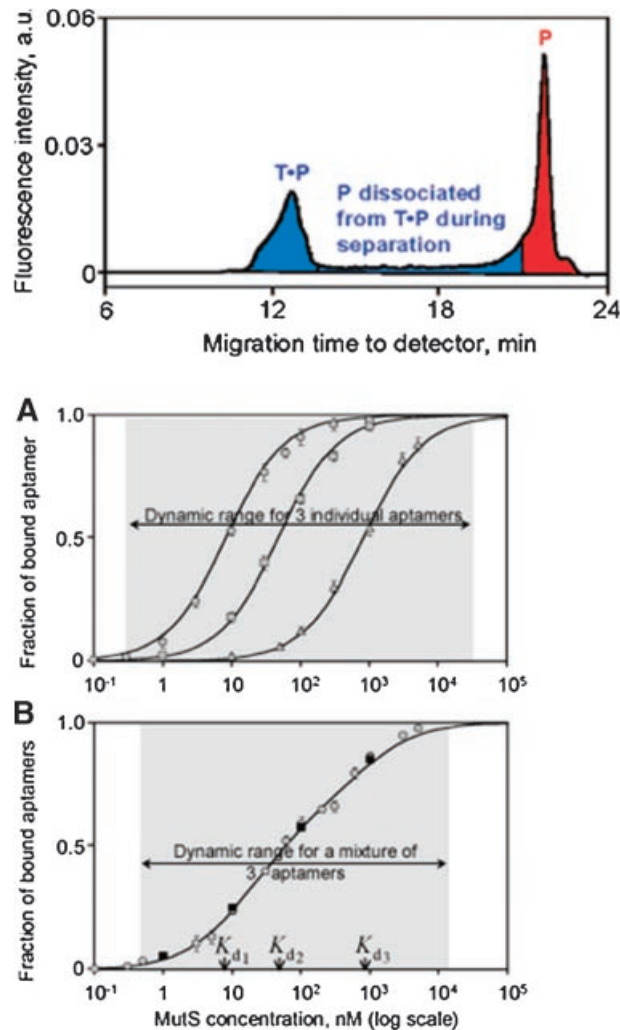


Figure 3. (Top) Determination of the fraction f of bound aptamer in aptamer based affinity analysis of MutS protein using the NECEEM method. The blue and red areas in the electropherogram represent the amounts of bound and unbound aptamer, respectively, in the equilibrium mixture. The fraction of bound aptamer is calculated as a ratio between the blue and the sum of the blue and red areas corrected for migration times of corresponding species. (Bottom) Concentration dynamic range for the affinity analysis of MutS protein using 1 nM of three individual aptamers with K_d values of 7.6, 46, and 810 nM (A), and a mixture of 1 nM of these aptamers (B). Solid lines were calculated with Eq. (3) using the above values of K_d and concentrations. Gray points correspond to analyses of MutS in the bare buffer (50 mM Tris-acetate, pH 8.2), while black squares correspond to analyses of MutS in the presence of 2.5% FBS in the buffer. (Reprinted with permission from Ref. 26.)

Enantiomeric separations are not new in CE as many papers have detailed the use of chiral additives to separate a myriad of species.^{41–44} Drug enantiomers frequently differ in their pharmacological and toxicological activity thereby resulting in stereoselective clinical responses. Providing patients with a drug that has even minor enantiomeric impurities can cause severe toxic side effects. Hence, it is critical to design efficient analytical methodologies that can detect trace amounts of one enantiomer in the presence of excess of the other. Ruta et al.⁴⁵ described an aptamer-based enantioselective assay which allowed for the detection of 0.01% of the minor enantiomer in a nonracemic mixture. In this work an ACE-based competitive assay was designed using the anti-D-arginine L-RNA aptamer as a target-specific receptor. NMR or conventional separation techniques can only attain 0.1–1.0% detection limits.⁴⁶ Figure 4 shows the principle of the CE-based competitive binding assay. This work can potentially be integrated onto lab-on-chip platforms thereby allowing for parallel runs in high-throughput fashion.

Polymer–Polymer and Other Interactions

There is a growing interest in hydrophobically modified water-soluble polymers and in their interactions with surfactants due to their potential use in biological applications. The level of polymer–surfactant interaction is directly related

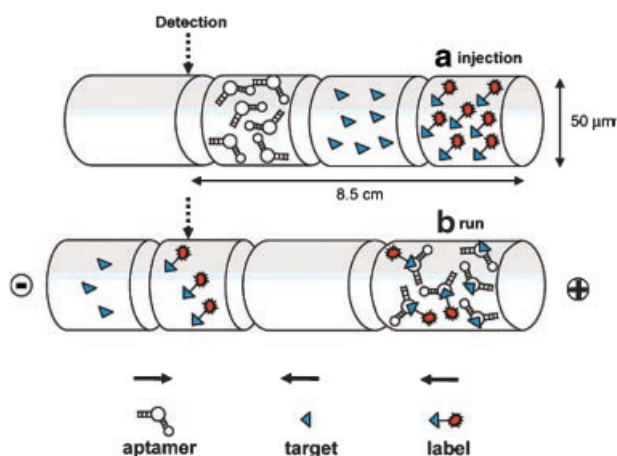


Figure 4. Principle of the CE-based competitive binding assay using the on-capillary mixing of the different species. Arrows indicated the migration direction of the interacting species when the electric field is applied. (Reprinted with permission from Ref. 45.)

to the extent of hydrophobic modification. Few studies in ACE have focused on investigating polymer–surfactant interactions. Hashidzume et al.⁴⁷ have employed frontal analysis continuous CE (FACCE) to examine the binding of Triton X-100 (TX) to amphiphilic copolymers of sodium 2-(acrylamide)-2-methylpropanesulfonate and *N*-dodecylmethacrylamide (C_{12}) ($p(A/C_{12}(x))$), where x is the mol% content of C_{12} . In a typical FACCE experiment, $p(A/C_{12}(x))$ is initially injected in the absence of TX to yield a single peak. In the presence of TX and on increasing its concentration, the migration time for the complexes decreases due to an increase in the average friction of the monomers and bound surfactant micelles. Using the signal intensities (absorbances) in the FACCE data the total concentrations of TX molecules existing as free (unbound) micelles and free molecules (unimers) in the bulk phase and of TX molecules in polymer-bound micelles can be calculated. Cooperative binding of TX micelles to the polymer was found to be cooperative.

Macromolecules that are light-sensitive have potential applications in controlled drug-delivery and sol–gel materials. Pouliquen et al.⁴⁸ have studied the binding of azobenzene-modified polyacrylate (AMP) to β -cyclodextrin (CD) and CD-containing polymers (PolCD) in aqueous solution and their photoresponse by CE and UV–vis spectrometry. They found that AMP interacts more tightly with PolCD than any CD monomer tested including α -CD, β -CD, and hydropropyl- β -CD. Increasing the hydrophobicity of azobenzene side-groups and increasing the density of azobenzene in chains increases the extent of binding.

Amphiphilic molecules or polymers containing appropriate chromophores, when exposed to light, can be used to achieve photoresponses including precipitation, aggregation and self-assembly. Khoukh et al.⁴⁹ demonstrated photoresponsive association between azobenzene-modified poly(acrylic acid)s (AMPs) and the surfactants tetraethylene glycol monododecyl ether and octadecyl ether (C12E4 and C18E4) in dilute aqueous solutions. CE was used to determine the amount of C12E4 bound per polymer chain. They found that increasing hydrophobicity of AMP strengthened the association with C12E4 in the dark. Exposure to UV light converted the azobenzene to the more polar *cis* isomer weakening the interaction with surfactant. The highest degree of binding was observed by using a spacer between the backbone of AMP and the azobenzene, in

more modified AMP species and at high salt concentration.

Virus infection initially requires attachment of the virion to structures at the host cell membrane. Eukaryotic cells have complex membranes containing a myriad of proteins attached to or imbedded in the lipid bilayer. Analysis of virus binding to a specific protein is therefore complicated. Bilek et al.⁵⁰ have instead chosen to mimic the cell membrane and studied one protein at a time by use of liposomes. In their work they have examined the binding between a minor group human rhinovirus (HRV) and its cognate receptors by CE. In their work liposomes were prepared from lipids with a nitrilotriacetic acid (NTA) group and, in the presence of Ni^{2+} , served as anchor for the his6-tags of recombinant derivatives of the very-low-density lipoprotein (VLDL) receptor. They showed using CE-LIF that HRV2 binds specifically to the receptor-decorated vesicles. Changes in electrophoretic mobility of the liposome proved binding of the virus.

Electrophoresis in microchips is now well known and many applications have been detailed in the past 10 years. The majority of these studies have utilized T- or cross-shaped branches for sample injection. These types of microchannels limit the number of separation units that can be integrated into the microchip since three or four reservoirs are required per unit. Inoue et al.⁵¹ have demonstrated electrophoresis in I-shaped microchannels that can integrate 12 parallel microchannels with electrodes onto a 3 cm × 2 cm area poly(dimethylsiloxane) (PDMS)-glass hybrid microchip. They demonstrated the efficacy of the I-shaped microchannels by performing two studies. In the first, they separated a double stranded DNA (dsDNA) ladder (100–1000-bp) in the 12 microchannels simultaneously using hydroxyethylcellulose (HEC) as the sieving matrix. This work formed the basis for the separation of polymerase chain reaction (PCR) products of the wild-type *K-ras* gene and its point mutant using a probe DNA–poly(dimethylacrylamide) conjugate. Probe DNA was immobilized to the sieving matrix that bound single-stranded DNA (ssDNA) that was the sample electrophoresed in the microchannel. This work details a unique use of ACE on a microchip with the potential for high-throughput electrophoretic analyses.

The use of UV–vis detection schemes are the norm in many ACE studies since the majority of biological interactions are chromophoric. In cases

where neither species is active modifications in the detection scheme are warranted. Jensen et al.⁵² have utilized contactless conductivity detection to probe the binding of inorganic cations (Li^+ , NH_4^+ , and K^+) and organic cations (2-propylisoquinolinium and propylpyridinium) to the charged polymer dextran sulfate. They showed that identical association constants were obtained using CCD and UV-detection schemes. The analytical technology has potential in drug development.

DNA–Polymer and Other Interactions

Single-nucleotide polymorphisms (SNPs) are DNA sequence variations that occur when a single nucleotide (A, C, T, or G) in the genome sequence is altered. Diseases that develop after middle age, for example, are considered to be caused by the interaction between genetic polymorphisms and life style. Technologies that are sufficiently high-throughput and sensitive to examine these interactions are warranted. Han et al.⁵³ have developed a rapid and reliable SNP detection method on a microchip. Using ACE, ssDNA can be separated by interaction with a probe oligonucleotide immobilized to the sieving polymer matrix. Unfortunately, separation of longer samples (>100 bp) was problematic due to steric hindrance induced by formation of an intramolecular secondary structure. To remedy this issue, unlabeled oligonucleotides (helpers) were used to offset the formation of these secondary structures. The helpers were annealed to regions near the probe-binding site. Specifically, the sample ssDNA is electrophoresed into a polymeric region of the microfluidic chip where the probe oligonucleotide is immobilized to the poly(dimethylacrylamide) (PDMA). They were able to demonstrate separation of fully matched and mismatched strands by a single-base difference. This work has great potential in the analysis of other DNA fragments based on probe hybridization.

Maeda followed up his earlier work on single-nucleotide polymorphism (SNP)⁵⁴ by developing an ACE method to detect single-base differences of ssDNA using poly(ethylene glycol)-oligodeoxyribonucleotide block copolymers (PED-*b*-ODN) as affinity ligands. In this work a mixture of 20 mer ssDNA (normal ssDNA) and a single-base-substituted 20 mer ssDNA (mutant ssDNA) were electrophoretically separated. The sequence of the ODN segment was complementary to part of

the normal ssDNA thereby impeding its migration through the capillary relative to the mutant ssDNA which did not interact with the copolymer. Studies on length of ODN segment, magnesium concentration and capillary temperature proved separation was accomplished by using reversible hybridization of normal ssDNA with PEG-*b*-ODN. Kimura et al.⁵⁵ detailed similar work on SNPs using the same copolymer.

The expression of DNA occurs through interactions with large biomolecules or metal ions. Stettler et al.⁵⁶ have used ACE to examine the interactions between ssDNA and several divalent cations (Ca^{2+} , Mg^{2+} , Mn^{2+} , and Ni^{2+}). Aggregation constants were obtained and the role of the buffer components (3-(*N*-morpholino)propanesulfonic acid (MOPS) and tris(hydroxymethyl)aminomethane (Tris)) was assessed. They found that the choice of buffer was important and that these buffers do bind transition metals by introducing additional equilibrium in the solution phase.

Novel variations in ACE are important given the diversity of interactions that require study. Whereas traditional ACE techniques (buffer containing a charged ligand is incrementally increased thereby causing a shift in the migration time of the receptor) have been successful in examining a paucity of noncovalent interactions, these techniques are not universal for all interactions. Stellwagen et al.⁵⁷ have developed replacement ion (RI) ACE to measure the binding of monovalent cations to random sequence, double-stranded (ds) DNA. The RI method measures weak binding affinities by CE using minute quantities of material. In this technique, the ionic strength is held constant by replacing a nonbinding ion in the solution with a binding ion and subsequently measuring the mobility of binding and nonbinding analytes as a function of binding ion concentration. In this work the binding of lithium, sodium, potassium, ammonium, and Tris to dsDNA were evaluated. They found that the cationic species bound weakly to dsDNA. No significant binding to single-stranded (ss)DNA.

The interactions between antibodies (Ab) and antigens (Ag)/haptens are well-known specific molecular recognition phenomena but they are not immune to nonspecific interactions. Cross-reactivity of Abs ranges from broad promiscuity to group specificity. Hence, techniques that can select and identify molecules of interest from complex mixtures are warranted. Miksa et al.⁵⁸ have utilized fluorescence line narrowing spectroscopy (FLNS) and flow through partial filling

affinity capillary electrophoresis (FTPFACE) to separate four diastereomeric anti-benzo(*a*)pyrene diol epoxide (BPDE)-derived deoxyguanosine (dG) adducts using 8E11 mAb raised against BP-DNA conjugates. In the FTPFACE work, a mixture of the four isomeric BPDE-dG adducts was injected followed by a 1-min delayed injection of a plug of 8E11 mAb. Differences in electrophoretic mobilities cause the mAb plug to pass through the mixture of adducts. The extent of interaction between the mAb and adducts is different resulting in four separate peaks. Differentiation of the stereoisomeric adducts was also accomplished with FLNS.

Cyclodextrin–Small Molecule and Other Interactions

Cyclodextrins have found great utility in CE and especially in enantiomer separations. CDs form guest–host inclusion complexes with many small molecules and have been used as solubilizing, masking and protecting agents of guest molecules of interest in the pharmaceutical, health care and fragrance industries. Several groups have further developed the use of CDs in CE by employing them in ACE studies. Recently, Francois et al. used ACE to characterize the complex formation between several alkyl(methyl)methylimidazolium-based ionic liquid (IL) cations and eight CDs. Similar to previous studies, the majority of complexes formed followed a 1:1 complexation stoichiometry model but four cases adopted 1:2 (IL:CD) stoichiometry.⁵⁹ They determined that the most important factor dictating the strength of the interaction was the length of the alkyl side chain on the imidazolium ring. In addition, the size of the CD cavity affects the stability of the complex. These studies provide further impetus for synthesizing other derivatized CDs and in examining their complexation to a host of other small molecules and drugs. Figure 5 shows a representative series of electropherograms demonstrating the mobility shift of 1-octyl-3-methylimidazolium cation (C8MIM) as a function of α -CD concentration.

Terekhova and Scriba⁶⁰ have used cyclodextrins (CDs) as buffer additives to separate the pyridine derivatives pyridoxine, pyridoxal, nicotinamide, nicotinic acid and isonicotinic acid. Both nicotinic and isonicotinic acid formed 1:1 complexes with α -CD and hydroxypropyl- α -CD (stability constants ranged from 3 to 33 kg/mol). Calorimetric and UV-spectroscopic techniques were also used to study the interactions.

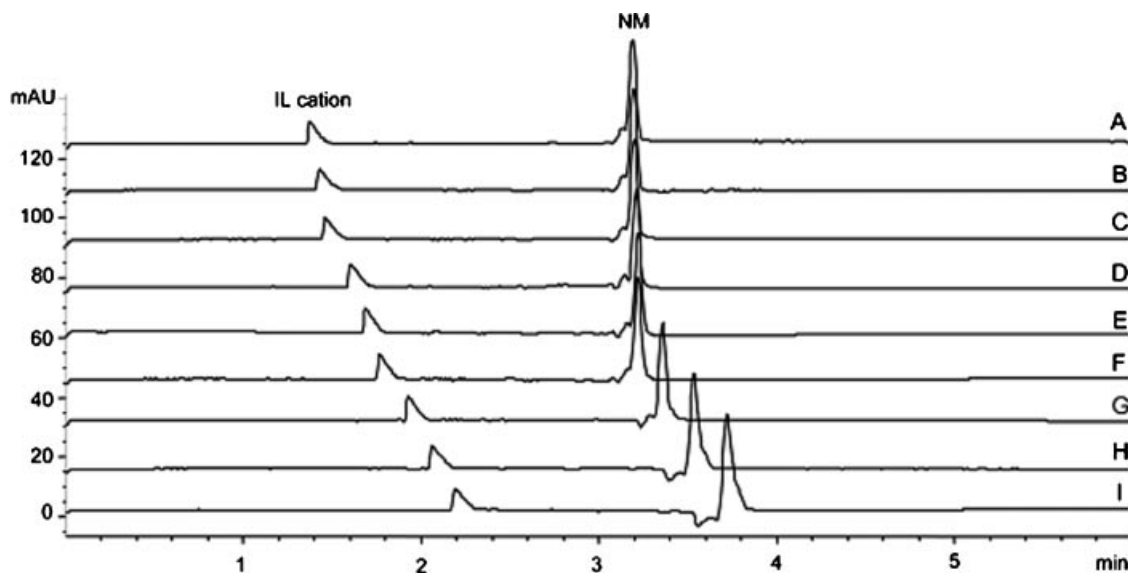


Figure 5. Electropherograms showing the mobility shift of 1-octyl-3-methylimidazolium cation (C8MIM) as a function of α -CD concentration in the running buffer. (A) Zero (neat background buffer); (B) 1 mM; (C) 2 mM; (D) 3 mM; (E) 5 mM; (F) 10 mM; (G) 20 mM; (H) 30 mM; (I) 50 mM. Experimental conditions: bare fused-silica capillary, 50 μ m i.d. \times 35 cm (detection cell, 26.5 cm). Running electrolyte: 30 mM sodium acetate buffer, pH 5.0 (ionic strength, 30 mM) containing α -CD at various concentrations (A–G). Applied voltage: 20 kV (current intensity: 50 μ A). Temperature: 25°C. Sample: 2 mM C8MIM Br in the sodium acetate buffer, pH 5.0. NM: neutral marker. (Reprinted with permission from Ref. 59.)

Cyclodextrins (CDs) are robust in their ability to complex with a potpourri of small molecules. Holm et al.⁶¹ have examined the interaction of natural β -CDs and chemically modified β -CDs to the bile salts (BS) taurocholate, tauro- β -muricholate, taurodeoxycholate, taurochenodeoxycholate, glycocholate, glycodeoxycholate and glycochenodeoxycholate by ACE. Binding constants obtained varied from 2×10^3 to 4×10^5 M⁻¹. The presence or absence of hydroxyl groups at positions C-7 and C-12 affected the affinity of the BS for the CDs. Modification of the CD did not affect the extent of interaction.

Stern et al.⁶² used CE-inductively coupled plasma (ICP)-mass spectrometry (MS) to measure binding constants (K) for rare earth elements complexed with humic acids. An increase in log K is obtained with a decrease in ionic radius for rare earth element–humic complexes.

Theoretical and Statistical Studies on Binding Interactions

The use of the retention factor k , also known as the mass distribution coefficient or capacity factor, is

one of the most universally used parameters in liquid chromatography (LC). Its use allows for the comparison between retention data obtained in different chromatographic systems. In ACE measurement of the shift in migration time of a receptor on changes in concentration of a ligand in the running buffer is widely used and is considered a form of a mobility shift assay. The ligand can also be considered the pseudo-stationary phase thereby defining a two-phase system and is termed affinity electrokinetic chromatography (EKC). Recent work²⁸ incorrectly applied the well-known equation for the retention factor ($k = (t_r - t_0)/t_0$) to capillary electrophoretic affinity studies assuming a two-phase system. Ostergaard has carefully examined why the use of the retention factor in determining affinity parameters is not appropriate.²⁹ The retention factor in electrokinetic chromatography can be expressed in terms of migration times that can be related to electrophoretic mobility (Eq. 1)

$$\mu = \left(\frac{l_c l_c}{V} \right) \left(\frac{1}{t} - \frac{1}{t_{eof}} \right) \quad (1)$$

In electrokinetic chromatography, the retention factor can be defined as in Eq. (2)

$$k = \mu_A - \frac{\mu_0}{\mu_{ps}} - \mu_A \quad (2)$$

Here μ_A , μ_0 , and μ_{ps} are the effective electrophoretic mobility of an analyte and the analyte in the absence and presence of a pseudo-stationary phase, respectively.

From Eqs. (1) and (2), Eq. (3) can be obtained which is very different from the equation for k used in LC

$$k = \frac{t_A - t_{eof}}{t_{eof}(1 - t_A/t_{ps})} \quad (3)$$

It was concluded that mobility shift ACE should be used to examine biological interactions.

Peak broadening and distortion caused by operating conditions are two major reasons; separation efficiency in CE can be comprised. Like other modes of CE, ACE can also suffer from analyte adsorption onto the capillary walls thereby yielding or inaccurate values for binding constants. Additionally, the injected sample concentration and plug length can also be a source of systematic error in this constant. Fang et al.⁶³ have developed a two-dimensional (2D) simulation of ACE to model affinity interaction and wall adsorption simultaneously. SIMulation of Dynamic Complexation Capillary Electrophoresis (SimDCCE), previously used to simulate affinity interactions in CE, was utilized to consider wall adsorption. In fact, the systematic errors originating from finite sample plug length and wall adsorption are quantified for the first time. Binding isotherms for ACE experiments were generated in the presence of wall adsorption. An important outcome of this work is that a procedure has been detailed to compensate for wall adsorption and plug length. This is a seminal piece of work that requires follow-up with real ACE experiments to demonstrate how accurate the SimDCCE model is.

Many cancers, their initiation in particular, can be traced back to an interaction of protein, sugar or peptide to DNA. Hence, their study by any means is integral in determining how cancers develop and in their cure. Araya et al.⁶⁴ have summarized some recent developments in the use of CE in the analysis of DNA–ligand interactions dividing it into free solution CE and instances where polymers are in the matrix. CE interfaced with a mass spectrometer and footprinting are also described.

CONCLUSIONS

ACE continues to be a versatile technique to assess the extent of interactions between a myriad of species including proteins, DNA, sugars, antibiotics, and other biological compounds. The aforementioned papers attest to this. Recent developments in the use of ACE has expanded on and developed new ACE methodologies that have broadened the tools available to the researcher working in the area of molecular recognition. Some of these techniques are of the high-throughput type and require even less quantities of material than previously necessary in earlier ACE studies. The future is bright for ACE. For example, recent work detailing the integration of ACE onto a microfluidic format is exciting and promises to be an area much researched in the future.

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