Review

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Bioanalysis

Recent advances in affinity capillary electrophoresis for binding studies

The present review covers recent advances and important applications of affinity capillary electrophoresis (ACE). It provides an overview about various ACE types, including ACE-MS, the multiple injection mode, the use of microchips and field-amplified sample injection-ACE. The most common scenarios of the studied affinity interactions are protein–drug, protein–metal ion, protein–protein, protein–DNA, protein–carbohydrate, carbohydrate–drug, peptide–peptide, DNA–drug and antigen–antibody. Approaches for the improvements of ACE in term of precision, rinsing protocols and sensitivity are discussed. The combined use of computer simulation programs to support data evaluation is presented. In conclusion, the performance of ACE is compared with other techniques such as equilibrium dialysis, parallel artificial membrane permeability assay, high-performance affinity chromatography as well as surface plasmon resonance, ultraviolet, circular dichroism, nuclear magnetic resonance, Fourier transform infrared, fluorescence, MS and isothermal titration calorimetry.

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Many biochemical processes are regulated by the interaction of hormones, drugs and other 'messenger' molecules with receptors that are either embedded in the cell membrane (membrane-bound) or present in the cytoplasm (soluble receptor) or the nucleus of the cell. Changes in receptor density and a disturbed balance in the (in)activation of these receptors give rise to the development of diseases and an understanding of this can aid subsequent treatment. Receptor screening methodologies that can be based on either the determination of a functional response (e.g., cell proliferation), the production of second messengers (e.g., Ca^{2+}) or the interaction of a ligand with its receptor are therefore of major interest.

Affinity capillary electrophoresis (ACE) was first reported in 1992 as a CE separation technique [1] for the study of ligand and receptor interactions. The technique has grown up over the years, the number of published articles is increasing to reach currently more than 1000 articles (from 1992 to 2014) according to SciFinder which reflects the increase in popularity of this method for studying affinity interactions in many fields including bioanalysis, drug design, pharmaceutical and industrial [2].

ACE is used to measure the binding affinity of receptors to neutral and charged ligands. ACE experiments are based on differences in the values of electrophoretic mobility of free and bound receptor [3,4] based on the change in the respective charge-to-mass ratios. The binding constant of these interactions can be estimated as well [5].

Since the electrophoretic mobility change is always superimposed by the electroosmotic flow (EOF) in ACE [6], this parameter needs to be carefully estimated and considered [7]. Therefore the use of EOF neutral marker (e.g., acetanilide [6]) is necessary to avoid the measurement errors that are coming from EOF fluctuation [4,8]. Accordingly, it is more accurate to describe the migration behavior of receptor or ligand as mobility ratio which can be expressed as shown in Equation 1 [4,9].

$$R = t_{eof} / t_{prot} \qquad \text{Equation 1}$$

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Key term

Circular dichroism: Differential absorption of left and right circularly polarized light.

Where R refers to mobility ratio, t_{eof} is the migration time of acetanilide (neutral marker), t_{prot} is the migration time of the receptor (e.g., protein).

Moreover, the conformational change of a target protein after binding with a given ligand is also observed as change in peak shape [10] as shown in Figure 1 for the study of β-lactoglobulin metal ion interaction.

Using this parameter, now confidence intervals can be estimated to understand if a ligand–receptor interaction is significant or not (Equation 2):

$$\text{cnf}\left((R_i - R_f)/R_f\right) = \left((R_i - R_f)/R\right) \pm \left(t_{\alpha/2,n_1+n_2-2} \cdot \hat{\sigma}_{total} \cdot \sqrt{\frac{2}{n_1 + n_2}}\right)/R_f$$

Equation 2

R_i and R_f are the mobility ratio with and without ligand addition, respectively, n_1 and n_2 are the two series with same data numbers of R_i and R_f. The value $n_1+n_2.2$ represents the degrees of freedom, *total* σ is the total standard deviation. If the $\Delta R/R_f$ value exceeds 0.01, the interaction is often significant [4].

Additionally, ACE can be used to calculate the binding constant of receptor–ligand interaction by analyzing data using either one of four mathematical plotting models [4,6]: the linear regressions (x-reciprocal, y-reciprocal, double-reciprocal) and the nonlinear regression. Linear regression is commonly used when receptor and ligand bind at 1:1 ratio [10]. However, this 1:1 binding stoichiometry is not common since most of the bimolecular interactions exhibit multiple binding sites. Thus it is considered best and most accurate to analyze the data by nonlinear regression [6].

ACE can be conducted in three modes, which are chosen related to the kinetics involved. Fast, intermediate and slow kinetics have been observed, although the fast kinetics (complete equilibrium within milliseconds) clearly dominate.

The first mode is referred to as pre-equilibrium [9] (also called nonequilibrium electrophoresis of pre-equilibrated sample mixture [2]). In this mode, the two species receptor and ligand are premixed in the sample vial before electrophoresis [1,2]. This mode requires the kinetics data of interaction (association constant K_{on} and dissociation constant K_{off}) [1,9] in order to be able to investigate complex formation. It is, for example, suitable for analysis of DNA-related interactions (DNA with protein or small molecules) [1].

The second ACE mode is referred to as dynamic equilibrium [1,2], also called mobility shift [9]. In this mode one of the analysis species is located in the sample solution and the other in the electrophoresis separation buffer. The interaction then occurs during their contact inside the capillary, thus this mode is only useful for studying interactions with fast kinetics [1,2].

The third ACE mode is referred to as immobilized selectors, here the ligand is fixed to the capillary wall and the receptor pass through in the solution [1]. This type is called electrokinetic affinity chromatography [2].

Since all human physiological functions and pathological disorders depend on the interactions between receptors, enzymes, transporter proteins and nucleic acids with various numbers of ligands [1], it is very important to study these interactions for drug discovery and development, pharmacodynamics, pharmacokinetics and drug interactions. This increases the need for fast, sensitive and precise methods of analysis [2]. ACE is continuously developed in order to be able to employ this technique rapidly and precisely for different binding

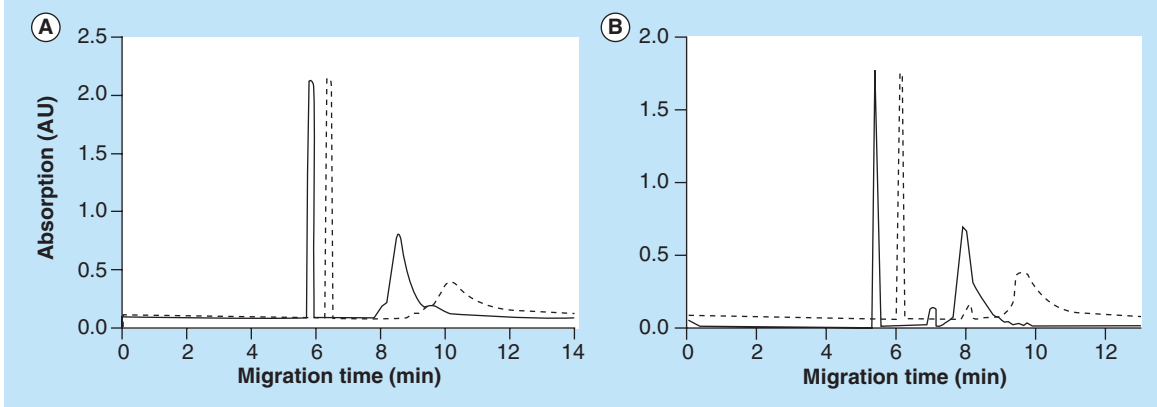

Figure 1. Electropherograms presenting peak shape changes and sample heterogeneity of β-lactoglobulin through metal ions. (A) acetanilide and β-lactoglobulin without barium chloride (solid line), acetanilide and β-lactoglobulin with 250 μM barium chloride (dashed line). **(B)** Acetanilide and β-lactoglobulin without calcium chloride (solid line), acetanilide and β-lactoglobulin with 250 μM calcium chloride (dashed line).
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studies [4]. Despite the presence of many techniques to study affinity parameters such as MS [11], nuclear magnetic resonance (NMR) [12], spectometry [13], Stop-flow [14] and HPLC [15], ACE offers advantages over the other techniques as we will comprehensively discuss in the next sections. ACE only requires a very small sample amount, provides high speed of analysis [16,17] and high efficiency [2], in addition to good precision [9], high selectivity and low cost [16]. In particular, the possibility to directly inject impure samples [4,6] and the ability to study mixtures of analytes in the same solution [18] facilitate the analysis of biological samples.

Application fields

ACE is currently applied in multi-disciplines, thus many ACE models are used to accommodate for each discipline, including partial filling ACE, frontal analysis, multiple injection affinity CE, pressure-mediated ACE, microchips ACE, dynamic ligand exchange ACE, etc. In the following subsection, the most common scenarios of receptor and ligand interactions are presented.

Protein–drug interactions

The behavior of a drug during its life cycle inside the body is attributed to its interaction with various body proteins, starting from association with plasma proteins, binding to its target receptor to give the desired pharmacological response and finally termination of its action under the influence of metabolizing enzymes. In pharmaceutical and medical research, study of such interactions plays an important role in the discovery and development of new drug substances [5,19].

Over the last two decades, ACE has been extensively used in this field because it has many advantages over the other alternative methods. ACE can be applied with the aim of measuring the affinity and the binding constant K_b between the drug and the associated protein, determination of the rate constant of association (K_{on}) and dissociation (K_{off}) and investigation-binding stoichiometry. The knowledge of these three points is essential for evaluation of drug affinity and understanding its mechanism of action once bonded to the receptor [19,20].

Moreover, ACE has been used as a physicochemical tool to demonstrate binding properties of the physical interactions including the strength of the interaction and to estimate the overall charge of the complex formed [4,21]. ACE was also used in combinatorial studies to find a lead compound by screening a library of candidates at the same time [19,20].

Protein–single drug

More than 1000 articles have been published since the year 2000 (as estimated by SciFinder) showing the use of ACE for the estimation of binding constants between various drugs and a number of body proteins [22,23]. The first model system used carbonic anhydrase (CA) as the receptor protein and aryl sulfonamide as the binding ligand [20]. Aryl sulfonamides are neutral molecules of low molecular weight: when they interact with the protein, they cause minor changes in net charge that cannot be detected by simple methods. Competitive ACE is a suitable method to estimate the binding constant in such a situation. The idea depends on generating a competition between the small neutral ligands and another charged ligand with known affinity to that protein [20]. In one example, competitive binding assays used a flow-through partial filling ACE (FTPFACE) to estimate the K_b of CA–aryl sulfonamide complex [23]. A sample of CA and aryl sulfonamide with two standards was initially prepared and allowed to pass through a capillary partially filled with negatively charged ligands. A new complex is formed between CA and the negatively charged ligands, causing changes in the relative migration time of the complex [23]. For many other examples, see [24].

To increase the sensitivity of ACE methods, coupling with more sensitive detectors can be highly valuable. For example as in the case of high binding constants ($> 10^5$/M) as the binding of trimethylin to human serum albumin (HSA), ACE was coupled with inductively coupled mass spectrometry (CEICPMS) [21]. For lower values of K_b ($<10^5$/M), ACE was coupled to a high sensitivity cell (capillary with increased light path length at the detection window) as in binding of retinoic acid with HSA and bovine serum albumin (BSA) [22]. However, the selection of detector is not only influenced by binding strength but also other issues like the lack of chromophore part in receptor for UV absorption.

Zhang *et al.* used ACE and fluorescence method to study the interaction of flouroquinolones (FQ) and HSA [25]. The results of both methods were congruent, but in ACE less FQ and HSA were consumed and no buffer modifiers were added. In a previous study on FQ interaction with BSA, UV-Vis absorption spectometry, fluorescence spectometry and circular dichroism spectometry were used [26]. Similar results were obtained showing that the secondary structure of BSA changed in the presence of FQ and that hydrophobic interactions strongly contribute in the interaction.

Liu *et al.* studied the interaction of kedarcin (potent anticancer antibiotic) with apoprotein. The results showed that by increasing the concentration of organic solvent, the affinity between kedarcin-chromophore and apoprotein decreased [19].

Protein–enantiomeric drugs

The US FDA has put restrictions on marketing of enantiomeric drugs, ensuring the release of preparations that contain only the effective enantiomer. This step was

adopted after encountering many problems due to the use of racemic forms of drugs containing both enantiomers. It is well known that often only one of the enantiomers is responsible for the pharmacological effect while the other one may cause undesirable side effects or even extremely hazardous teratogenic effects [20]. To lower the toxicity of drugs, several methods have been employed for enantiomeric characterization, including HPLC, capillary electrochromatography (CEC) and ACE [27].

ACE is a high selective method for chiral separation using various types of chiral selectors. For separation and determination of binding constants of enantiomers, ACE can be accomplished with partial filling technique using field-amplified sample injection [22]. The idea of separation depends on different affinities between the enantiomers and the chiral selector generating a disparity in electrophoretic mobility that finally results in separation [20]. By this method, not only separation of enantiomers is possible, but also their differences in binding affinity can be estimated by the respective mobility differences.

Different chiral selectors can be used, examples of these include cyclodextrins (CDs), chiral surfactants [27], antibiotics (vancomycin and restocetin are enantioselective due to their sizes and sterical arrangement of functional groups) [28], oligo- and poly-saccharides (chondroitin sulfate), proteins and peptides [27].

Most proteins exhibit a degree of enantiospecificity and can be used for enantiomeric separation. The use of proteins as selectors is expected to lower the sensitivity of the applied method due to high background UV absorbance. To overcome this, the partial filling technique can be used [29,30]. Examples of proteins that were successfully used in enantiomeric separations include HSA, BSA, streptavidin, α1-glycoprotein, egg white avidin [30], flavoproteins and various enzymes, including trypsin, cellobiohydrolase I and lyzozyme [31]. ACE is the preferred method when the chiral selectors are proteins, since immobilization of proteins is not needed which may alter their tertiary structures causing changes in the behavior of these proteins [31].

Using HSA as a chiral selector, M-Pla *et al.* studied the enantioselectivity of propranolol, ofloxacin and verapamil by ACE. The β-blocker propranolol has one chiral center, the S-enantiomer has greater activity (100-times) than the R-enantiomer [27]. Propranolol was separated by HAS and BSA, ofloxacin was separated only in the presence of BSA, while verapamil by HSA.

Protein–multiple drugs

Co-administration of drugs may reveal another mode of protein–drug interactions in which more than one drug compete for the same target protein. Drug–drug interaction occurs when two drugs compete for the same plasma protein, the same receptor or the same degradation enzymes. Protein bounded drug (A) is displaced by another drug (B) of higher affinity, thus causing an increase in the free concentration of drug A, possibly leading to more toxic effects. ACE can be used to predict the drug behavior and the possible drug interactions that may occur.

Li *et al.* used ACE to measure the binding constants and to study the competitive binding properties of ibuprofen and salicylic acid when they interact with serum albumin (BSA or HSA). Borate buffer solution (pH = 8.5) containing different concentrations of serum albumin (0–20 μM) was incorporated into uncoated fused silica capillary. Results showed that ibuprofen has higher affinity than salicylic acid toward the protein and can displace it from its low and high affinity binding sites [32].

Another method called vacancy ACE (VACE) was used for the study of competitive interaction of co-administered drugs toward a given protein. Initially, a solution containing buffer, protein and drug is introduced in to the capillary that causes high detector signal. When a neat buffer is injected and the voltage is applied, two negative peaks (vacancies) will appear in the electropherogram, these belong to the protein and the drug. They appear as vacancies due to local deficiency of the free protein and drug, respectively. Changes in the concentration of protein and drug will lead to corresponding changes in AUC, while any changes in migration time are correlated to the degree of complexation [5]. Erim and Kraak used VACE successfully to study the displacement of warfarin from BSA by furosemide and phenylbutazone [33].

ACE can also be applied in combinatorial library screening where a group of compounds are tested together instead of testing them one by one, to reveal their binding affinities toward one of the targets (mainly proteins) in a way that saves time and costs. In drug discovery research, coupling of ACE with mass spectometry is valuable for screening of such libraries [19]. The wide range of genomic sequence opens up prospects for identification of more and more of potential antimicrobial targets, examples including virulence gene products, membrane transporters, cofactor biosynthesis, chorismate biosynthesis and fatty acid biosynthesis [34].

High-throughput ACE has also been used to identify hits that can bind to YihA (a novel antimicrobial target against tenacious strains of bacteria like methicillin-resistant *Staphylococcus aureus* [MRSA]) [34]. When a ligand is allowed to pass through a capillary filled with a solution of the target, it will impart a change in mobility due to changes in the charge or the conformation. Custom software detect ACE migration changes

and mark the compound as a hit [34]. Hits are classified as weak binders ($K_d \geq 100$ μM), moderate binders ($K_d \geq 10$ μM) and strong binders ($K_d \geq 10$ nM). Binding interactions of the target or ligand can be detected at low concentrations by using laser-induced fluorescence (LIF). A library of 44,440 compound was screened to identify if these compounds have binding activity against the target protein (YihA).

Protein–metal ion interactions

It is well known that a third of all known proteins are metalloproteins, this means: various metal ions contribute to their vital function in living organisms [35,36]. The metal ions have different functions, but many of them have catalytic role and can activate many enzymes [37–39]. Some metal ions (copper and zinc) may also contribute to the mechanism of several diseases like Parkinson's disease, Alzheimer's disease and cancer [10].

Protein–metal ion interactions may occur during transport and storage of these metals as transferrin (Fe^{3+} transport), ceruloplasmin (Cu^{2+}transport) and metallothionin (Zn^{2+} storage) [40]. ACE has been widely used to identify the interactions between proteins and charged ligands including metal ions, cationic pharmaceuticals and anions [9–10,41].

When a charged ligand binds to a given protein, it will alter the electrophoretic mobility of that protein. Other changes in mobility may result from fluctuation in the EOF due to adsorption of proteins and cations to the inner capillary wall. The use of neutral markers and mobility ratios is essential to compensate for such changes of the EOF [4]. Another important issue is to preserve the protein's structure and to prevent its aggregation or denaturation [10,41]. The native and denatured form of a protein can be easily distinguished through the change in mobility and peak shape [42].

Changes in the protein stability during analysis contribute to inaccurate results, if one salt changes, this may alter the protein binding properties resulting in significant errors. Any changes in protein surrounding environment appears as a change in the charge, the size or the conformation of that protein [10,41]. Various factors affect the stability of the protein including the pH of the media, ionic strength and the concentration of the organic solvent. Extreme values of these factors may completely destabilize the protein [41].

For example, changes in the pH of the surrounding environment may alter the size of BSA. Dimers of BSA are found at low pH, at high pH little peak broadening occurs, while at neutral pH, the radius of BSA decreases. Strong hydrogen bonding between water molecules and the surface of protein will cause a change in protein size [10]. Various methods were used to detect the changes in the hydrodynamic radius of proteins including dynamic light scattering, pulsed-field gradient NMR or atomic force microscopy [10,41].

Addition of amino acids like arginine or lysine can often prevent protein aggregation [10]. Optimization of separation conditions for each method reduces the errors resulting from alteration in any of the previously mentioned parameters [4,10,41].

Protein–metal cations

For each protein there are many binding sites for different metal ions. More than one amino acid is required for binding these ions, resulting in different interactions with unequal strength. ACE detects changes in the electrophoretic mobility of the protein that result from changes in the charge-to-mass ratio when binding additional ions. These changes appear in the electropherogram as a shift in the migration time of the protein peak [10]. Other changes in the protein may cause a change in peak shape due to conformational change [41].

Redweik *et al.* studied the binding of metal ions (Ni^{2+}, Ba^{2+}, Mn^{2+}, Ca^{2+}, Cu^{2+}) to ovalbumin, β-lactoglobulin and BSA [10]. For a given concentration, the ionic strength was the same for each metal ion solution.

Proteins have unspecific metal binding sites, so they can bind several ions. The overall protein charge is the main motivator for its mobility. Protein mobility is attributed mainly to the changes in the overall charge after binding to a metal, not to the mass. In most cases, the interaction between proteins and metal ions results in longer migration time and subsequent reduction in mobility ratio. These were unexpected results, because when cations are added to proteins, the normal expected result is to make proteins less negative which leads to a decrease in migration time. However, the observed extended migration times was due to increasing negativity of the protein that can be attributed to the formation of further complexes. For example, binding of metal ions to thiol groups of cys66 and cys160 of β-lactoglobulin, primarily, thiol groups are deprotonated before binding to metal ions, then the complex formed will bind to other anions that increases the negativity of β-lactoglobulin [10,41].

It is worth noting that, not only thiol groups, also the amide nitrogen of the backbone can be deprotonated before binding some transition metals. The capillary length has an obvious impact on the mobility shift of a protein after binding metal ions. This is attributed to the various interaction times within different capillary lengths. AlHazmi *et al.* found that the strength of interactions between the proteins BSA and ovalbumin and the ligands Ni^{2+} and Ba^{2+} have been decreased when the capillary length is decreased [4]. Therefore,

decreasing the sample concentration could compensate the loss in interaction time [4].

Protein–anions

Several techniques are used to study interaction between anions and proteins including HPLC, dynamic light scattering, isothermal titration calorimetry and Fourier transform IR spectometry. ACE is a useful tool here as well, studying the interactions of various anions as in the cellular environment (phosphate, succinate and acetate) or in pharmaceuticals like aspirin and ibuprofen that present as anions of acetyl salicylate and iso-phenylpropionate, respectively at physiological pH. Xu *et al.* studied the anions succinate (important in citric cycle), glutamate (neurotransmitter), phosphate, acetate, nitrate, iodide, thiocyanate and pharmaceuticals (SA, aspirin and ibuprofen) binding to BSA, β-lactoglobulin, ovalbumin, myoglobulin and lyzozyme performing both mobility shifts and prequilibrium ACE modes [9]. The different anions exhibited different effects on the selected proteins. Since proteins under nondenaturating conditions exist in equilibrium between (almost two or three) different forms of monomer, dimer, trimer, octamers or even dodecamers, different anions could shift this equilibrium toward different forms as monomer-dimer, monomer-dimer-trimer or many other forms. These different forms showed different changes in their charge-to-mass ratio that results in different mobility shifts. Chaotropic anions (Cl-, Br-, I-) were also investigated, but no significant changes in proteins mobility were observed [9].

The overall interaction strength was lower for anions than for cations, which was not surprising for originally negatively charged proteins. However, these weak but significant interactions of anions can still have an important meaning, since these anions are often present in high concentrations within biological systems [9].

Other applications

Since ACE technique is characterized by ease of performance, low sample consumption, no necessity of sample purity and rapid analysis time, it is widely used in other applications including protein–protein interactions, protein DNA, protein–carbohydrate, peptide–peptide, DNA–dye, carbohydrate–drug and antigen–antibody interactions.

Protein–protein interactions

This type of interaction is of essential importance in many biological processes as in signal transduction pathways. Taga *et al.* studied the interaction between transferrin (TF), the protein which is responsible for iron transport and human serum proteins (albumin and α-globulin) [43]. Results detected at 200 nm showed that TF did not cause changes in migration time of these proteins. It is worth noting that peptide bonds between amino acid residues absorb at around 200 nm, whereas aromatic ring residues absorb at higher wavelength (280 nm). Thus the detection wavelength is more selective in principle at 280 nm. However, this wavelength also requires higher sample concentrations, which in turn make more contaminants visible. This is because TF has sialic acid residue that prevents its binding to proteins. The method was repeated using de-sialyated TF (obtained by sialidase digestion under slightly acidic conditions). Now a significant change in migration time of α-globulin was detected. In an attempt to investigate the effect of iron (II) that is present in TF, they used apo-TF and de-sialyated apo-TF, changes in migration time were obtained as well, but K_a values were reduced emphasizing that iron participate in binding of the proteins [43].

Protein–DNA interactions

Gene expression, DNA replication, transcription, recombination and repair of damaged DNA can be classified as DNA interactions [23,44]. Various types of cancer and heritable diseases can result from mutations in DNA, thus it is important to study these interactions [5]. The traditional method used for DNA separation is capillary gel electrophoresis which separates nucleic acids according to their size. Furthermore, gel-free ACE methods can be used to separate nucleic acids in free solutions [5,45], measuring mobility shifts and determining the extent of complexation between protein and DNA [44].

ACE is a useful approach for quantitative assessment of protein–DNA interactions, in order to understand transcription as a first step in cellular function and study gene regulation [44]. IR spectometry is usually used in addition to demonstrate conformation and the binding of biomolecules and drugs to proteins, DNA and RNA.

Malonga *et al.* used ACE-UV/Vis and FTIR spectroscopic methods to study the interaction between calf thymus DNA and HSA (a major target for DNA binding), measured the binding constant and characterized the biopolymer secondary structure in DNA–HSA complex [44] under physiological conditions. Results of FTIR analysis showed that the interaction occurs through the guanine carbonyl bases of HSA and the backbone phosphate of DNA, no changes observed in the secondary structure of HSA (remain stable) while DNA structure show minor changes upon binding.

Zou *et al.* used ACE coupled with LIF to study binding stoichiometry and specific recognition between

methyl CPG-binding domain (MBD2b) protein and methylated DNA an interaction which is involved in gene transcription [46].

The binding stoichiometry of the protein depends on DNA length, as the length of both methylated and unmethylated DNA increases the complex formed will increase too. However, a decrease in the dissociation constant of the complex will be more significant in methylated form of DNA. More than one protein (up to 4) can bind to one DNA molecule; each protein can bind up to 20 nucleotides in DNA molecule. When multiple proteins bind the same DNA molecule, the specific recognition of the protein to the methylated form of DNA will be decreased. Binding stoichiometry of 1:1 has higher affinity toward methylated DNA (form more stable complexes) while complexes with unmethylated DNA dissociate rapidly.

Higher binding stoichiometry will enhance nonspecific interactions with unmethylated DNA as a result of electrostatic interactions between positively charged residue of the protein and the phosphate backbone of DNA.

In another work Shen *et al.* used affinity probe capillary electrophoresis coupled with LIF to study the interaction of recombinant erythropoietin-a (used in treatment of anemia) and fluorescently labeled specific ssDNA aptamer [47].

Protein–carbohydrate interactions

These interactions are essential for growth control, cell adhesion, cell differentiation [7], apoptosis, fertilization, cell proliferation and morphogenesis [48]. Moreover, they have a critical role in various diseases and physiological disorders like autoimmune diseases, viral infection, inflammation [10], parasitic infection and tumor metastasis [48,49].

Gotti *et al.* used ACE to determine the correlation between binding constant and heparin HS (anticoagulant, highly sulfated glucosaminoglycan) structure or its biological activities when it binds to the serine proteinases antithrombin (III) (AT) [50]. In this study, PVA-coated capillaries were used, employing various concentrations of heparins from different origins (porcine, bovine and ovine mucosa). The heparins were introduced in the background electrolyte and a sample of AT was allowed to pass through the capillary in physiological conditions. Results showed that the complex formed has lower migration time than the free AT and the AT peak appeared later using a high concentration of heparin sulfate, thus confirming the presence of interaction between AT and HS. Heparin bound to the pentasaccharide-binding site, a specific binding site of the AT protein. Heparin sulfate has a higher dissociation constant than heparin since it lacks the sulfated pentasaccharide sequence. The measured binding constant indicated that the complex formed has *in vitro* activity as anti-factor Xa and IIa.

In a previous work, Varenne *et al.* used ACE to investigate the interaction of Fucoidan (anticoagulant-sulfated polysaccharide of marine algae) and antithrombin [48]. The binding stoichiometry for this interaction was 1:1. The dissociation constant was measured K_d >1 μM confirming weak noncovalent interactions, the obtained results were the same when frontal analysis was applied as an alternative method.

Kuhn *et al.* studied lectin–sugar interactions which are involved in cell recognition, enhancing lymphocyte movement and plays a role at purification and localization of sugar-containing molecules such as glycoproteins, proteoglycan and glycolipids [49]. Lectin is a protein obtained from animals or vegetables and show high affinity to low molecular weight carbohydrate, for example, L-(-)-Fructose-1-phosphate. In this study, three peaks of lectin appeared, these peaks were altered by fructose complexation. Addition of calcium ions to the buffer enhanced complex formation with the three lectins. Another family of adhesion molecules (selectins) that bind negatively charged ligands were identified based on association constant determination. These selectins are essential for leukocyte binding to the endothelium at inflammatory sites [49].

Carbohydrate–drug interactions

ACE is a useful tool in characterizing the interaction of drugs with various carbohydrates, examples are the interaction of starch degradation products (maltooligosaccharide) and β-blocker propranolol. This interaction is the cause of transport retardation across lipid membranes [5]. Interactions with cyclodextrins are other examples of carbohydrate–drug interactions. Different forms of cyclodextrins can be used in pharmaceutical preparations for many purposes. They can form host–guest complexes (inclusion complexes) with many drugs to increase their water solubility, their stability or to obtain sustained-release preparations as for example with the interaction with ethylated β-CD. Lemesle-Lamache *et al.* applied ACE to determine binding constants for inclusion complexes of β-CD and ethylated β-CD (Et-β-CD) with the β2 agonist salbutamol [51]. Complexation between β-CD and salbutamol modifies the release pattern of salbutamol leading to a sustained-release formulation. With β-CD, the total release was achieved after 1 h, while it required 8 h in case of 6-Et-β-CD, 8 h is the time required to release only 20% of salbutamol from complexes with 10-Et-β-CD. The effect of several parameters including buffer pH, temperature, voltage and viscosity of solution on binding constants were studied.

By increasing the CD concentration, a reduction in both the electrophoretic mobility toward the anode and the EOF toward the cathode was observed due to increased solution viscosity. This increase in viscosity also resulted in a decrease in electrical conductivity. Results showed that salbutamol migrated with the velocity of EOF at pH 7.5–9.5, but salbutamol becomes anionic at pH >9.5. Thus the electrophoretic mobility toward the anode increased and the migration time also increased, at pH <7.5 salbutamol gained positive charge leading to enhanced migration toward the cathode. The applied voltage has little influence on the binding constant. Increasing the temperature showed slight increase in the binding constant. The salbutamol–β-CD interaction is weak and can be enhanced by the ethylation of CDs.

In another work, Darwish *et al.* studied the interaction between sugammadex (gamma CD) and penicillin by ACE. Sugammadex can form strong complexes with the muscle relaxant rocuronium, thus reverses its neuromuscular blocking effect after surgical operations. Coadministration of sugammadex and penicillins could attenuate the pharmacological effects of both drugs [51]. Mobility shifts appeared upon complexation of amoxicillin, ampicillin, oxacillin, dicloxacillin and azlocillin with host molecules, no complex formed between sugammadex and penicillin G or pipracillin. This variation in interaction between different penicillins and sugammadex is attributed to difference in structure of these penicillins, especially in the R side chain [52].

Danel *et al.* studied the complexation of risperidone (Risp) and 9-hydroxyrisperidone (9-OH-Risp), atypical antipsychotics with seven cyclodextrins (CDs) (native and hydroxypropylated [HP] CDs and methyl [Me]-β-CD) using ACE and NMR spectometry [53]. ^{1}H NMR spectometry showed 1:1 binding stoichiometry. ACE showed that the highest binding constants with β-CD and Me-CD, β-CD cavity size can fit with the analyte while α and γ-CD cavities were not suitable for inclusion formation. Decreasing the hydrophobicity of analyte by 9-hydroxylation increased complexation.

Peptide–peptide interaction

Several articles reported the use of ACE to investigate the interactions between various peptides. The most tested peptides are the glycopeptide antibiotics [54,55]. Zavalenta *et al.* used multiple injection ACE to study the affinity and measure the binding constant of vancomycin, ristocetin and teicoplanin when interacting with the bacterial cell wall precursor terminus D-Ala-D-Ala [56].

Silverio *et al.* used on column ligand synthesis coupled with ACE to study the interaction between glycopeptides antibiotic (teicoplanin) and D-Ala-D-Ala terminus of bacterial cell wall precursor [57]. Results showed that teicoplanin has higher affinity to the D-Ala-D-Ala terminus than vancomycin. Colton *et al.* used ACE to screen small library of peptides that have affinities toward vancomycin [21]. Caladwell *et al.* developed an ACE method for screening peptides that can interact with extracellular binding domain of the erythropoietin receptor [58].

Heegard *et al.* used ACE coupled with laser-induced fluorescence to detect the mobility changes of the recombinant major histocompatibility complex II when it binds to a peptide fragment of influenza virus hemagglutinin [59].

DNA–drug interactions

These interactions are important in targeting tumor growth and various infectious diseases (bacterial, viral and fungal). He *et al.* studied the interaction between netropsin and a 14mer dsDNA by ACE and capillary zone electrophoresis (CZE) [60]. Netropsin is positively charged and found to cause reduction in charge-to-mass ratio of the negatively charged DNA. Increasing netropsin concentrations led to retardation in the migration of DNA, since the fraction of DNA-netropsin that contributed to the total dsDNA was increased. Van-der-Waals forces, electrostatic interaction, hydrogen bonding and hydrophobic interaction, all can share to some extent in the interaction between netropsin and DNA. The structural arrangement of DNA changed to be more fitted with the crescent shape of netropsin.

Other results showed that the use of CZE is preferred in studying such interactions, since fewer reagents are required. CZE showed larger binding constants than that obtained by ACE, since different quantitative models are used as the peak height. Different ranges of receptor-to-ligand ratios were obtained by the two methods, probably more investigation is required to judge which method is more accurate for this particular interaction.

Antigen–antibody interaction

ACE was successfully used in the characterization of antigen–antibody interactions (immune ACE [IACE]) [24]. Polyclonal and monoclonal antibodies as well as antibody fragments (Fab) were studied by ACE.

In 1994, Heegard was the first one to use ACE for the determination of the interaction between a monoclonal antibody and a negatively charged ligand phosphoserine [59]. Lin *et al.* studied the characterization of the polyclonal antihapten antibodies against haptens (dinitrophenols) [61]. At low hapten concentrations, one peak was obtained for the contribution of different antibodies, gradual split in the peak was obtained when the hapten existed in excess (32- to 1024-fold

molar excess of the initial concentration used). Results showed that all antibodies were of low-to-moderate affinity.

In a previous study, Lin *et al.* used ACE to determine the dissociation constant of phosvitin-anti-phosphoserine (monoclonal antibody) [62]. The results obtained were close to those obtained by other methods as ELISA [62]. Guzman *et al.* used IACE in the determination of inflammatory biomarkers. Cytokines were investigated in the cerebrospinal fluid of a patient with head trauma, neuropeptides like substance P, vasoactive intestinal enzymes and calcitonine gene related peptide were also studied in addition to a cyclosporine A metabolite as biomarkers [63].

ACE technique improvement

There has been many attempts to improve the conditions that give the best precision and sensitivity for ACE.

Improved precision

Precision is an important issue for each analytical technique. In ACE it depends on many factors, the main one being the stability of EOF (the EOF velocity may change as a result of receptor adsorption on the capillary surface, change in heat or use of high voltage [6] leading to false results). Precision can be improved by improving rinsing procedures, using the term mobility ratio to describe the mobility changes, acetanilide is usually used for this purpose because it is easily solubilized and remains neutral under the used conditions, control the protein concentration range, the temperature, the drug plug length and the applied voltage [6]. In 2010, AbdElHady *et al.* studied the effect of all the mentioned factors and developed a rinsing protocol that gave precision corresponding to an RSD of <5% (for n = 300–600). The effect of the applied voltage was also investigated at 5kV giving a precision of RSD = 1.53% and improved to be RSD = 0.48% at voltage 20 kV [6]. As protein concentration increased, the viscosity of the buffer increased as well and the precision decreased (due to the change of migration time of the neutral marker). This was overcome by the use of mobility ratio as an expression to the change in migration time [6,22]. In 2012, Redweik *et al.* investigated the interactions of various metal and pharmaceutical ions with many proteins obtaining precise methods with RSD below 2 for 90% of the interactions and RSD below 1 for 79% of the interactions by the use of an appropriate rinsing protocol (described also in AbdElHady *et al.* [6]) and the use of mobility ratio [41]. In ACE experiments, joule heating is an important factor since the temperature affects the mobility of the analyte and the binding equilibrium, and consequently affects the precision. Alhazmi *et al.* [4] investigated the effect of temperature on precision. Modern CE instruments reduce the generated heat by cooling systems for the capillary, but they differ in efficiency and in the percentage of capillary coverage. Until now there is no CE instrument which cooling system covers the whole capillary length. Flowing through the capillary, the sample will thus pass through different temperature areas (starting from hot inlet to the cold middle part and last to the hot outlet). Therefore the sample will undergo different interaction behaviors. In order to reduce this problem, Alhazmi *et al.* improved the precision by pushing buffer in the capillary directly after sample injection [4].

Improved rinsing protocols

Regularly rinsing the capillary is essential to remove adsorbed proteins, in order to maintain a stable EOF and obtain a better precision. Originally, it can be achieved by flushing the capillary with 0.1 M NaOH before each sample injection [64]. Lloyd *et al.* flushed the capillary with a solution containing SDS. SDS binds to proteins and being negatively charged, it increases electrostatic distraction between the proteins and the negatively charged silanol groups at the fused silica surface. Thus, SDS facilitates the desorption of proteins. The use of SDS in submicellar concentration in the running buffer itself can also keep the capillary wall clean but may denature proteins, so SDS was used in higher concentrations as washing solution. This rinsing was as effective as 1M HCl or 1M NaOH washing [64]. AbdElHady *et al.* improved another procedures to remove the adsorbed proteins, this protocol gave precision of RSD less than 0.5% in long-term measurements n = 300–600, by rinsing the capillary after each run with 0.1 M NaOH for 2 min at elevated pressure of 3000 mbar, then by water for 3 min at 3000 mbar and after 30 consecutive runs flushing the capillary with buffer for 25 min at 3000 mbar to avoid the formation of noise in the background [6] as shown in Figure 2 stable baseline was obtained.

Redweik *et al.* further optimized the protocol of AbdElHady [6] by decreasing the pressure to 2500 mbar and increase the pushing time to 3.5 min for 0.1 M NaOH, then the water for 5.5 min, at the end of analysis day capillary flushed with 0.1 M NaOH at 1000 mbar for 5 min then by water for 10 min and after 30 consecutive runs flush the capillary with buffer for 25 min at 2500 mbar [10,41]. In 2014, Alhazmi *et al.* studied the differences in rinsing protocols between short and long capillaries. Results showed that it requires the same procedures but shorter rinsing times for shorter capillaries [4].

Key terms

Fluorescence polarization (FP): The inverse relation between molecule size and its rate of rotation in the liquid media. Measurements provide information on molecular orientation and mobility, and processes that modulate them; for example, receptor–ligand interactions.

Chemiluminescence (CL): The emission of luminescence through a chemical reaction between chemiluminescence reactants.

Improved sensitivity

Most ACE experiments employ UV detectors [65–68]. These are typically sensitive to detect concentrations in the microgram per milliliter range. For lower concentrations, there is often the need for coupling ACE with more sensitive detectors.

Coupling with fluorescence detector

Fluorescence is a special character of some substances, when a substance absorb energy it transmit from ground state to the excited state, to return to ground state it loses this energy as fluorescence. An optical fiber light-emitting diode [69] or laser-fluorescence [70] can be used as a source for excitation. ACE coupled with fluorescence detectors is important in studying the nucleic acids (DNA, RNA) and protein interactions because it is very sensitive and able to detect ligands at trace level [71].

Zou *et al.* used this technique to study the interaction of MBD2b protein and dsDNA, in order to investigate the effect of DNA length on this interaction. DNA which is not fluorescent by its nature, was labeled at their 5′ ends by TMR (tetramethylrhodamine). Helium-neon laser was used as energy source to excite fluorescence, and photomultiplier tube was used to detect this fluorescence. Current-to-voltage converter was applied to convert the signal output from photomultiplier tube to voltage signal. The obtained electropherograms are shown in Figure 3 [46].

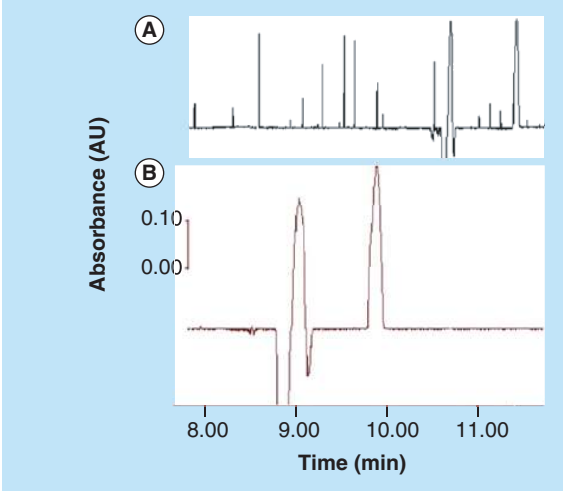

Figure 2. The effect of rinsing protocols on the mobility of Ac and Trp after 30 consecutive runs.
(A) Without further flushing and **(B)** with further flushing by phosphate buffer for 25 min under pressure 3000 mbar.
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Mass spectometry is a very sensitive, selective [7,72] and fast [7] technique, aiming to identify the analyte composition according to very precise molecular weight determinations [72]. MS can provide much better resolutions than one atomic mass unit. Detectors based on MS are widely implicated to determine the binding kinetics between protein and ligand [73–75] as also coupled with other techniques as ACE [18,76].

ACE/laser-induced fluorescence polarization

Coupling **fluorescence polarization (FP)** to ACE is used to study the interactions of tested molecules and to investigate these interactions as changes in migration time and fluorescence polarization [77]. It is a powerful and very sensitive analytical tool to study protein–peptide [78], protein–protein [79] and protein–DNA interactions [80]. Song *et al.* [77] used this technique to detect the human thrombin in the sub-nanomolar level using specific aptamers as probes (labeled with fluorescently TMR) and to study the dissociation constant K_d and dissociation rate constant K_{off} and the effect of some metal cations on the binding. The use of fluorescence polarization provides special advantages. It can distinguish between free and complexed thrombin, even if it is not well separated by ACE, using the FP value as shown in Figure 4. The FP value also helps in differentiating between the complexes with different stoichiometry. The FP is calculated as the difference of the emission light intensity parallel ($I_{||}$) and perpendicular ($I_{\perp}$) to the excitation light plane normalized by the total fluorescence emission intensity based on Equation 3 [81].

$$FP = (I_{||} - I_{\perp}) / (I_{||} + I_{\perp})$$

Equation 3

ACE with chemiluminescence detection

This type of detector allows the detection of compounds that are present in traces, thus improves the sensitivity of the method [82]. High sensitive **chemiluminescence** (CL) allows online detection of low volumes (in nanoliter range), with minimum background noise and peak broadening [82–84]. Substances that can be detected by CL include CL reactants, catalysts, enhancers or inhibitors of CL reactions or CL coupling reactions [82]. Examples of different CL reactants include luminal, peroxyoxalate, acridinium, ruthenium (II) and permanganate [82]. Biomarkers usually

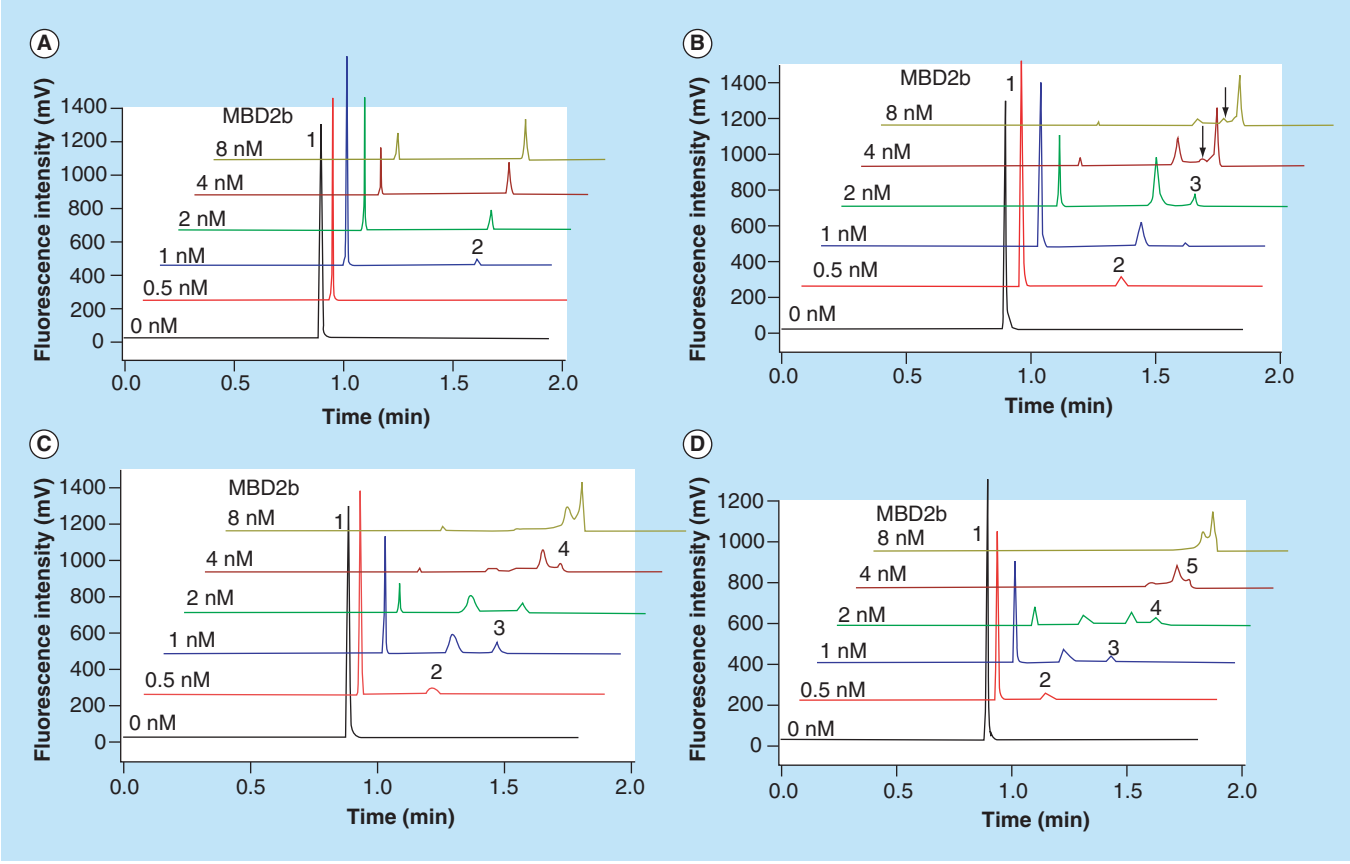

Figure 3. Capillary electrophoresis analysis of mixtures containing TMR-labeled DNA probes (2.0 nM) and MBD2b protein (0–8.0 nM). Peak 1 is the free DNA, and peaks 2–5 correspond to the DNA–MBD2b complexes with corresponding stoichiometries of 1:1, 1:2, 1:3 and 1:4: **(A)** M-20mer*, **(B)** M-40mer*, **(C)** M-60mer* and **(D)** M-80mer*.
Reproduced with permission from [41] © Ameican Chemical Society (2012).

exist in traces in biological fluids, so the need of high sensitive detection is an issue in the analysis of these compounds. Lin *et al.* used high sensitive CL detection and boronate ACE to investigate the positional isomers of benzendiols in urine [82]. These isomers are catechol, resorcin and hydroquinone which are the o-, m- and p-benzendiol, respectively. They can be found in urine after exposure to benzene or any product that contain one of these isomers as dyestuff and pesticides. Spiked urine samples of the three isomers were used, three separate peaks were obtained, these results emphasize that CL is an accurate (recoveries >97.2%), sensitive (LODs about 3×10^{-8} mol/l) and reproducible (RSDs% > 5.7%) method since it detects traces of different benzendiols despite of having similar structures [82]. CL can also be applied in immunoassays.

ACE–MS

MS may be divided according to the type of ionization into fast atom bombardment (FAB), chemical ionization (CI), APCI, ESI and MALDI, where the ESI is the most predominant one. ESI can be combined with MS in two ways: sheath liquid interfacing or sheathless interfacing. The sheath liquid interface is commercially available and most widely used for CE-MS. For good CE-MS coupling, there are some requirements as to have very low flow rate (nanoliter per minute), and to have closed electrical circuit to maintain high voltage across the capillary (which achieved by suitable interfaces) [85].

CE-MS can be applied in fields such as proteomics, metabolomics, pharmaceutical, forensic science and food analysis. For example it is used in the analysis of biomarker in the body fluids (urine, CSF, plasma) in disease diagnosis [86–88].

Mironov *et al.* compared between ACE-UV, ACE-MS and DIMS in studying the interaction between eight drugs and β-cyclodextrin [18], the ACE-MS system provided several advantages over the other systems. ACE provided the separation ability and MS provided the very sensitive molecular weight and structure detection ability in the same system, it does not need special labeling for substances to be able to measure the mass in fluorescence detectors. So it can detect more substances. MS can determine the structure of all analyte components including the known analyte, unknown components

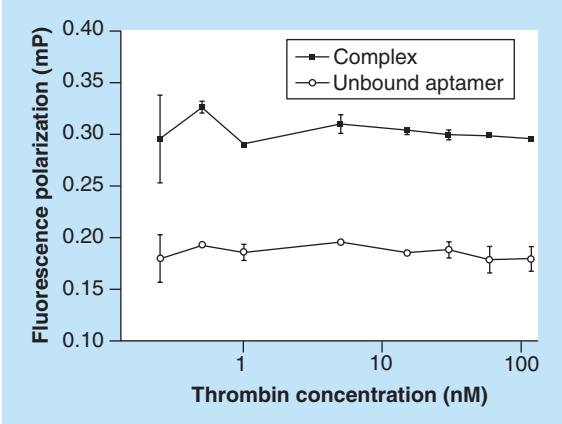

Figure 4. The change of FP value obtained from the samples containing 5 nM TA35 and varying concentration of thrombin. The FP values are the mean obtained from three measurements. The error bars were the standard deviations from the mean. Reprinted with permission from [72] © Elsevier (2005).

and impurities. Thus the interactions of multiple analytes in the same experiment can be studied in the same experiment [18]. MS can distinguish which compound really exhibit affinity for the protein. The interaction occurs approximately under physiological conditions, even though the detection does not. Mass detection reduces the error in K_d calculations and determine the K_d even for small molecules which have the same mobility as the cyclodextrin and its complex (overlapped peaks).

Inductively coupled plasma MS is a type of mass spectometry which depends on ionization of the sample by inductively coupled plasma (plasma is the state of the matter where the gas is ionized) which is ionized by heating the gas in an electromagnetic field contain ions and electrons. It has high efficiency and sensitivity to detect all metals in very small concentrations and can also detect several nonmetals. Coupling ACE-ICP-MS provide low detection limits and particularly small sample sizes [89]. This coupling requires a specific interface to ensure the lock of the CE electric circuit and to regulate the flow rate in the outlet of the electrophoresis to be suitable for the inlet of the detector [90]. Claveranne-Lamolère *et al.* studied the uranium–colloid interaction in carbonated soil by ACE-ICP-MS because of its high efficiency to detect trace metals [90]. In 2012, Sun *et al.* used ACE-ICP-MS and NECEEM-ICP-MS to study the binding constant of organometallic compounds (TMT, TPrT, TBT, TphT) with HSA, the Ace results obtained from ACE-ICP-MS are more precise and accurate for calculating the binding constant using the four linear regressions equations (isotherm, x-reciprocal, y-reciprocal and double reciprocal) [89]. The change in electrophoretic mobility of each metal was monitored as the concentration of HSA and buffer additives increased as shown in Figure 5.

ACE hyphenating with high sensitivity cell

The low sensitivity of ACE-UV is the result of the short light path within the UV detector (which is equal to the internal diameter of the capillary). Using a high sensitivity cell, the light path is elongated and hence the sensitivity is increased. AbdElHady *et al.* coupled ACE with high sensitivity cell to study the interaction of retinol and retinoic acid with HSA and BSA and calculate the binding constants. The displacement of retinol and retinoic acid on HSA and BSA in the presence of ibuprofen was investigated. A high sensitivity cell with a light path made of blacked fused silica was used in order to minimize the deflection of light and to define the aperture for diode-array detection [8].

In another study chiral capillary electrophoresis CCE (using cyclodextrins as receptors) was coupled to a high sensitivity cell to study the catechin (C) and epicatechin (EC) in human plasma. This coupling improved the time-corrected peak area by ten-fold over standard cell and fivefolds over a capillary with bubble cell. The same design of the high-sensitivity detection cell for Agilent 3DCE system (Figure 6) was used [8,16] in both studies.

ACE & field-amplified sample injection or large volume sample stacking

Field-amplified sample injection (FASI) is an online sample pre-concentration method to improve the sensitivity of ACE in the detection of samples in the nano-scale depending on the use of electrolyte buffers with high conductivity to fill the capillary before the injection of sample with low conductivity. This leads to a higher electric field in the sample zone than in the electrolyte zone. Hence, the sample focuses with high velocity before reaching the electrolyte zone and stacks at the interface between sample and electrolyte buffer (for each analyte type individually) and reaches the detector individually stacked leading to improved sensitivity. This can be achieved either by reducing the sample conductivity through the addition of organic solvents as acetone, acetonitrile or alcohol, or dilute the sample by pure water. However, this can also be achieved by increasing the conductivity of the electrolyte buffer through increasing the electrolyte concentration [91]. For further improvement of the stacking, a water plug can be injected in front of the electrolyte buffer [91,92]. The field-amplified sample injection provided advantages over other stacking methods because the injection volume is not principally limited [91]. Danger *et al.* compared the results of three methods for sample stacking: FASI, LVSS and normal sample stacking in the reaction of α,α-dialkylated amino acids and NCAs (N-carboxyanhydrides), FASI gave an enhancement factor of 20 and RSD of 11% of the

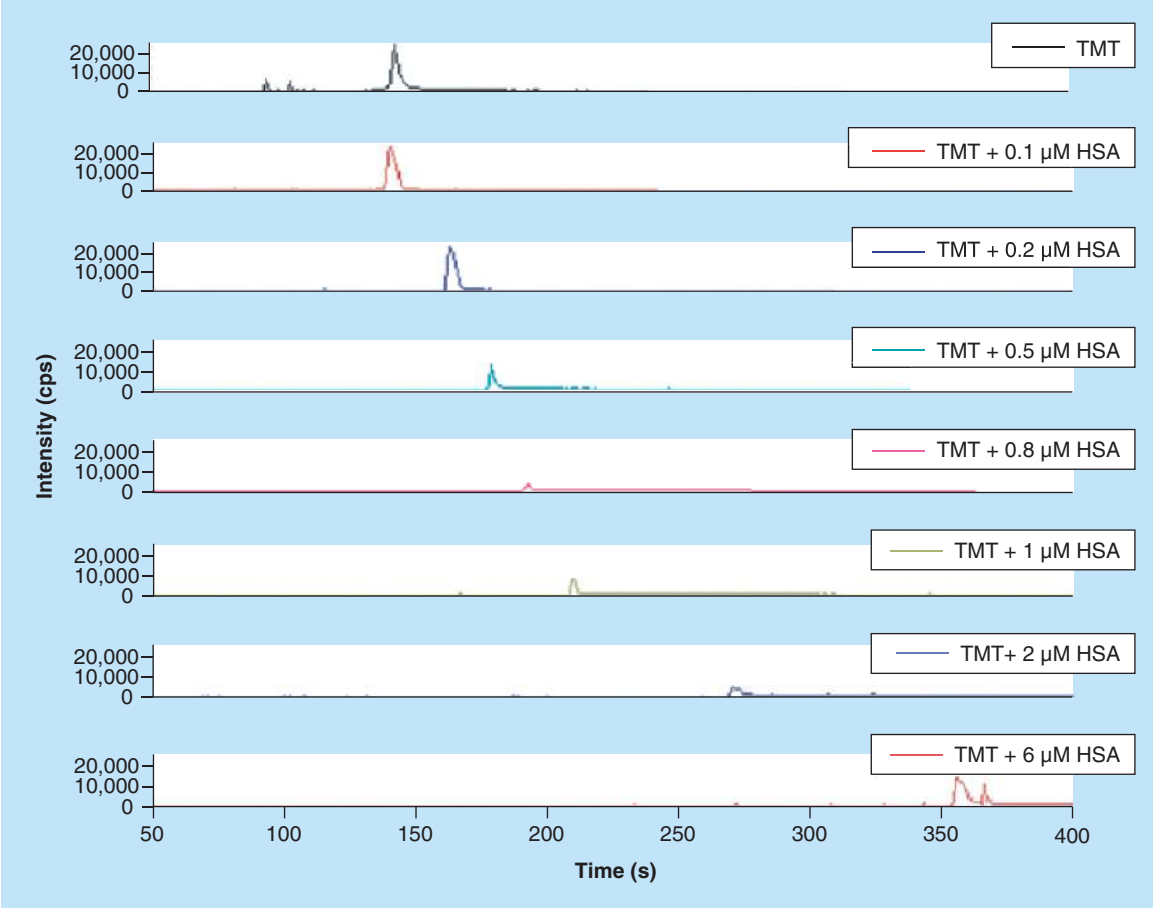

Figure 5. Affinity capillary electrophoesis electrophoregram about the mobility shift of the organo-tincompound obtained with background buffers containing various concentrations of human serum albumin. Separation condition: uncoated-fused silica (75 µm id × 50 cm); applied voltage: 20 kV; hydrodynamic injection (20 mbar, 5 s); background electrolyte: 10 mmol/l phosphate buffer (pH = 7.4) containing various concentrations of HSA (0–6 _mol/l).
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time-corrected peak area for five samples [91]. FASI methods were successfully used with other CE techniques to improve the sensitivity [93,94]. Therefore FASI can be beneficially used for ACE as well. However, please note that the nonlinear processes during focusing will complicate the precise calculation of binding constants. When the sample concentration is very low conventional and ACE methods are not sensitive to these limits, on-line sample preconcentration methods can be useful to decrease the LOD [95–102]. Then large volume sample stacking can be employed, which is characterized by the ability to inject sample up to 95% of the total capillary length [91].

Immunoassays by CE & ACE

Immunoassays by CE are certainly related to ACE [103]. Even though these assays often serve different purposes, they can of course also be used to screen for binding properties and estimate binding constants. Since this is a well-defined area as such, we recommend the reader to the respective entries in databases. Certainly a review related to this subject can be expected in the near future as well.

IACE is strongly related to immunoassays in general. In addition, Mangru and Harrison used chemiluminescence (CL) detection to investigate the binding of mouse IgG Fab fragment conjugated with horseradish peroxidase (catalyst for CL system) on microchip ACE [84]. Zhang *et al.* developed a method in which light emitting diode irradiation (LED) was used to induce photoxidation of luminal to generate CL. LED-CL is regarded as a fast high sensitive method with good reproducibility [83].

Modification of ACE modes

Multiple-injection ACE

Multiple-injection ACE (MIACE) is a highly reproducible developed ACE approach to calculate the binding constants. Advantages include saving of sample volume and the analysis time. The sample solution containing neutral marker and receptor is

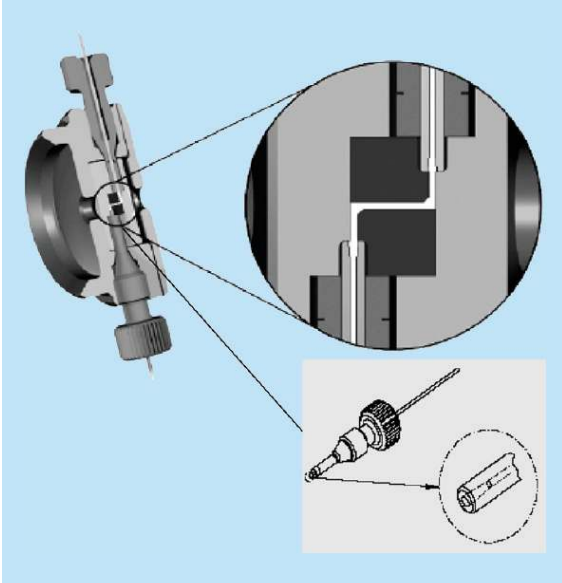

Figure 6. Schematic illustration of the high-sensitivity detection cell for Agilent 3DCE systems and capillary connection.

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injected as a plug and electrophoresed in a buffer contained known concentration of ligand, this step was repeated in the same run and the same capillary but with different ligand concentrations from low to high, increase in the ligand concentration until having alternative arrangements of the sample plug and the ligand plug generating a single electropherogram containing a series of individual sample plugs superimposed on environments of buffer containing increasing concentrations of ligand. The binding constant was calculated from the changes in relative migration time ratio (RMTR) as a function of ligand concentration [56,104]. MIACE has already been used to determine the binding constants between several peptides and glycopeptide antibiotics [56].

Partial-filling ACE

Partial filling technique is especially useful when the available sample amount is very small, as if it is difficult to synthesize or isolate, or when it is expensive [7,105–107]. A sample plug is injected to partially fill the capillary instead of running sample solution in the classical ACE [108]. There are numbers of modified PFACE techniques including flow-through PFACE(FT-PFACE) [108,109], competitive flow through PFACE (CFTPFACE), on-column ligand synthesis PFACE(OCLSPFACE) and multiple-step ligand injection PFACE (MSLIPFACE) [104,108].

Key term

Amperometric detection: Detection of ions in a solution based on electric current or changes in electric current.

Pressure-mediated ACE

Pressure-mediated ACE (P-ACE) is a method developed to accelerate the ACE process, shorten the total analysis time and preserve the accuracy in determination of protein-drug binding constant for weak interactions (k_b <10^5 M^{-1}). Like ACE, P-ACE can be used to determine the mobility of the analyte and the change in electrophoretic mobility due to protein interactions. This modified approach offers many advantages over the conventional ACE. Using gas pressure to force the sample toward the detector speeds up the process, and also pushes the sample directly into the thermostated section, mitigating temperature variation effects on the separation accuracy [110].

Dynamic ligand exchange-ACE

Dynamic ligand exchange-ACE (DLE-ACE) is a method used to study protein interactions with small ligands that do not affect the protein mobility. By determination of the relative stability of the apo\holo protein state upon unfolding (protein apo state refers to the free protein while protein holo state refers to protein–ligand complex). DLE-ACE helps in determine the binding constant, apparent ligand dissociation constant (K_d), holoprotein conformational stability (ΔG^o_u) and cooperativity parameters [111].

Microchip ACE

ACE can be conducted on microchips to help reduce the sample volume and time of analysis while keeping the ability to calculate the binding constant. Microchip ACE (MC-ACE) can be equipped with UV or amperometric detection. The microchip is usually fused silica divided into separation channels with (25 mm, 80 cm) length for UV and amperometric detector, respectively, 50 µm width, and 20 µm deep. It is good noticeable that the separation pathway in microchips is much shorter than that in capillaries which result in very short analysis time. Stettler and Schwarz [112] compared the ACE in capillary and on microchip and found that MC-ACE allows to distinguish between the isomers and gives the same electrophoretic migration as in capillary. The separation in capillary was preferred because it is more precise and gives narrower peaks. The wide peak using chips resulted from the high injection volume relative to the separation channel length and the system recording the electropherogram. However, the microchip is preferred as it is faster and consumes less buffer. Microfluidic chip interfacing to ACE was also successfully applied for immunoassay studies. The application of multichannel device allowed rapid mixing, reaction and separation for simultaneous direct immunoassays [113].

Using dual markers

One of the most important issues while applying ACE technique is to serve a stable EOF during analysis. Noninteracting standards can be added to compensate for any fluctuation in EOF that may occur. Mobility ratios of the standard and the receptor before and after complexation can be used by scatchard analysis to measure the binding constant of the receptor–ligand complex. Furthermore, the use of two standards (dual markers) has proved its value in ACE studies [114]. Since more accurate binding constants can be obtained by using the RMTR:

$$RMTR = (t_r - t_{s'})/(t_{s'} - t_s)$$

Where t_r, t_s and t_s are the migration time of the receptor and two markers. Furthermore, another term used to increase the accuracy of results obtained through the use of dual marker is the time average ratio (TAR) which explains the migration time of the receptor (t_r) divided by the average of the migration times of standards (t_s and $t_{s'}$).

$$TAR = (t_r)/((t_s + t_{s'})/2)$$

Subsequently, the binding constant K_b can be calculated from scatchard analysis by measuring the change in TAR as a function of the concentration of the ligand.

$\Delta TAR_{R,L}/[L] = K_b \Delta TAR_{R,L}(max) - K_b \Delta TAR_{R,L,}$ where $K_b \Delta TAR_{R,L}$ (max) is the greatest value obtained by full saturation. In a work for Villareal *et al.*, standard ACE, (FTPFACE) and on-column derivatization coupled to ACE were applied to investigate the reproducibility of TAR in measuring the binding constant for different model systems including binding of carbonic anhydrase B to its inhibitor (arylsulfonamide) and vancomycin to the D-Ala-D-Aladipeptide. Results proved that more accurate values for K_b were obtained by using the TAR form. These values were close to K_b values obtained by applying the RMTR approach [114].

Software to support ACE data evaluation

These programs provide mathematical models that allow the measurement of binding constants for receptor–ligand interactions, determination of the strength of interaction and understanding the structural geometry of the complex formed by calculating the intra-atomic distances between atoms that share in the interaction and determining their orientation in space.

Peak shape modeling by Haarhoff-Van der linde function

To obtain more precise results for the migration time of a compound from the apex of the peak, this peak must be symmetric. The peaks obtained from ACE are intermediate from Gaussian to triangular due to the influence of electromigration dispersion (EMD). Triangular peaks give negative or positive errors in the migration time, thus accurate values cannot be obtained from the peak apex. EMD can result from alteration in migration velocity of an analyte due to changes in pH, pK_a and conductivity. To obtain elegant peak shapes, EMD must be minimized by optimizing background electrolyte composition or the use of small amount of the substrate. However, in some cases EMD cannot be completely avoided (e.g., using FASI or P-ACE, see section 3.3 and 3.4). It was reported that triangular peaks can be well fitted by a Haarhoff-Van der linde function (HVL) [115]. Several parameters contribute to HVL including peak area, center of Gaussian component, SD of Gaussian component and peak distortion measurement. HVL allows the estimation of migration times from the central value of Gaussian component in cases of strong triangular peaks. Saux *et al.* evaluated the use of HVL to modify the fronting and the triangular shape of the peak in two concentration levels of naproxen and β-cyclodextrin [115].

ACE & molecular modeling

The use of the density functional theory (DFT) with ACE was reported by Ehala *et al.* [116–119]. In one study [119] the complexation between metal cations (Rubidium and Cesium) and hexaarylbenzene-based receptor was investigated. The binding constants of the complexes of metal cations and the receptor were estimated by ACE, using a nonlinear regression analysis, then the structural characteristics were determined by DFT calculations. Results showed that the free receptor has C3 symmetry and a bowel-shaped cavity formed by the aromatic bottom (aromatic ring) and the six ethereal oxygen atoms (from aryl groups). The depth of the cavity and its diameter were calculated before and after complexation by simulation programs, showing an increase in the depth of the cavity of the receptor after complexation. In a previous work, Ehala *et al.* used DFT to investigate the structural binding property of benzo-18-crown-6-ether complexes, which are sensors for organic ions and protecting groups in organic synthesis, with the ammonium cation. Binding constants calculations of the complex were obtained using ACE [117]. Complementary DFT calculations and ACE were also used to investigate the binding of the lithium ion to anhexaarylbenzen-based receptor [118] and valinomycin [119].

Nachbar *et al.* found that the use of semi-empirical methods like PM7 can also be employed with ACE [Nachbar M, Mozafari M, AlHazmi H *et al.*, Manuscript in Preparation]. The complexation of calcium ions by a serine-rich peptide obtained from digestion of galectin-3 (CBPep) was investigated. This peptide was

Key term

Equilibrium dialysis: A technique used to measure the binding of a microsolute or ions to a macrosolute placed inside a dialysis bag suspended in the solution containing the microsolute.

found to bind Ca^{2+} ions exceptionally strong in MS experiments [120]. However, it was wondered if the binding properties would be as strong in aqueous solutions.

The only interaction partner for the calcium ions is probably the peptide backbone because of the otherwise mainly hydrophobic residues. The weaker binding of CBPep and Ca^{2+}-ions in water derived by ACE confirmed this assumption [Nachbar M, Mozafari, M., AlHazmi H. *et al.*, Manuscript in Preparation]. The semi-empirical calculations for the CBPep–calcium complex made this more obvious. The complex shows cation–π interactions between the calcium ion and the peptide carbonyl oxygens (charge solvation) [121]. Therefore, the hydrophobic parts of the peptide face the surroundings. This is possible in the MS experiment considering vacuum environment, but in water this state is energetically not favored [122] (Figure 7).

Other questions can be answered by the combination of molecular modeling and ACE as well [Nachbar M, Mozafari M, AlHazmi H. *et al.*, Manuscript in Preparation.] Dehydrins are unstructured, herbal proteins which are mainly expressed during the embryogenesis of plants or when they suffer from water stress such as drought, cold and high salinity. It is reported, that they can bind some metal ions and that through metal ion complexation, α-helices were assembled [123,124]. Unfortunately no crystal structures are available for proteins of the dehydrin family. Therefore, homologous models were designed from the amino acid sequence of the dehydrin AtHIRD11 using the software Molecular Operating Environment (MOE) [125] and Phyre2 [126] which explain their structure and the observed binding properties toward transition metal ions. Additionally, the interactions between the binding domain D6 and Cu^{2+} as well as Zn^{2+} were investigated by ACE and semi-empirically modeled to give a better insight into the complexation at this binding site [Nachbar M, Mozafari M, AlHazmi H *et al.*, Manuscript in Preparation].

Comparison of alternative techniques to ACE

Many techniques have been developed to measure ligand–receptor interactions. These techniques could be classified into separative and nonseparative techniques [126]. The separative group involves the separation of the free ligand from the bound species and is used to determine directly either the unbound ligand or the bound ligand concentration. The second nonseparative group relies generally on the detection of a change in a physicochemical property of either the ligand or the receptor because of the binding.

Separative techniques include equilibrium dialysis (ED), parallel artificial membrane permeability assay (PAMPA), high-performance affinity chromatography (HPAC) and ACE (ACE). As an ideal assay should cumulate the properties of specific, sensitive, easy to perform, reproducible, cheap, rapid and suitable for automation and the possibility to trace quantify multiple analytes in a single assay, the HPAC and the ACE systems should preferably be interesting for scientists. The number of publications in the last five years (2008–2013) (cumulated by Scopus as in Table 1) reflected this fact. In HPAC, the receptor (e.g., protein) is immobilized on a support and the interacting ligand is injected into the column. It is therefore important to consider the extent to which the support-bound receptor will model the behavior of the receptor in its soluble form. This is of crucial concern because the immobilization process can affect receptor activity by denaturation, improper orientation or steric hindrance at the binding sites to be studied [Nachbar M, Mozafari M, AlHazmi H *et al.*, Manuscript in Preparation.] Furthermore, the relatively high cost of the column manufacturing, the improper stability conditions of immobilized receptors which are in most cases apart from physiological conditions and the difficult treatment of data handling could limit the use of HPAC. On the other hand, ACE has several advantages as described in the aforementioned sections such as high separation efficiency, low sample volume, no need to a highly purified sample, low buffer consumption, ease and low cost of automation and ability to work with huge numbers of receptors under physiological conditions. Furthermore, ACE data measurements are precisely handled in a short analysis time (by using excel spread sheet as explained in our previous work [6]) and consequently binding constants are adequately calculated. Moreover, ACE experiments can be performed in parallel because of the multiplexed CE instruments available. However, at present the number of HPAC publications still exceeds the corresponding number for ACE. Possibly ACE inherits the situation of CE related to HPLC. HPLC is much more popular than CE, due to the vast experience available with the first mentioned technique. There are a variety of possible manufacturers, offering very reliable instrumentation to competitive prices and also a huge knowledge base about HPLC principles in text books and technical articles are available. HPLC principles are widely taught to graduated and postgraduated students, and there is a high number of training workshops available for HPLC basic operations and troubleshooting all over the world. From our experience the main reasons

for this are because of the marketing but scientifically CE have several advantages motivating its use in biomolecular interaction studies and analyses in complex matrices. Thus, ACE may well close up to HPAC or even overtake.

The nonseparative group could be classified into two subclasses as radioactive and nonradioactive assay formats. Nonseparative techniques include spectroscopic approaches (including ultraviolet spectrophotometry (UV), circular dichroism (CD), NMR, fluorescence, mass spectometry and Fourier transform infrared (FTIR)), potentiometry, isothermal titration calorimetry (ITC) and surface plasmon resonance (SPR). Over the past years, a shift from radioactive to nonradioactive-based detection of receptor–ligand interactions was observed to reduce health risks and environmental pollution as well as costs. As shown in Table 1, most of useful nonseparation technologies in ligand-binding studies are fluorescence, NMR, SPR and ITC. Fluorescence spectometry helps in identification of the binding site of a drug and can also be used to calculate the binding distance between the fluorophore on the protein and the drug [126]. NMR spectometry indicates which groups or parts of a protein molecule are involved in the binding process. SPR-based techniques involve immobilization of receptor on a surface and monitoring its interaction with a ligand in solution that flows over the surface [126,127]. Basically, an SPR detector monitor changes in the refractive index that occur as molecular complexes break during the binding reaction at the sensor surface anchoring one of the interaction partners. Mathematical treatment of the signal obtained gives the binding data.

A major drawback of SPR is the need to label and/or immobilize one or both of the interacting partners like in HPAC with the possibility of altered binding characteristics and specificity. Moreover, the refractive index change per molecule is related linearly to the molecular weight of the compound that binds the immobilized molecule. Therefore, when working with small molecular weight drugs that bind to high molecular weight proteins, it might be difficult to obtain reliable data without specific equipment [118]. ITC is the calorimetric approach most used to investigate biomolecular interactions. Typically, ITC is based on the successive additions of a drug to a solution of protein contained in a reaction cell. Each addition of ligand to the protein sample leads to the formation of a specific amount of ligand–protein complex which can be evaluated by monitoring the heat release [126,128]. Low throughput of ITC may also be an issue, because the time required to run a full titration experiment is at least 2.5 h and usually even longer [126]. As for all nonseparation techniques, the samples have to be highly purified. Table 2 cumulated the most features (advantages and disadvantages) of different technologies for ligand-receptor binding studies. It is therefore difficult to have a universal nonradioactive mix-and-read assay format for all receptors currently known, which makes the classic radioreceptor assay still the method of choice in drug screening. Furthermore, long analysis

Table 1. The number of publications in the last 5 years (2008–2013) cumulated by Scopususing: key is the name of technology between parentheses searched in title/abstract/keywords, document type is article or review and subject area is life science.

Technique	Number of publications during 2008–2013 year
Separative techniques	
High performance affinity chromatography (HPAC)	1973
Affinity capillary electrophoresis (ACE)	411
Equilibrium dialysis(ED)	342
Parallel artificial membrane permeability assay (PAMPA)	77
Nonseparative techniques	
Fluorescence(FL) and binding	21,649
Nuclear magnetic resonance (NMR) and binding	10,496
Surface plasmon resonance (SPR) and binding	4304
Isothermal titration calorimetry (ITC) and binding	2069
Mass spectometry (MS) and binding	2039
Ultraviolet spectrophotometry (UV) and binding	1299
Potentiometry and binding	638
Fourier transfer infrared (FT-IR) and binding	180
Circular dichorism (CD) and binding	7

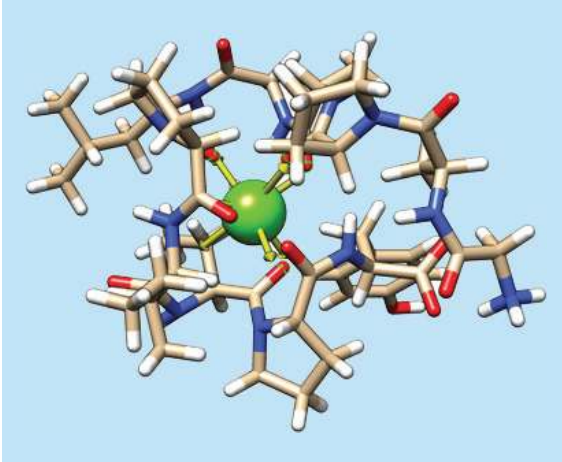

Figure 7. Charge solvated complex of CBPep and Ca^{2+} with hydrophobic residues on the surface. The Ca^{2+}-ion is bound by five carbonyl oxygens derived from the peptide bonds. Molecular graphics and analyses were performed with the UCSF Chimera package. Chimera is developed by the Resource for Biocomputing, Visualization and Informatics at the University of California, San Francisco (supported by NIGMS P41-GM103311) [117].

time in SPR, large amount of samples and the need of highly purified reagents in spectroscopic and calorimetric approaches could limit significantly their using in future.

The suitable choice of the method for ligand–receptor interactions is a hard decision (Table 2) and is based on more information about the physicochemical properties of the studied compounds. The amount of material available may also condition this choice. For example, if the solubility of a sample in aqueous medium is low, spectroscopic and calorimetric approaches may fail whereas HPAC or SPR may be adequate. A limitation of PAMPA could be the analysis of very lipophilic drugs that might stay trapped in the membrane. When material is scarce, ACE should be considered, as it consumes only minimal amounts of sample. The classical methods (as ED) are not suited to study interactions between compounds of approximately the same size, because they are based on differences in molecular size. Typically, protein–protein interactions cannot be analyzed by such systems. SPR that senses changes proportional to the mass of bound material on the sensorchip may be a fine alternative. Samples of low purity require separation techniques, such as HPAC or ACE, unless a purification step is performed before.

By taking an overlook on the available techniques for the study of ligand and receptor binding, ACE has had great attention in the last years. Meanwhile, there are still drawbacks with ACE, including its inability to yield validated complex stoichiometry, lack of kinetic information and lack of knowledge about the conformational structure of the binding receptor (Table 2). In order to validate the stoichiometry of a complex formed, one needs to perform further experiments using UV spectometry and NMR. SPR is ideal for obtaining kinetics information that might be of great importance in understanding some biological phenomena. Furthermore, through the NMR experiments, especially 2D correlation spectometry (COSY) experiments, valuable information about molecular structure could be provided.

Several trials as described before in this review were performed for the hyphenation of ACE with other non-separative techniques to enrich the information about ligand-receptor binding or to improve the throughput of its performance. For example, the hyphenation of ACE with laser-induced fluorescence and mass spectrometry (MS) detection systems were commonly discussed, nowadays. Unfortunately, because only few molecules possess native fluorescence, labeling is often required in LIF which can interfere with the binding interactions. For ACE–MS, the essential use of MS-compatible buffers can also alter the binding process. In this case, a sheath–liquid interface for the ACE–MS coupling is useful. The composition of the sheath liquid is very critical as it may have a huge influence on the stability and intensity of the observed signals. To detect receptor–ligand interactions, the sheath liquid should not contain high concentrations of organic and acidic/basic components. Probably sheathless interfacing could become a very prominent option to circumvent these difficulties.

Therefore, because of the complementary nature of the techniques, often only a combination of different approaches enables the scientist to catch a glimpse into ligand–receptor interactions [22]. In other words, there is no single 'magic bullet' technique for hit-to-lead ligand-receptor binding progression in the current state. To obtain detailed information on binding, it is best to use a combination of techniques, each having its own advantages and limits, together with computational tools, and later cross-validate data.

Conclusion & future perspective

This review gives an overview on the recent developments of ACE assay technology for the study of ligand and receptor interactions. The measuring principles, advantages and disadvantages of ACE are discussed and highlighted on some practical examples. Over the past 10 years, recent improvements 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 bioanalysis. Nowadays, several improvements in ACE validation were achieved to get precise, sensitive and robust bioanalyti-

Table 2. The most common features of different techniques used in the receptor-ligand binding studies.

Parameter	Separative techniques				Nonseparative techniques				
	ACE	HPAC	ED	PAMPA	FL	NMR	SPR	ITC	Spectro
Sample purity	Not required	Not required	Highly pure	Highly pure	Highly pure	Highly pure	Highly pure	Highly pure	Highly pure
Sample volume	nanoliter	ZE: 10 μl FA: ≥1000 μl	500–1000 μl	300 μl	Low milliliter	Low milliliter	D: 30–500 μl	200–1400 μl	Low milliliter
Order of concentration	10(-6) mol/l	ZE: D: 10(-6) mol/l FA: D: 10(-6) mol/l	10(-6) mol/l	10(-6) mol/l	10(-6) mol/l	10(-3) mol/l	D:10(-6) mol/l P: 50–100 μg	10(-3) mol/l	10(-6) or 10(-3) mol/l
Physiological conditions	Yes	Yes	Yes	Yes	Difficult	Difficult	Yes	Difficult	Difficult
Immobilization	No	Yes	No	No	No	No	Yes	No	No
Separation efficiency	High	Moderate	Low	Low	NA	NA	NA	NA	NA
Simplicity and analysis time	Simple and rapid	Moderate	Long	Rapid	Long	Rapid	Long	Long	Rapid
Multiple binding equilibria	Yes	Yes	Yes	No	No	No	No	Difficult	No
Kinetic data	Rare	Rare	No	No	Yes	Yes	Yes	No	Yes
Receptor-ligand affinity rate	High and moderate	ZE: high and moderate FA: low	Moderate and low	Moderate	Moderate and low	Moderate and low	High to low	Moderate and low	Moderate and low
Receptor-ligand stoichiometry	Difficult	Difficult	No	No	Yes	Yes	Difficult	No	Yes
Validity of binding constants	Yes	Yes	Poor	Poor	Yes	No	No	No	poor
Conformational structure	No	No	No	No	No	Yes	Yes	Facilitate	No
Cost	Inexpensive	Column cost	Inexpensive	Membrane cost	Expensive	Expensive	Expensive	Inexpensive	Inexpensive
Others	96-capillary format possible adsorption on capillary wall	Binding percentage	Donnan effect Nonspecific adsorption Volume shifts	96-well technology	Labeling requirement	High cost of reagents and gases	Kinetic stability information	Thermodynamic parameters	Low sensitivity False negatives

ACE: Affinity capillary electrophoresis; D: Drug; ED: Eequilibrium dialysis; FA: Frontal analysis; FL: Fluorescence; HPAC: High-performance affinity chromatography; ITC: Isothermal titration calorimetry; NA: Not available; NMR: Nuclear magnetic resonance; P: Protein; PMPA: Parallel artificial membrane permeability assay; Spectro: Spectroscopic approaches; SPR: Surface plasmon resonance; ZE: Zonal elution.

cal data. The parameters that could affect the robustness of ACE include the capillary manufacture batch, type of temperature controlling tool and the purity of running buffer constituents.

The race toward automation, miniaturization and parallelization is likely to continue. In this case, the point venue between the experts in ACE with others in sophisticated techniques such as SPR, MS and NMR is the leading step for the future progress in receptor-ligand studies. Future progress in SPR will follow the current exploratory array based developments of SPR imaging/microscopy. The sensitivity improvements of NMR have recently allowed the study of in-cell protein–ligand interactions, opening the door to the in-cell screening of drugs targeting specific intracellular proteins by NMR spectometry. Therefore, new developments in ACE hyphenated with other techniques are still needed for the study of membrane protein–ligand interactions, especially for structural studies. A lot of efforts in future will probably be dedicated to hyphenate and combine ACE with such techniques. The future holds much exploration for the use of ACE in bioanalysis.

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Executive summary

- Affinity capillary electrophoresis (ACE) in term of principles, advantages and disadvantages, recent advances and multidiscipline applications are covered.
- Different affinity interactions including protein–drug, protein–metal ion, protein–protein, protein–DNA, protein–carbohydrate, carbohydrate–drug, peptide–peptide, DNA–drug and antigen–antibody are discussed.
- Improvements of ACE in term of precision, rinsing protocols and sensitivity are described.
- The performance of ACE is compared with other techniques such as equilibrium dialysis, parallel artificial membrane permeability assay, high-performance affinity chromatography as well as surface plasmon resonance, ultraviolet spectrophotometry, circular dichroism, nuclear magnetic resonance, Fourier transform infrared, fluorescence and mass spectrometry and isothermal titration calorimetry.
- Significant improvement in ACE validation were reported to get precise, sensitive and robust bioanalytical data.
- The parameters that could affect the robustness of ACE include the capillary manufacture batch, type of temperature controlling tool and the purity of running buffer constituents.
- Successful application of computer simulation programs to support data evaluation is highlighted. The race toward automation, miniaturization and parallelization is likely to continue.
- The point venue between the experts in ACE with others in sophisticated techniques such as SPR, MS and nuclear magnetic resonance is the leading step for the future progress in receptor-ligand studies.
- Therefore, new developments in ACE hyphenated with other techniques are still needed for the study of membrane protein–ligand interactions, especially for structural studies.
- A lot of efforts in future will probably be dedicated to hyphenate and combine ACE with such techniques. The future holds much exploration for the use of ACE in bioanalysis.

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